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Développement de nouveaux outils de détection de la résistance à la colistine chez les bactéries à Gram négatif

Lucie Bardet

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MÉMOIRE
DU DIPLÔME D'ÉTUDES SPECIALISÉES
D'INNOVATION PHARMACEUTIQUE ET RECHERCHE

Soutenu le vendredi 29 septembre 2017

Par Mme **BARDET Lucie**
Née le 4 mai 1988 à Paris

Conformément aux dispositions de l'Arrêté du 04 octobre 1988
tenant lieu de

THÈSE
POUR LE DIPLÔME D'ÉTAT DE DOCTEUR EN PHARMACIE

----oOo----

TITRE :

**Développement de nouveaux outils de détection de la
résistance à la colistine chez les bactéries à Gram négatif**

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JURY :

Président : Monsieur le Professeur COLSON Philippe

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Docteur BORNET Charléric

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***« L'UNIVERSITE N'ENTEND DONNER AUCUNE
APPROBATION, NI IMPROBATION AUX OPINIONS EMISES
DANS LES THESES. CES OPINIONS DOIVENT ETRE
CONSIDEREES COMME PROPRES A LEURS AUTEURS. »***

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INTRODUCTION

L'émergence de Bactéries Multi-Résistantes (BMR) représente une préoccupation mondiale, notamment avec la description des Bactéries Hautement Résistantes (BHRe) qui a été classée comme prioritaire par l'Organisation Mondiale de la Santé (OMS). Il s'agit de bactéries pathogènes résistantes aux antibiotiques utilisés en dernier recours pour traiter les BMR, à savoir les Entérobactéries Produisant des Carbapénémases (EPC) et les Entérocoques Résistants aux Glycopeptides (ERG) (1). Les entérobactéries sont des bacilles à Gram négatifs souvent décrites comme des agents pathogènes responsables de maladies infectieuses en pathologie humaine, en particulier les espèces *Escherichia coli* et *Klebsiella pneumoniae*.

La diffusion mondiale de la résistance aux carbapénèmes a conduit à la réutilisation d'anciens antibiotiques, notamment les polymyxines (2). Leur utilisation thérapeutique en santé humaine avait en effet été abandonnée dans les années 1970, en raison de leurs néphrotoxicité et neurotoxicité. La colistine a en revanche été utilisée massivement dans le monde vétérinaire, et son utilisation croissante en clinique humaine au cours de la dernière décennie a conduit à la détection de pathogènes résistants chez les entérobactéries ainsi que chez les espèces non-fermentaires *Pseudomonas aeruginosa* et *Acinetobacter baumannii* (3).

Jusqu'à récemment, tous les mécanismes de résistance décrits étaient d'origine chromosomique, puis en novembre 2015, le premier gène de résistance à la colistine médié par un plasmide a été décrit et nommé *mcr-1* (4). Depuis, des variants de *mcr-1* (*mcr-1.2*, *mcr-1.3* ...) ainsi que les gènes *mcr-2*, *mcr-3* et *mcr-4* ont été décrits (5–9). Cette découverte récente est une source d'inquiétudes quant à l'augmentation et à la dissémination de la résistance chez les entérobactéries (10), et a conduit à de nouvelles recommandations pour le diagnostic *in vitro* et la prise en charge des patients par les cliniciens (11). En effet, ces gènes de résistance présentent une haute capacité de transfert permettant leur dissémination parmi les pathogènes bactériens. De plus, plusieurs études ont rapporté la détection du gène *mcr-1* dans des souches d'EPC (12–14).

L'apparition de la résistance aux antibiotiques d'intérêt clinique conduit habituellement au développement de nouveaux outils dans les laboratoires de microbiologie clinique (15). Actuellement, si la détection des bactéries productrices de carbapénémases est bien déterminée, combinant des milieux de culture spécifiques, un test de phénotypage, l'antibiogramme et la biologie moléculaire (16–18), un protocole doit également être mis en place pour détecter la résistance aux polymyxines.

Le problème majeur posé par cette résistance plasmidique aux polymyxines réside dans la difficulté de sa détection *in vitro*. En effet, la majorité des souches porteuses du gène *mcr-1* présentent un faible niveau de résistance, avec des Concentrations Minimales Inhibitrices (CMI) de colistine d'environ 4 µg/mL (4), ce qui est proche de la CMI critique déterminée par les systèmes experts (sensible ≤ 2 µg/mL et résistant > 2 µg/mL). D'autre part, l'évaluation de la sensibilité aux polymyxines nécessite le développement d'outils particuliers, car cette grosse molécule cationique ne peut pas diffuser efficacement dans le milieu de culture de référence : Mueller Hinton ajusté en cations (MH2) (19). Enfin, l'hétérorésistance à la colistine est un phénomène encore largement sous-estimé et nécessite une approche approfondie (20–22). La mise en place d'outils de dépistage rapides et fiables pour détecter et analyser tous les pathogènes résistants à la colistine, y compris les résistances plasmidiques, est actuellement une approche indispensable pour permettre aux cliniciens d'isoler le patient et d'adapter son traitement (33).

Ce travail de thèse va ainsi porter sur le développement d'outils spécifiques de la détection des souches résistantes à la colistine, en réalisant dans un premier temps une revue de la littérature afin de décrire les nouvelles méthodes de dépistage mises au point, incluant l'ensemble des méthodes existantes et adaptées à la détection de ces pathogènes. Dans un deuxième temps, le milieu de culture spécifique LBJMR a été développé et a donné lieu à un dépôt de brevet. Ensuite, l'efficacité du kit commercial UMIC Colistine permettant de déterminer la CMI a été évaluée. Enfin, mes travaux de thèse m'ont conduit à la description d'une souche de *K. pneumoniae* hétérorésistante à la colistine et d'une nouvelle espèce, *Pseudomonas massiliensis*.

I. FAMILLE DES POLYMYXINES

1) Structure et mécanisme d'action

La famille des polymyxines est constituée d'antibiotiques bactéricides décrits en 1947 et naturellement produits par *Paenibacillus polymyxa*. Ces molécules sont des polypeptides cationiques, représentées en clinique par la Polymyxine B et la Colistine ou Polymyxine E (Figure 1). Elles sont volumineuses (1155 Da), composées d'un cycle de 7 acides aminés et d'une chaîne latérale tripeptidique sur laquelle est fixée un acide gras. Elles diffèrent d'un acide aminé dans le cycle peptidique en position 6 (la D-phénylalanine de la polymyxine B est remplacée par la D-leucine chez la colistine).

