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MECHANISMS OF RESISTANCE

Deciphering Heteroresistance to Colistin in a *Klebsiella pneumoniae* Isolate from Marseille, France

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ABSTRACT Here, we report the description of a colistin-heteroresistant *Klebsiella pneumoniae* isolate fortuitously isolated from the stool sample of a patient with suspicion of tuberculosis in a public hospital of Marseille, France. In the colistin-resistant subpopulation, a mutation in the *mgrB* gene leading to a premature stop codon was found, and the hypermucoviscous phenotype was lost. Susceptibility to other antibiotics remained unchanged. To our knowledge, this is the first identification of such a colistin-heteroresistant *Klebsiella pneumoniae* isolate in France.

KEYWORDS *Klebsiella pneumoniae*, heteroresistance, colistin, *mgrB*

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Klebsiella pneumoniae is a significant human pathogen belonging to the *Enterobacteriaceae* family (1). The increase of multidrug-resistant *Enterobacteriaceae* led to the renewed use of colistin as a treatment of last resort (2), conducive to the emergence of colistin resistance among *K. pneumoniae* strains worldwide (3). Colistin has bactericidal action against Gram-negative pathogens, targeting the lipid A moiety of lipopolysaccharide (LPS) and leading to cell membrane disruption (4). The main known mechanisms of colistin resistance induce lipid A modifications through the addition of 4-amino-4-deoxy-L-arabinose or phosphoethanolamine (5), mostly mediated by mutations in the two-component system PmrA/PmrB or PhoP/PhoQ (6) or its negative regulator, MgrB (7–9), or by acquisition of the *mcr-1* plasmid-mediated gene (10), resulting in reduction of the polymyxin affinity to LPS.

Heteroresistance can be defined as the presence of subpopulations with various susceptibilities to an antibiotic within an isolate (11). Few reports of colistin heteroresistance exist, because it cannot be assessed by the recommended MIC testing methods (12). Here, we isolated a colistin-heteroresistant *Klebsiella pneumoniae* strain from the stool sample from a patient in the public hospital Hôpital Nord of Marseille, France. This isolation was fortuitously performed during research on *Mycobacteriaceae*, and the isolate was detected after 7 days by culture on an innovative selective medium containing the BBL MGIT PANTA antibiotic mixture (BD, USA), which included 2.38 μg/ml of polymyxin B. (PANTA includes polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin.)

Colistin heteroresistance was detected by MIC testing using colistin Etest strips (bioMérieux, Marcy l'Étoile, France), where colonies were observed within the clear zone of inhibition (Fig. 1A). Two subpopulations were separated by subculture on Columbia agar plus 5% sheep blood (COS [bioMérieux, Marcy-l'Étoile, France]). Both were identified as *Klebsiella pneumoniae* by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Microflex; Bruker Daltonics, Bremen, Germany) (13). They exhibited different colony morphotypes, and their colistin MIC was assessed by broth microdilution method and interpreted according to the EUCAST guidelines (http://www.eucast.org). The colistin-resistant subpopulation, named LB1,

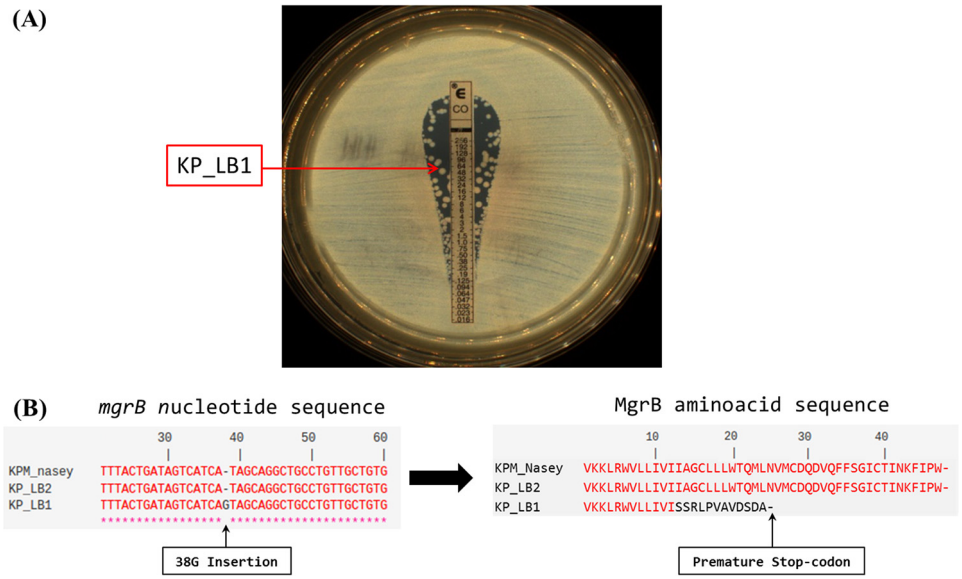

FIG 1 (A) Colistin MIC determination using a colistin Etest strip. The arrow indicates the colistin-resistant subpopulation LB1. (B) Alignments of *mgrB* nucleotide sequences and amino acid MgrB protein sequences of the two subpopulations of colistin-heteroresistant *Klebsiella pneumoniae* with KPM_Nasey retrieved from GenBank (accession no. KF852765).

