

Acknowledgements

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Transparency declarations

None to declare.

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Emergence of colistin resistance in *Klebsiella pneumoniae* from veterinary medicine

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Sir,
Colistin is among the very few antimicrobials that retain activity against MDR Gram-negative bacteria. It binds to the negatively charged LPS, leading to the disruption of the membrane.¹ Since its discovery, colistin has been used in veterinary medicine, in particular in cattle and swine, usually collectively through prophylactic or metaphylactic practices but also as individual treatment.^{2,3} On the other hand, colistin is a last-resort antibiotic to treat multiresistant bacteria in human medicine, although colistin-resistant clinical isolates have recently been reported. This raises concerns about the possibility that colistin-resistant strains might be selected in animals and then might be the sources of human infections.

Colistin resistance in *Klebsiella pneumoniae* is related to modifications of LPS by addition of cationic charges, such as addition of 4-amino-4-deoxy-L-arabinose to lipid A, which decreases the affinity between colistin and its target.⁴ The modification of LPS is mediated by the *pmrHFIJKLM* operon, regulated by the

PhoPQ and PmrAB two-component systems.⁵ It has been demonstrated that MgrB, a small transmembrane protein, negatively regulates the PhoPQ system by interaction with the sensor kinase PhoQ in the periplasmic domain, preventing activation of the *pmrHFJKLM* operon.⁶

It was recently shown that insertional inactivation of the *mgrB* gene in *K. pneumoniae* could result in up-regulation of the PhoPQ system, leading to overexpression of the *pmrHFJKLM* operon, resulting in colistin resistance due to addition of positive charges to lipid A.^{7,8} This was demonstrated in a KPC-producing isolate from Italy and then among a series of clonally unrelated isolates with worldwide origins. Interestingly, different genetic events were identified at the origin of this resistance, being either the insertion of different types of IS at different locations into the *mgrB* gene or the occurrence of a premature stop codon in the MgrB coding sequence. All these genetic events led to truncations of the *mgrB* gene and consequently impaired the production of a functional MgrB protein.

The aim of this study was to evaluate the occurrence of colistin resistance among *K. pneumoniae* isolates recovered from bovine mastitis, a disease that can be treated by intra-mammary application of colistin (mostly in combination with penicillins) in France, and to decipher the corresponding mechanism(s). Ninety-seven non-duplicate *K. pneumoniae* isolates that caused mastitis were recovered through the Resapath network (www.resapath.anses.fr) in 2013 and sent to Anses (Lyon) for further analysis.

Antimicrobial susceptibility testing was performed by broth microdilution according to the EUCAST recommendations, using cation-adjusted Mueller–Hinton broth.⁹ MICs of colistin were determined by Etest[®] (bioMérieux, La Balme-les-Grottes, France) and breakpoints were those recommended by the EUCAST: >2 mg/L, resistant; and ≤2 mg/L, susceptible. Only 1 of the 97 isolates (isolate NK34373) showed resistance to colistin, with an MIC of 8 mg/L. MLST, performed as previously described¹⁰ and interpreted using the public MLST web site (<http://bigsdw.web.pasteur.fr/klebsiella/klebsiella.html>), identified isolate NK34373 as being ST37. This isolate was susceptible to all other antibiotics tested, including broad-spectrum cephalosporins, all aminoglycosides, quinolones, fluoroquinolones, chloramphenicol and tetracyclines.

A PCR specific for the *mgrB* gene was performed using primers mgrB-Kp-F (5'-TTAAGAAGGCCGTGCTATCC-3') and mgrB-Kp-R (5'-AAGGCGTTCATTCTACCACC-3'). It revealed a larger amplicon compared with a WT *mgrB* amplicon (data not shown) and sequencing showed that the *mgrB* gene was interrupted by a 1057 bp IS903B element (98% nucleotide identity) belonging to the IS5 family (<https://www-is.biotoul.fr>) (GenBank accession number X02527). The IS was inserted into the *mgrB* gene between nucleotides 67 and 68 and was bracketed by a 9 bp target site duplication (5'-ACTCAGATG-3'), likely being the signature of a transposition event. In parallel, sequence analysis of the *pmrCAB* operon as previously described¹¹ identified WT genes.

Complementation experiments were then performed as previously described using recombinant plasmid pTOPO-mgrB or pTOPO-pmrB, encoding WT *K. pneumoniae* MgrB and PmrB proteins, respectively.¹⁰ Electro-transformation was performed by electroporation into the colistin-resistant isolate NK34373 and electro-transformants were selected onto Mueller-Hinton agar plates supplemented with 100 mg/L zeocin (resistance marker of cloning vectors). Complementation with plasmid pTOPO-mgrB fully restored

susceptibility to colistin, while the MIC of colistin remained unchanged upon transformation with plasmid pTOPO-pmrB.