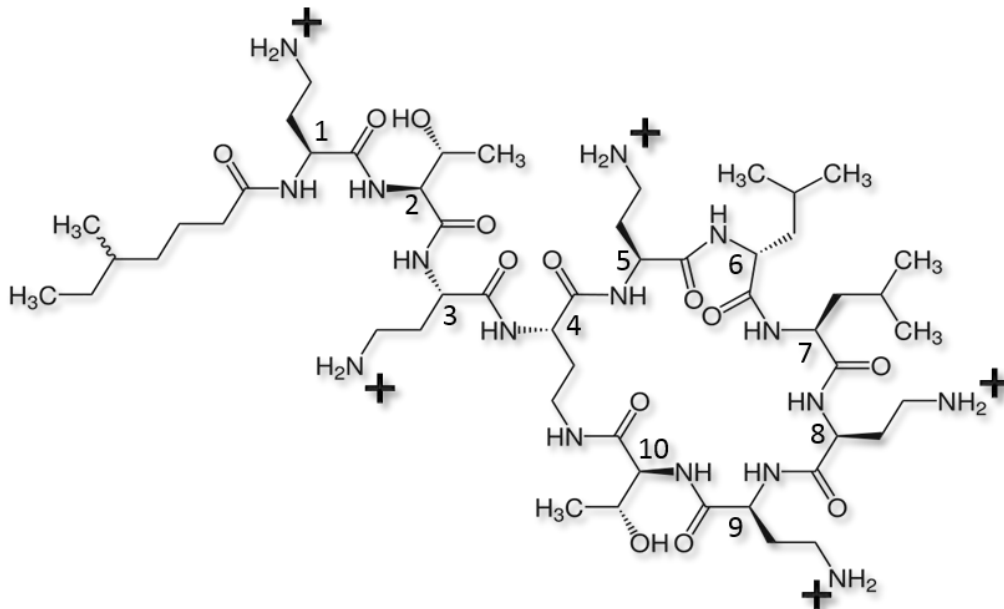


Figure 1. Structure chimique de la colistine (polymyxine E).

La colistine est la plus utilisée en clinique humaine et cible le lipopolysaccharide (LPS), principal constituant de la membrane externe des bactéries à Gram négatif. Son mécanisme d'action n'est encore pas totalement élucidé, et semble lié à l'effet polycationique conféré par ses résidus acides L-α-γ-diaminotériques en position 1, 3, 5, 8 et 9 (Figure 1), lui donnant une forte affinité pour la membrane chargée négativement. Elle déplace ainsi les cations Ca^{2+} et Mg^{2+} situés au niveau des phosphates du lipide A, partie hydrophobe et transmembranaire du LPS permettant son ancrage à la membrane externe. Cette interaction entraîne une désorganisation du LPS induisant l'augmentation de la perméabilité membranaire et la fuite du contenu cytoplasmique entraînant la mort bactérienne (23). Un autre mécanisme pourrait être la neutralisation de l'endotoxine (lipide A) ou encore la formation de radicaux libres (24).

2) Formes pharmaceutiques et indications

Actuellement la colistine est utilisée en clinique sous la forme de colistiméthate sodique (CMS), une prodrogue activée *in vivo*, et est indiquée chez l'enfant et l'adulte sous deux formes pharmaceutiques : par voie injectable dans le traitement des infections sévères dues à des bactéries aérobies à Gram négatif sensibles chez des patients pour qui les options thérapeutiques sont limitées, ou par voie inhalée dans la prise en charge des infections pulmonaires chroniques dues à *Pseudomonas aeruginosa* chez les patients atteints de mucoviscidose. La Polymyxine B est quant à elle utilisée sous forme active en voie locale (vaginale, auriculaire ou oculaire) et toujours en association avec un aminoside et dans la plupart des cas avec un corticostéroïde. Les polymyxines sont exprimées en Unités Internationales (UI) : 80 mg de CMS = 1 MUI = 30 mg de colistine active (25).

Les entérobactéries γ sont généralement sensibles (*E. coli*, *K. pneumoniae*, *Enterobacter* sp) à l'exception des genres *Providencia*, *Proteus*, *Serratia* et *Morganella*. Chez les non fermentaires, les espèces γ sont également généralement sensibles (*Acinetobacter* sp., *Pseudomonas aeruginosa*, *Stenotrophomas maltophilia*, *Citrobacter freundii*) à l'exception du genre *Burkholderia*. D'autres espèces de bactéries à Gram-négatif présentent des résistances naturelles comme les espèces des genres *Campylobacter*, *Brucella*, *Legionella*, *Neisseria* ou encore *Chryseobacterium meningosepticum* et *Vibrio cholerae*.

3) Pharmacocinétique et effets indésirables

Les polymyxines sont peu absorbées au niveau du tractus intestinal et nécessitent donc une administration par voie parentérale (25). L'effet bactéricide est concentration-dépendant sur les bactéries sensibles et les concentrations plasmatiques doivent être maintenues au-dessus des CMI critiques (2 $\mu\text{g/mL}$) pour éviter d'une part la sélection de clones résistants et d'autre part la survenue de toxicité rénale. Chez les sujets sains, 60 à 70% du CMS sont excrétés sous forme inchangée dans les urines en 24 heures. L'effet antibactérien est rapide ($C_{\text{max}} = 2\text{h}$). La demi-vie de la colistine est évaluée autour de 3 à 4h, et est prolongée jusqu'à 18h en cas d'insuffisance rénale sévère. La posologie doit donc être adaptée dans ce cas. Il n'y a pas d'effet post-antibiotique.

Les principaux effets indésirables sont l'insuffisance rénale, généralement réversible et imposant la surveillance de la fonction rénale au cours du traitement, et la neurotoxicité en cas de surdosage (paresthésies péri-buccales et des extrémités, désorientation temporo-spatiale, syndrome confusionnel, blocage neuromusculaire). Il n'existe pas d'antidote.

II. RÉSISTANCE ACQUISE A LA COLISTINE

La résistance à la colistine est principalement due à la modification de la cible chez les entérobactéries, *Pseudomonas aeruginosa* et *Acinetobacter baumannii* (26). L'ajout de sucres chargés positivement sur les charges négatives du lipide A entraîne la perte d'affinité des polymyxines pour le LPS (Figure 2). Ces sucres sont la phosphoéthanolamine (PEtN) ou le 4-amino-4-arabinose (L-Ara4N).

Les autres mécanismes de résistance décrits sont la surexpression des pompes à efflux AcrAB-TolC et KpnEF ou encore l'hyperproduction de la capsule de *K. pneumoniae* (27, 28).