had a colistin MIC of 128 μg/ml and exhibited gray and plate colonies on COS medium, while the colistin-susceptible subpopulation, named LB2, had a colistin MIC of 1 μg/ml and exhibited white and hypermucoviscous colonies, with a positive string test (14). Their stability was assessed by 15 daily subcultures on COS medium, and both the morphologies and the colistin susceptibilities were maintained.

Susceptibility testing of the two subpopulations to 22 antibiotics was performed by the disk diffusion method, and their growth rates were compared with those of colistin-susceptible (15) and *mcr-1*-positive *K. pneumoniae* strains as controls (3, 16). The two subpopulations presented identical growth rates (see Fig. S1 in the supplemental material) and had the same susceptibility patterns, except for colistin, for which the isolates were not defined as multidrug-resistant pathogens (Table 1). Faced with this phenotype similarity, we explored the existence of heteroresistance by comparative methods, including comparison of MALDI-TOF MS mass spectra (17), pulsed-field gel electrophoresis (PFGE) with XbaI digestion (18), and multilocus sequence typing (MLST) analysis, using the Pasteur Institute database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). Comparison of the MALDI-TOF MS mass spectra and PFGE gave identical protein profiles (Fig. 2), and the sequence type found by MLST was ST86, which had been reported as a hypermucoviscous (hypervirulent) strain, causing life-threatening infections (14, 19). These results confirmed that we had isolated a colistin-heteroresistant *Klebsiella pneumoniae* isolate, as LB1 and LB2 were isogenic strains.

Colistin resistance genes *mgrB*, *pmrA*, *pmrB*, *phoP*, and *phoQ* were amplified and sequenced from the two subpopulations (3), and the presence of the recently described *mcr-1* gene was investigated by reverse transcription-PCR (RT-PCR) (20). Mutation was absent in *pmrA*, *pmrB*, *phoP*, and *phoQ*, and the amplification of *mcr-1* remained negative. However, sequence analysis of *mgrB* showed a single nucleotide insertion (38G) in colistin-resistant subpopulation LB1, leading to a premature stop codon and, based on the predicted protein sequence, an inactive MgrB regulator (Fig. 1B). MgrB mutations following colistin exposure were reported (21, 22) as an *in vitro* mutant selection (23) and also without previous exposure (24). To our knowledge, the patient did not receive previous polymyxin treatment, but as the isolate was detected on a polymyxin-containing medium, the mutation of the resistant population could be induced (23) or the subpopulation selected, allowing the growth of the bacteria (25, 26).

TABLE 1 Antibiotic susceptibility testing of LB1 and LB2 isogenic isolates by the disk diffusion method[a]

Antibiotic disk content (μg)	Breakpoint(s) from CA-SFM,[b] 2013 (mm)	Inhibition diam (mm)		Susceptibility
		LB1	LB2	
AMX (25)	16–21	6	6	R
AMC (30)	16–21	14	14	R
TIC (75)	22–24	6	6	R
TIM (85)	22–24	20	20	R
TZP (85)	17–21	25	25	S
FEP (30)	21–24	33	33	S
CRO (30)	23–26	32	32	S
CTX (30)	23–26	36	36	S
FOX (30)	15–22	26	26	S
IPM (10)	17–24	32	32	S
ETP (10)	26–28	31	31	S
ATM (30)	21–27	35	35	S
NAL (30)	15–20	21	21	S
CIP (5)	22–25	28	28	S
OFL (5)	22–25	36	36	S
AMK (30)	15–17	20	20	S
GEN (15)	16–18	20	20	S
TOB (10)	16–18	23	23	S
FOF (50)	14	19	19	S
SXT (25)	13–16	6	6	R
NIT (300)	15	12	12	R
CST (50)	15	9	17	R (LB1)/S (LB2)

[a]Both isolates were resistant (R) to amoxicillin (AMX), amoxicillin-clavulanate (AMC), ticarcillin (TIC), ticarcillin-clavulanate (TIM), nitrofurantoin (NIT), and trimethoprim-sulfamethoxazole (SXT) and susceptible (S) to piperacillin-tazobactam (TZP), cefepime (FEP), ceftriaxone (CRO), cefotaxime (CTX), cefoxitin (FOX), aztreonam (ATM), imipenem (IPM), ertapenem (ETP), nalidixic acid (NAL), ciprofloxacin (CIP), ofloxacin (OFX), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), and fosfomycin (FOF). LB1 was resistant to colistin (CST), while LB2 was susceptible.