These results demonstrated that the loss of functional MgrB was responsible for the colistin resistance trait observed in the *K. pneumoniae* isolate. To the best of our knowledge, this is the first description of a mechanism responsible for colistin resistance in a veterinary strain. Notably, it corresponds to a mechanism that is identical to that identified among human *K. pneumoniae* isolates, i.e. the inactivation of the *mgrB* gene. As opposed to those colistin-resistant isolates that have been identified among human isolates, this isolate was susceptible to all other antibiotics. The routes of selection of colistin resistance in this isolate remain unknown. It may result from local injection of colistin in the infected udder, but colistin resistance might also have been selected in the farm environment as a consequence of oral administration of colistin to calves, then further eliminated in the faeces. Owing to the wide use of colistin in veterinary medicine, these results suggest that more extensive epidemiological surveys should now be conducted to evaluate the prevalence and molecular features of colistin-resistant isolates in animals.

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Transparency declaration

None to declare.

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***Neisseria gonorrhoeae* isolates with high-level resistance to azithromycin in Australia**

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Sir,

Antimicrobial resistance in *Neisseria gonorrhoeae* is a major public health concern globally. Over time, gonococci have developed resistance to most antimicrobial agents used for first-line treatment and declining susceptibility to the extended-spectrum cephalosporins is emerging worldwide.^{1–3} Consequently, dual antimicrobial therapy consisting of ceftriaxone (250 or 500 mg by intramuscular injection) together with azithromycin (1 or 2 g orally) is now recommended as standard treatment for gonorrhoea in the USA, the UK and Europe and is now gradually being adopted in major clinics throughout Australia.^{4–7} Monotherapy with 2 g of azithromycin is an alternative treatment in patients who are allergic to cephalosporins.⁴ These treatment regimens are threatened by the emergence of azithromycin resistance in gonococci, particularly high-level azithromycin resistance. High-level azithromycin resistance in *N. gonorrhoeae*, defined by an azithromycin MIC >256 mg/L, is associated with mutations in the peptidyltransferase loop in domain V of the 23S rRNA gene, principally A2059G, and a single base pair deletion (A) in the promoter region of the *mtrR* gene.^{8,9} Isolates of gonococci displaying high-level azithromycin resistance have been reported from the UK, Italy, Argentina, the USA and Sweden.^{10–14} Here, we provide the first report of gonococci in Australia with high-level azithromycin resistance.

The Australian Gonococcal Surveillance Programme (AGSP) monitors gonococcal antimicrobial resistance to a wide range of antimicrobials, which is achieved through the referral of gonococcal isolates from clinical diagnostic laboratories to nominated state or territory reference laboratories. These reference laboratories together form the National *Neisseria* Network (NNN) of Australia, through which ~3500 *N. gonorrhoeae* isolates are tested for antimicrobial resistance annually. Since 2011, six isolates of gonococci submitted to NNN laboratories were found to exhibit high-level azithromycin resistance: one isolate from the state of New South Wales, two isolates from the state of Victoria and three isolates from the state of Queensland. Details are summarized in Table 1.

Briefly, identification was confirmed as *N. gonorrhoeae* on the basis of Gram stain, oxidase, superoxol and carbohydrate utilization tests or MALDI Biotyper[®] (Bruker Daltonik, Bremen, Germany). Breakpoint MICs were initially determined by agar plate dilution using the method of the AGSP.¹⁵ Azithromycin MICs were confirmed by Etests (bioMérieux, Marcy-l'Étoile, France). In addition, the 23S rRNA and *mtrR* genes of each isolate were

Table 1. Characteristics of the first strains of *N. gonorrhoeae* with high-level resistance to azithromycin from Australia

Isolate/state	Year	Gender/age (years)	Site	MIC (mg/L) (susceptibility category)					23S rRNA mutation (no. of alleles)	NG-MAST
				azithromycin	ceftriaxone	ciprofloxacin	PPNG	TRNG		
1/QLD	2011	male/44	urethra	>256 (R)	0.016 (S)	8 (R)	yes	yes	A2059G (4)	ST5309
2/NSW	2011	male/39	urethra	>256 (R)	0.016 (S)	4 (R)	yes	yes	A2059G (4)	ST10572
3/VIC	2013	female/48	vagina	>256 (R)	0.016 (S)	8 (R)	no	yes	A2059G (4)	ST8917
4/QLD	2013	male/24	urethra	>256 (R)	≤0.008 (S)	≤0.03 (S)	no	no	A2059G (4)	ST649
5/QLD	2013	male/26	urethra	>256 (R)	0.016 (S)	≤0.03 (S)	no	no	A2059G (4)	ST649
6/VIC	2013	female/44	cervix	>256 (R)	0.016 (S)	8 (R)	yes	yes	A2059G (4)	ST10133

NSW, New South Wales; QLD, Queensland; VIC, Victoria; PPNG, penicillinase-producing *N. gonorrhoeae*; TRNG, tetracycline-resistant *N. gonorrhoeae*; S, susceptible; R, resistant.