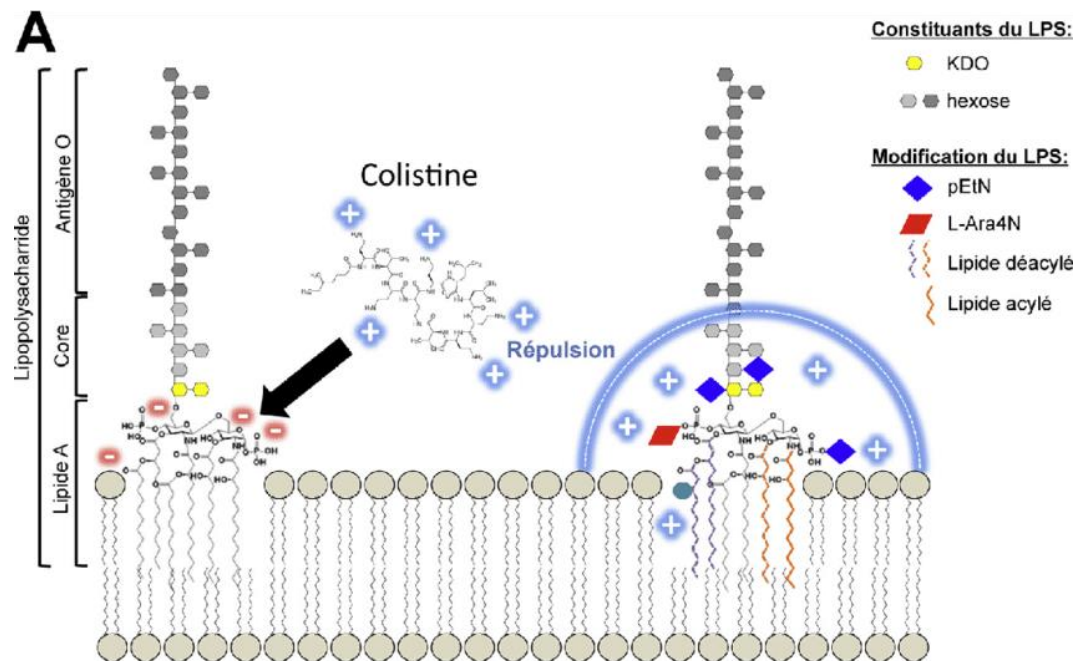


Figure 2. Modifications du lipide A entraînant la répulsion des polymyxines.

(Issue de Dortet *et al.*, J. Antiinfectieux, 2016) (24)

1) Modification du LPS par mutations chromosomiques

La synthèse et la fixation des sucres sont régulées par des systèmes membranaires à deux composants : PmrAB et PhoPQ (26), par l'intermédiaire de l'opéron *arnBCADTEF* pour le L-Ara4N ou d'une phosphoéthanolamine transférase (PmrC chez toutes les entérobactéries, EptB et EptC chez *E. coli* et *Salmonella* spp.) pour la PEtN.

Ces régulateurs vont être activés par différents stimuli environnementaux, en présence de polymyxines ou par des mutations activatrices sur leurs gènes respectifs situés sur le chromosome bactérien (Figure 3 et Table 1).

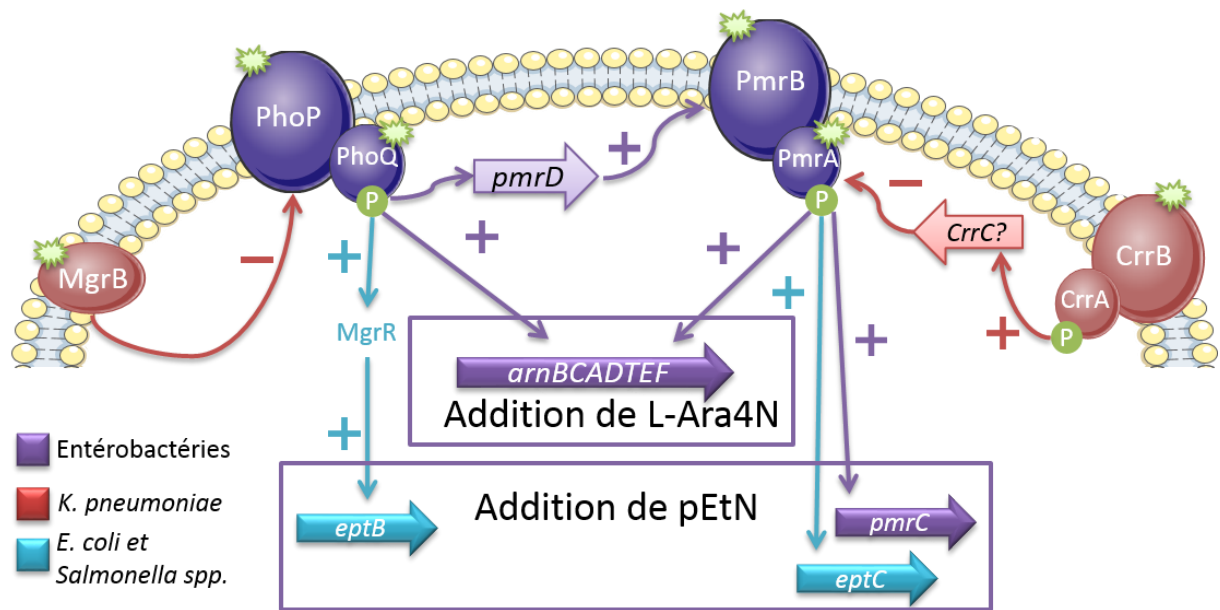


Figure 3. Mécanismes moléculaires de la résistance aux polymyxines.

Chez *K. pneumoniae*, le principal mécanisme de résistance décrit est l'inactivation de MgrB, un régulateur négatif de PhoPQ, induisant une surexpression de l'opéron *arnBCADTEF*. Cette espèce comporte un système à deux composants supplémentaire : l'opéron CrrAB qui semble réguler négativement PmrAB (Figure 3).

P. aeruginosa possède 3 systèmes supplémentaires ParRS, ColRS et CprRS qui induisent l'expression de l'opéron *arnBCADTEF* (29, 30). *A. baumannii* peut perdre totalement son LPS par la biosynthèse des gènes *lpxA lpxC* et *lpxD* induisant des altérations du lipide A (31).

L'ensemble des gènes dont les mutations sont connues pour induire la résistance aux polymyxines chez chacune de ces espèces bactérienne est résumé dans la Table 1 (32).

Table 1. Gènes induisant la résistance acquise à la colistine chez les Gram-négatifs.

Espèce bactérienne	Gènes dont les mutations ont été décrites
<i>Klebsiella pneumoniae</i>	<i>pmrA, pmrB, phoP, phoQ, mgrB, crrB</i>
<i>Escherichia coli</i>	<i>pmrA, pmrB, phoP, phoQ</i>
<i>Enterobacter aerogenes</i>	<i>pmrA</i>
<i>Salmonella enterica</i>	<i>pmrA, pmrB</i>
<i>Pseudomonas aeruginosa</i>	<i>pmrA, pmrB, phoP, parR, parS, colS, cprS</i>
<i>Acinetobacter baumannii</i>	<i>pmrA, pmrB, lpxA, lpxC, lpxD</i>

D'autres mécanismes sont impliqués dans les modifications du LPS, comme le régulateur intrinsèque RamA chez *K. pneumoniae* ou les gènes *lpxM, pagP* et *pagL* qui entraînent l'ajout d'acides gras sur le lipide A (24, 32).