[b]Comité de l'Antibiogramme—Société Française de Microbiologie (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM2013vjuin.pdf).

As in a recent report highlighting the association between biofilm formation and heteroresistance (27), we described different stable morphotypes of the colonies exhibiting different susceptibilities to colistin, without previously described reversion (7, 28). The loss of hypervirulence and fitness of strains after acquisition of colistin resistance was previously demonstrated for hypermucoviscous *K. pneumoniae* ST23 strains (29).

There are only 2 reports of genomic analysis of colistin-heteroresistant *K. pneumoniae* isolates, which were multidrug-resistant bacteria: one OXA-48 carbapenemase-producing strain with a mutation in protein PhoP (7) and five extended-spectrum β-lactamase (ESBL)-expressing isolates with mutations in the *phoQ*, *lpxM*, and *yciM* genes, as well as two with mutations on the *mgrB* gene (the presence of an insertion sequence and deletion) (30). This is the first description of a colistin-heteroresistant *Klebsiella pneumoniae* isolate in France and the first description of a truncated MgrB protein after an insertion of a single nucleotide.

Colistin-heteroresistant *K. pneumoniae* isolates among isolates that had been classified as susceptible in clinical practice showed higher prevalence rates than isolates classified as colistin resistant in some studies (26, 28). These findings may raise concern about the choice of antimicrobial susceptibility testing methods to assess heteroresistance, as the prevalence of colistin-resistant isolates is widely underestimated (31). Indeed, in clinical practice, the MICs are often assessed with automated techniques such as Vitek2 (bioMérieux, Marcy l'Étoile, France), and the only reliable method to assess the colistin MIC is broth microdilution according to EUCAST (http://www.eucast.org/ast_of_bacteria/warnings/#c13111), but these techniques have been reported as unreliable in detecting heteroresistance (32, 33). Currently, only agar-based methods, especially Etest, can detect heterogeneous populations and could be used concomitantly as screening tests (7, 11, 34). These studies highlight that heteroresistance should

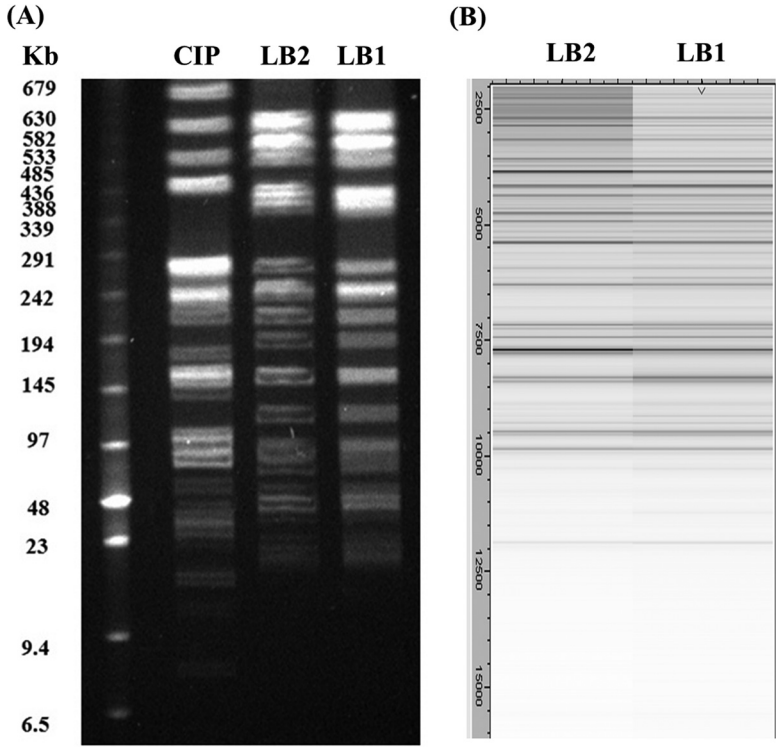

FIG 2 (A) PFGE profiles of XbaI-digested genomic DNAs showing genomic relatedness among the CIP 82.91, LB2, and LB1 *K. pneumoniae* strains. DNA sizes of the low-range pulsed-field gel marker (Biolabs, New England) are indicated in kilobases on the left. (B) MALDI-TOF MS spectra from LB1 and LB2 strain comparison with GelView using Flex Analysis software.

be further studied, as the resistant population can be selected and lead to rapid development and therapeutic failure (12). Because this strain was isolated fortuitously, we believe that screening of colistin-resistant strains from fecal samples should be performed at least in intensive care units in order to isolate such patients.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00356-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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We have no conflicts of interest to declare.

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