2) Résistance médiée par un plasmide

La problématique de la résistance à la colistine a évolué avec la découverte en novembre 2015 du premier gène de résistance porté par un plasmide, nommé *mcr-1*, dans une souche d'*E. coli* en Chine (4). Depuis, les gènes *mcr-2*, *mcr-3*, *mcr-4* et les variants *mcr-1.2* à *mcr-1.7* ont été décrits (5–7). Ces gènes codent pour une phosphoéthanolamine transférase et induisent l'ajout de PEtN sur le lipide A. Contrairement aux mécanismes d'origine chromosomique, ils confèrent des niveaux bas de résistance aux polymyxines, avec des CMI de l'ordre de 4 µg/mL, proche de la CMI critique.

Un dépistage rétrospectif a eu lieu au niveau mondial après cette découverte, et le gène *mcr-1* a été découvert dans un nombre important de souches dont le mécanisme de résistance aux polymyxines était jusqu'à présent inconnu. Actuellement, sa présence a été signalée dans près de 50 pays dans des échantillons d'origines diverses, tant animales qu'humaines, présentés dans la Figure 4 (33–39). La prévalence de *mcr-1* est notamment importante chez les animaux d'élevage (porcs et poulets principalement) corrélée à l'utilisation massive de colistine en clinique vétérinaire (40).

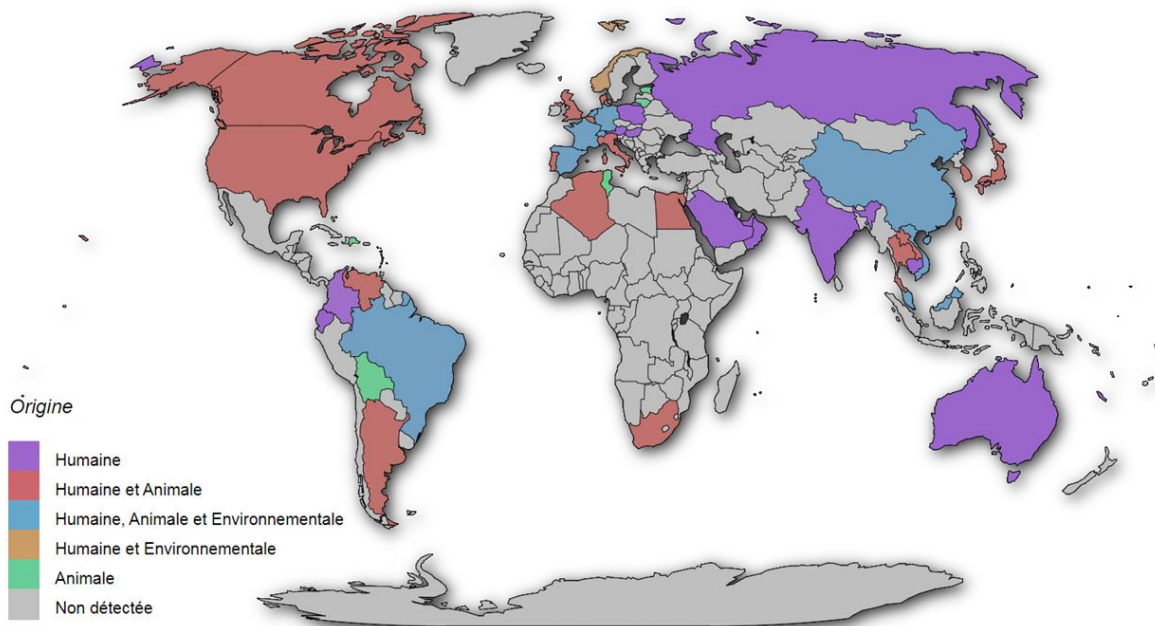


Figure 4. Dépistage mondiale du gène *mcr-1*.

Ce gène a été le plus souvent détecté dans des souches d'*E. coli* mais également dans de nombreuses espèces bactériennes telles que *K. pneumoniae* (41), *Enterobacter aerogenes*, *Enterobacter cloacae* (42, 43), *Salmonella* sp. (44), *Shigella sonnei* (45), *Citrobacter freundii* (46), *Kluyvera ascorbata* (47), *Cronobacter sakazakii* (48).

Si la découverte des gènes plasmidiques est récente, il semble que le phénomène ne soit pas nouveau, car une analyse rétrospective a pu détecter *mcr-1* dans des échantillons de poulets des années 1980 en Chine (49).

Cette émergence est d'autant plus inquiétante que le gène *mcr-1* a souvent été dépisté dans des souches productrices de bêta-lactamases à spectre étendu (BLSE) (50–52), plus rarement de carbapénémases (32), voire les deux (53). Dans la plupart des cas les souces étaient isolées de prélèvements humains.

Ainsi les recommandations françaises préconisent de tester la résistance à la colistine et rechercher la présence du gène *mcr-1* chez toute souche d'EPC chez un patient ayant été hospitalisé à l'étranger au cours de l'année écoulée ou dans le cas de l'initiation d'un traitement par la colistine (54).

Mcr-1 est un gène de 1626 paires de base (pb) localisé dans un transposon de 2607 pb contenant des insertions de séquence *ISApII* (55) aux deux extrémités du gène, qui lui confèrent une grande transférabilité (56–59). En particulier, Li et al ont identifié l'habilité du transposon à se circulariser en passant de la cassette *ISApII-mcr-1-orf-ISApII* à la forme *ISApII-mcr-1-orf* ce qui lui confère une grande transférabilité (60). Ceci est reflété par la description de *mcr-1* dans différents types de plasmides : Inc2, IncX4, IncHI2, IncH1, IncP, IncY, IncF, IncX3 et IncK (61) et de *mcr-2* dans IncFII et IncX4 (62). Cette large distribution montre la capacité des gènes *mcr* à disséminer. La présence de *ISApII* en amont du gène semble être un composant clé de ce mobilome mais sa présence n'est pas systématique (63), suggérant que l'insertion du gène *mcr-1* peut être stabilisée.

3) Hétérorésistance

L'hétérorésistance est un cas particulier de résistance qui est largement sous-estimé pour les polymyxines car également difficile à détecter par la méthode de référence (64). Il s'agit d'une souche présentant des sous-populations ayant des sensibilités différentes à un antibiotique (65) et dont les morphologies peuvent différer (66). Elle a été décrite chez *K. pneumoniae* (67–69), *Enterobacter* sp. (28, 70, 71), et *A. baumannii* (72–74). Jusqu'à présent, l'hétérorésistance à la colistine n'a pas été corrélée à la présence des gènes *mcr*.

Ce phénomène est problématique car si la sous-population résistante n'est pas détectée la souche peut être classée comme sensible lors de l'antibiogramme or elle doit être considérée comme résistante *in vivo*. L'hétérorésistance peut être suspectée dans la méthode de référence en cas de résultats ininterprétables en présence de multiples sauts de puits (71).

III. DÉTECTION DE LA RÉSISTANCE À LA COLISTINE

1) Méthodes qualitatives

a. Milieux de culture sélectifs

La culture est la méthode de référence pour la détection de germes en laboratoire de microbiologie clinique par leur isolement à partir d'échantillons cliniques. Des milieux sélectifs adaptés sont continuellement développés pour la détection de germes résistants (15, 75, 76). Jusqu'à récemment, les milieux commercialisés contenant des polymyxines étaient utilisés dans la plupart des cas pour éviter la croissance des entérobactéries et ne sont pas adaptés à la détection des souches résistantes de bas niveau à la colistine, soit en raison d'une concentration trop élevée, soit de la présence d'autres antibiotiques (Table 1). Seul le milieu commercial ANC (Acide Nalidixique - Colistine), normalement utilisé pour détecter des bactéries à Gram positif, a pu détecter une souche de *E. coli* porteuse du gène *mcr-1* (77) et une souche de *K. pneumoniae* (78, 79).

Pour ces raisons, un nombre important d'études ont mis au point des milieux de culture "maison" afin de dépister les souches porteuses des gènes *mcr*, en ajoutant des concentrations faibles de colistine (2 ou 4 mg/L) à des bases d'agar, parfois associés à d'autres anti-infectieux pour inhiber la croissance de bactéries à Gram-positifs ou de levures Table 1 (7, 80–91). Ces milieux ont été utilisés dans le cadre d'un dépistage mais leur sensibilité de détection n'a pas été évaluée.

Le milieu SuperPolymyxin (Elitech, Signes, France) a été développé et évalué pour la détection des souches porteuses du gène *mcr-1* (92). Sa composition est décrite dans la Table 2. Il permet une reconnaissance visuelle facilitée des souches d'*E. coli* qui vont se différencier par un éclat métallique (vert scarabée), mais n'a pas été évalué sur des échantillons cliniques. Il a été mis au point pour s'insérer dans les protocoles de recherche de germes résistants à la colistine : les bactéries isolées à partir un milieu spécifique pour détecter les BLSE ou EPC sont repiquées sur ce milieu, pour détecter les souches résistantes à la colistine puis les gènes *mcr* sont recherchés par biologie moléculaire.

Il est donc nécessaire de développer un milieu de culture spécifique de la détection des bactéries à Gram négatif résistantes à la colistine permettant également l'isolement des souches porteuses des gènes *mcr*.

Table 2. Composition des milieux de culture contenant des polymyxines.

Bactéries ciblées	Milieu de culture	Antibiotiques (µg/mL) contre:				
		Gram-négatifs		Gram-positifs	Levures	
		Polymyxines	Autres			
Gram-négatifs ColR	LBJMR ^a (développé au cours de ce travail)	4 (C)		Vancomycin 50		
Gram-positifs VancoR						
Résistants à la colistine	SuperPolymyxin (92)	3,5 (C)		Daptomycine 10 BM 65 Éosine 400	5 (AB)	
Gram négatifs	<i>Neisseria sp.</i>	Martin-Lewis Agar (93)	7,5 (C)	5 (T)	Vancomycine 4	20 (A)
		Thayer-Martin Agar (94)	7,5 (C)		Vancomycine 3	2,57 (N)
		MTM ^b Agar (95)	7,5 (C)	5 (T)	Vancomycine 3	2,57 (N)
		NYC ^c Agar (96)	7,5 (C)	3 (T)	Vancomycine 2	20 (A)
	<i>Bulkhoderia cepacia</i>	Cepacia Medium (97)	30 (B)		Ticarcelline 100	
		OFFPBL ^d Agar (98)	30 (B)	Bacitracine 3		
		Bulkhoderia Cepacia Agar (97)	17.8 (B)	5 (GEN)	Ticarcelline 100	
		Bulkhoderia Cepacia Selective Agar (99)	71.4 (B)	10 (GEN)	Vancomycine 2.5	
	<i>Legionella sp.</i>	BCYE ^e Selective Agar with:				
		GVPC ^f (100)	9,4 (B)	Glycine 3000	Vancomycine 1	80 (CH)
		CCVC ^g (101)	16 (C)		Vancomycine 0,5 Cefalotine 4	80 (CH)
		GPVA ^h (102)	11,9 (B)	Glycine 3000	Vancomycine 1	80 (A)
		PAV ⁱ (103)	4.76 (B)		Vancomycine 0,5	80 (A)
		PAC ^j (104)	9.52 (B)		Céfamandole 2	80 (A)
		DGVP ^k (105)	8 (B)	Glycine 3000	Vancomycine 1	
<i>Campylobacter sp.</i>	Campylobacter Agar :					
	Butzler (106)	0.33 (C)	Bacitracine 338	Novobiocine 5 Céfazoline 15	50 (CH)	
	Skirrow (107)	0.25 (B)	2.5 (T)	Vancomycine 5		
	Blaser-Wang (108)	0.125 (B)	2.5 (T)	Vancomycine 5 Cefalotin 15	2 (AB)	
<i>Brucella spp.</i>	Preston (109)	0.125 (B)	5 (T)	Rifampicine 5	50 (CH)	
	Brucella selective medium (110)	1 (B)	Bacitracine 500		100 (CH)	
<i>Vibrio sp.</i>	CPC ^l (111)	66.34 (C) 11.9 (B)				
Gram positifs	<i>Streptococcus sp. et Gram positifs</i>	ANC ^m (112)	10 (C)	A.Nalidixique 10		
	<i>Listeria monocytogenes</i>	Oxford medium (113)	20 (C)	Fosfomycine 10	Cefotetan 2 Acriflavine 5	400 (CH)
		Modified Oxford (114)	10 (C)		Moxalactam 15	
	<i>Listeria spp.</i>	PALCAM ⁿ (115)	10 (B)		Ceftazidime 8 Acriflavine 5	
	<i>Bacillus cereus</i>	MYP ^o (116)	10 (B)			
	<i>Mycobacteriaceae</i>	Middlebrook 7H11 (117)	25 (B)	20 (T)	Carbénicilline 50	10 (AB)
	<i>Clostridium perfringens</i>	SPSP ^p Agar (118)	10 (B)	Sulfadiazine 120		
TSN ^q Agar (119)		20 (B)	Néomycine 50			
SFP ^r Agar (120)		3,57 (B)	Kanamycine 12			

(B) Polymyxine B, (C) Colistine, (AB) Amphotéricine B, (A) Anisomycine, (CH) Cycloheximide, (MB) Bleu de méthylène, (N) Nystatine, (GEN) Gentamicine et (T) Triméthoprime.

^aLBJMR : Lucie Bardet et Jean-Marc Rolain, ^bMTM : Modified Thayer-Martin, NYC^c : New York City, ^dOFFPBL : Oxydation/Fermentation, Polymyxine B, Bacitracine et Lactose, ^eBCYE : Charbon tamponné (Buffered Charcoal) et Extrait de Levure (Yeast Extract), ^fGPVC : Glycine, Polymyxine B, Vancomycine et Cycloheximide, ^gCCVC : Cephalotine, Colistine, Vancomycine et Cycloheximide, ^hGPVA : Glycine, Polymyxine B, Vancomycine et Anisomycine, ⁱPAV : Polymyxine B, Anisomycine et Vancomycine, ^jPAC : Polymyxine B, Anisomycine et Céfamandole, ^kDGVP : Colorants (dyes), Glycine, Vancomycine et Polymyxine B, ^lCPC : Cellulose, Polymyxine B, Colistine, ^mANC : Acide Nalidixique et Colistine, ⁿPALCAM : Polymyxine B, Acriflavine, Lithium, Ceftazidime, Esculine et Mannitol, ^oMYP : Mannitol, Jaune d'oeuf (Egg Yolk) et Polymyxine B, ^pSPS : Sulfite, Polymyxine B et Sulfadiazine, ^qTSN : Trypticase, Sulfite et Néomycine, ^rSFP : Shahidi Ferguson Perfringens.

b. Nouvelles techniques phénotypiques rapides

Le Colispot est une technique qui a été développée pour les laboratoires vétérinaires et qui consiste au dépôt d'une goutte de solution de colistine calibrée (8 µg/mL) sur la gélose ensemencée lors d'un antibiogramme par la méthode de diffusion des disques (121). La résistance à la colistine est révélée par l'absence de zone d'inhibition.

Le Rapid polymyxin NP test (Elitech, Suresnes, France) est un simple test de pH, basé sur l'acidification du milieu avec la fermentation du glucose. Il a été évalué directement sur des hémocultures (40, 41). Une revue récente propose un plan diagnostique intégrant ce test pour confirmer la résistance à la colistine des isolats ayant poussé sur un milieu de culture sélectif (124). Ce test a récemment été évalué en comparaison de la méthode de référence et a montré une excellente sensibilité pour détecter les souches porteuses des gènes *mcr* (125, 126).

L'essai Micromax (Halotech DNA SL, Madrid, Spain) est basé sur la détection par fluorescence des fragments d'ADN témoignant de l'endommagement de la paroi cellulaire après incubation de la bactérie en présence de colistine. La résistance est détectée en cas d'absence de fluorescence. Cette méthode est rapide, mais n'est pas spécifique du type de résistance. Il a été évalué chez *Acinetobacter baumannii* (127).

c. Techniques spécifiques pour la détection de *mcr-1*

Actuellement, il n'y a pas d'étude publiée sur l'utilisation du Matrix-Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS) pour détecter la résistance à la colistine, mais il a récemment été mis en évidence l'existence d'un pic spécifique pour *mcr-1*, après la description de celui du lipide A à 1796.2 m/z (128), et la possibilité de détecter des modifications du lipide A (129). L'utilisation du MALDI-TOF MS est prometteuse étant donné que la majorité des laboratoires cliniques possèdent maintenant le matériel requis pour identifier les isolats cliniques en routine (130–133).

Le test Colistin-MAC consiste en une méthode de microdilution liquide basée sur la chélation de MCR-1 par l'acide dipicolinique (DPA), entraînant une réduction de la CMI de colistine de 8 dilutions en présence de DPA (134). La CMI de colistine est faite en parallèle sans DPA. Il a été évalué sur 74 entérobactéries incluant dont 61 portaient le gène *mcr-1* et seules les deux souches de *Klebsiella pneumoniae* testées n'ont pas été détectées.

2) Nouvelles recommandations sur l'antibiogramme

L'antibiogramme est nécessaire pour adapter le traitement car il permet d'évaluer la sensibilité d'une souche à un antibiotique en indiquant sa catégorie clinique : résistante (R), intermédiaire (I) ou sensible (S), voire sa Concentration Minimale Inhibitrice (CMI), la plus faible concentration inhibant la croissance bactérienne. Les résultats sont interprétés suivant les recommandations des systèmes experts European Committee on Antibiotic Susceptibility Testing (EUCAST) et Clinical and Laboratory Standard Institute (CLSI).

a. Méthode de référence

La détermination de la sensibilité aux polymyxines est délicate, car ces molécules cationiques diffusent mal dans le milieu de référence, adhèrent aux parois, et les souches porteuses des gènes *mcr* présentent des CMI basse. Les systèmes experts ont ainsi joint leurs recommandations en 2017 : la CMI doit être déterminée pour la colistine et la polymyxine B par la microdilution en milieu liquide (BMD) (Table 3).

Les méthodes de dilution consistent en l'ajout de la colistine dans le milieu de culture liquide ou solide de manière à obtenir une série de concentrations de raison 2. Ces méthodes sont préparées conformément au guide CLSI M07-A10 (135). La macrodilution diffère de la BMD uniquement par la taille du support : des tubes au lieu des microplaques de 96 puits.

Table 3. Recommandations 2017 sur l'évaluation de la sensibilité à la colistine.

Méthode de référence :	Microdilution en milieu liquide		
Préparation selon la norme ISO 20776-1 :	-	Milieu Mueller Hinton ajusté en cations (MH2)	
	-	Sulfate de colistine	
	-	Plaques de polystyrène dépourvu de prétraitement	
	-	Absence de polysorbate 80 ou de surfactant	
CMI critiques (µg/mL)	<i>Enterobacteriaceae</i>	<i>P. aeruginosa</i>	<i>Acinetobacter spp.</i>
EUCAST (136)	S ≤ 2 et R > 2	S ≤ 2 et R > 2	S ≤ 2 et R > 2
CLSI (137)	ECV* : WT ≤ 2 et NWT ≥ 4	S ≤ 2 et R ≥ 4	S ≤ 2 et R ≥ 4
Contrôles qualité (138) (µg/mL)	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	<i>E. coli (mcr-1)</i> NCTC 18853 [#]
Valeurs cibles	0.5-1	1-2	4
Limites acceptables	0.25-2	0.5-4	2-8

*Epidemiological cut-off values : Données cliniques et pharmacocinétiques insuffisantes pour évaluer une CMI critique pour les espèces suivantes : *E. aerogenes*, *E. cloacae*, *E. coli*, *K. pneumoniae*, et *R. ornithinolytica*. WT : Wild-Type, NWT : Non Wild-Type

[#]Recommandé par l'EUCAST, la CMI doit être à 4 et occasionnellement à 2 ou 8 µg/mL.

La quantité de sulfate de colistine est exprimée en Unités Internationales (UI) et doit être ajustée comme l'indique la Table 4A du manuel CLSI M100 S27 à partir des informations données par le fabricant (137). L'antibiotique est mis en solution dans de l'eau stérile puis dilué dans du milieu MH2 préalablement préparé avant d'être distribué dans les microplaques.

L'inoculum bactérien est de 5×10^5 UFC/mL (Unités Formant des Colonies) ou 5×10^4 UFC par puits, préparé à partir de l'inoculum standard de 0,5 MacFarland (correspondant à environ $1,5 \times 10^8$ UFC/mL). Les microplaques ensemencées sont incubées 18h à 37°C.

La lecture peut-être visuelle (voir Figure 5 : le trouble est le témoin de croissance), et facilitée à l'aide d'un indicateur coloré, ou par absorbance à l'aide d'un spectrophotomètre.

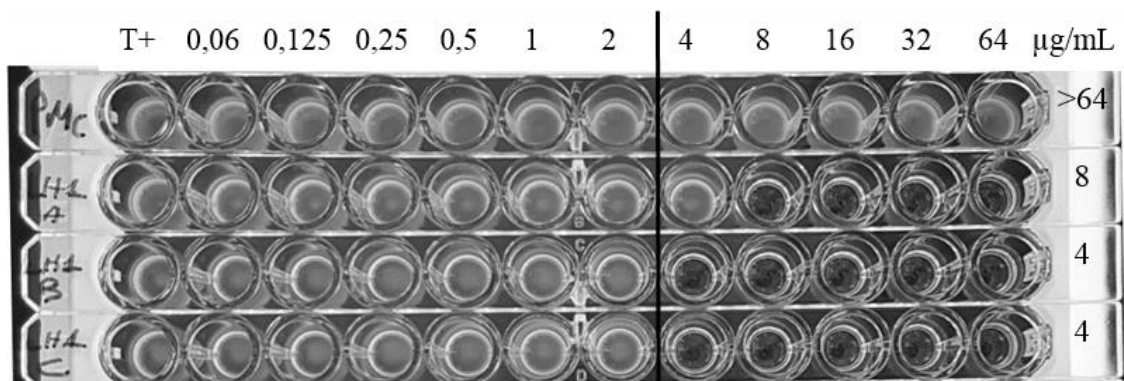


Figure 5. Exemple de microdilution en milieu liquide. Chaque barrette représente une souche dont la CMI est indiquée à droite. Le trait représente la CMI critique.

La BMD est longue et difficile à mettre en place en laboratoire de microbiologie clinique (75, 76), avec de nombreuses étapes, et la détection de l'hétérorésistance y est limitée. Il est donc nécessaire de mettre au point des systèmes faciles d'utilisation pour déterminer la CMI de colistine.

b. Dispositifs commercialisés pour évaluer la sensibilité aux polymyxines

Des kits basés sur la microdilution liquide ont été mis au point afin de faciliter l'accès à cette méthode en proposant des systèmes prêts à l'emploi. Ils présentent l'avantage d'éviter les étapes de préparations du milieu MH2 et des dilutions d'antibiotique dans la plaque.

Le kit UMIC Colistine (Biocentric, Bandol, France) se présente sous forme de tests unitaires associant : des barrettes en polystyrène contenant 11 concentrations de colistine déshydratée, des flacons contenant 2 mL de MH2, une feuille de résultats et une Umic Box pour l'incubation. Il suffit de diluer l'inoculum standard de 0,5 Macfarland dans un flacon de MH2 fourni dont le contenu sera distribué dans les 12 puits de la barrette ensuite incubée à 37°C. Une évaluation a mis en évidence son efficacité sur *Acinetobacter baumannii* (139).

Le kit Micronaut MIC (MERLIN Diagnostika Bornheim-Hersel, Allemagne) constitue un système similaire. Le même fabricant propose le système Micronaut-S, une microplaque standard contenant différentes concentrations de plusieurs antibiotiques. Son inoculation peut être automatisée avec l'appareil MICRONAUT ASTroID qui permet d'identifier simultanément la colonie analysée par son dépôt sur une cible de MALDI-TOF.

Le système Sensititre (Thermo Fischer Scientific, Waltham, MA, USA) proposent des panels constitués de plusieurs antibiotiques et peuvent être automatisés avec l'inoculation, l'incubation et la lecture basée sur la fluorescence après 18 à 24h d'incubation.

Le kit SensiTest Colistin (Liofilchem, Roseto degli Abruzzi, Italie) se présente sous la forme d'une plaque proposant 4 tests de 7 dilutions, perdant ainsi l'avantage du test unitaire.

Les systèmes automatisés ont été développés afin de réduire les délais d'obtention des résultats en augmentant la sensibilité, par l'incubation et la lecture en temps réel, et également dans le but d'éviter les biais de manipulation (173). Cependant, en combinant plusieurs antibiotiques, le nombre de concentrations à tester est limitée, ainsi ils ne permettent pas d'accéder réellement à la CMI.

Le système MicroScan WalkAway (Beckman Coulter Diagnostic, Brea, CA, USA) doit être inoculé manuellement et n'est pas disponible pour la polymyxine B.

Le Vitek 2 (Biomérieux, Marcy l'Etoile, France) se compose de cartes de réactifs lyophilisés, et l'identification est donnée en simultané de l'antibiogramme par un algorithme.

Le système BD Phoenix™ (Becton-Dickinson, Sparks, MD, USA) proposant l'identification simultanée, dans des plaques spécifiques de 84 puits.

c. Évaluation des méthodes d'antibiogramme

Elle se fait par comparaison à la méthode de référence avec les critères énoncés dans la Table 4 et suivant la norme International Standard Organization (ISO) 20776-2.

Table 4. Critères pour l'évaluation comparative d'une méthode d'antibiogramme.

Critère de validation	CMI obtenues / référence	Souches	Seuil
Agrément Catégorique (AC)	Même catégorie clinique (S, I, R)	Toutes	≥ 90%
Agrément Essentiel (AE)	±1 dilution log ₂	Toutes	≥ 90%
Écart Très Majeur (ETM)	S au lieu de R	Résistantes	≤ 3%*
Écart Majeur (EM)	R au lieu de S	Sensibles	≤ 3%
Écart Mineur (EMi)	R ou S au lieu de I, ou l'inverse	Toutes	

* Dans le cas d'un nombre peu important d'isolats résistants, ETM >3% est acceptable si l'AE est important, l'exactitude est alors considérée comme acceptable.

La Table 5 propose un échantillon d'évaluations comparatives de ces méthodes. Jusqu'en 2013, la dilution en agar était une méthode de référence (21, 22, 140–150), et présente en général un bon agrément avec la méthode BMD (19, 21, 151, 152).

Les méthodes de diffusion en agar (disques ou bandelettes) ne doivent plus être utilisées car les nombreuses évaluations ont paradoxalement obtenu des zones d'inhibition plus grandes que les limites critiques, donnant des ETM (125, 153–155). Elles présentaient néanmoins l'avantage de faciliter la détection des souches hétérorésistantes avec la présence de colonies dans la zone d'inhibition (67, 68).

Les systèmes automatisés Vitek 2 et Phoenix ont été couramment utilisés pour la détection de la résistance à la colistine dans les études ayant dépisté des souches porteuses du gène *mcr-I*, bien que les évaluations aient révélé qu'elles n'étaient pas assez sensibles, avec de hauts niveaux d'ETM obtenus pour la colistine (140, 154–156). (Table 5). Pour le système Phoenix, la majorité des erreurs arrivant proches de la concentration critique (2 au lieu de 4 µg/mL) (125, 126, 156, 157). En revanche, le taux de bactéries faussement sensible n'était pas si élevé pour le Vitek 2 avec la polymyxine B (154). Dans la même étude, le Microscan semblait ne pas être assez spécifique avec 15,8% de faux-résistants (154).

Enfin, les kits de BMD semblaient dans l'ensemble donner de bons résultats, en particulier le système Sensititre (153, 154), et le kit UMIC Colistine qui donnait le moins d'erreurs bien qu'un plus faible agrément que les autres kits testés (153).

Table 5. Évaluations comparatives de méthodes d'antibiogramme menées en 2017.

Souches étudiées	% R	Méthodes	Concentrations (µg/mL)	AC ≥90%	AE ≥90%	EM ≤3%	ETM ≤3%
14 <i>E. coli</i>	48%	Sensititre SEMPA1	< 0,06 - ≥ 64	94,7	96	10,2	0
18 <i>K. pneumoniae</i>		Micronaut S	< 0,06 - ≥ 64	89,3	96	15,4	5,6
21 <i>P. aeruginosa</i>		Micronaut MIC	< 0,06 - ≥ 64	90,7	99	12,8	5,6
22 <i>Acinetobacter spp.</i>		Sensitest	0,25 - 16	89,3	88	17,9	2,8
(153)		UMIC	< 0,06 - ≥ 64	92	82	7,7	8,3
117 <i>A. baumannii</i>	24,8%	Vitek 2 ASTXN05	≤ 0,5 - ≥ 16	89,7	88,9	1,1	37,9
(156)		Phoenix NMIC-96	≤ 1 - ≥ 4	88,9	91,5	1,1	41,4
		Dilution en Agar	< 0,06 - ≥ 64	87,2	93,2	15,9	3,4
123 entérobactéries	67,5%	Phoenix NMIC-93	≤ 1 - ≥ 4	91,8	ND	0	12,1
15 <i>mcr-I</i> , 1 <i>mcr-2</i>		Rapid NP	NA	98,3	NA	2,5	1,2
(126)							
76 entérobactéries	32,9%	Vitek 2 N315	≤ 0,5 - ≥ 16	88,2	93,9	0	36
21 <i>mcr-I</i>		Sensititre GNX3F	≤ 0,5 - > 4	90,1	89,5	11,8	4
(154)		Etest	≤ 0,64 - ≥ 256	92,1	75	5,9	12
		Microscan NM44	≤ 2, 4, ≥ 4	88,2	NA	15,8	4
Même étude avec la Polymyxine B	35%	Vitek 2	≤ 0,5 - ≥ 16	94,7	96,1	6,1	3,7
		Sensititre	≤ 0,5 - > 4	94,7	96,1	6,1	3,7
		Etest	≤ 0,64 - ≥ 1024	89,5	48,7	1,9	26,1

ND : Non déterminée, NA : Non Applicable.

3) Biologie moléculaire

a. Amplification de l'ADN

La PCR est la technique la plus sensible pour dépister la présence d'un gène ou encore la mutation d'un gène conférant la résistance à un antibiotique et est complémentaire des méthodes phénotypiques en confirmant le statut résistant de l'isolat. Son utilisation est limitée devant le nombre de gènes impliqués dans la résistance aux polymyxines et le fait que la résistance soit due à une mutation de ces gènes constitutifs, car ils doivent tous être amplifiés par PCR standard puis séquencés pour détecter une mutation.

La découverte du gène *mcr-1* justifie l'utilisation de la biologie moléculaire pour le dépistage en routine, car les gènes *mcr* sont acquis et peuvent être détectés par PCR en temps réel, une méthode rapide et quantitative. Ainsi, de nombreux systèmes ont été développés pour détecter rapidement la présence du gène *mcr-1* en créant des couples d'amorces pour PCR standard (58, 158–163), mais également pour qPCR (7, 63, 77, 83, 85, 89, 164–168), ainsi que pour le gène *mcr-2* (5, 91, 169–171). De manière intéressante, deux études ont développé des amorces universelles pour détecter à la fois *mcr-1* and *mcr-2* par PCR standard (169) et qPCR (79). Un seul système de PCR standard pour *mcr-3* (8) et *mcr-4* (9) existe à ce jour.

Des amorces spécifiques ont également été mises au point pour détecter la présence en amont du gène *mcr-1* de l'insertion de séquence IS*AplI* du transposon (172–174). D'autres ont également développé des amorces pour regarder directement dans le plasmide IncX4 portant les gènes *mcr* (7, 175, 176).

b. Développement de méthodes de détection rapides

Le kit Eazyplex SuperBug (Amplex Biosystems GmbH, Giessen, Allemagne) a été développé pour attester rapidement de la présence du gène *mcr-1* par amplification isothermique à médiation en boucle (177). Il peut également détecter le variant *mcr-1.2* mais pas le gène *mcr-2*. Il n'a pas été testé directement sur des échantillons cliniques.

Le système de puces à ADN Check-MDR CT103 (Check-points, Wageningen, The Netherlands) a été évalué pour la détection des gènes *mcr* : la sensibilité et la spécificité étaient excellentes pour *mcr-1* et ses variants (*mcr-1.2* à *mcr-1.7*) et *mcr-2*, mais n'était pas apte à détecter le gène *mcr-3* (178).

c. Nouvelle approche avec l'analyse directe du resistome

Le dépistage génomique représente une approche alternative pour étudier la résistance et permettre une meilleure compréhension du comportement des isolats bactériens (179). Le développement du séquençage de nouvelle génération (NGS), a conduit à une baisse des coûts et une réduction du délai de dépistage avec l'augmentation de la vitesse de séquençage associée à des bases de données à jour donnant accès à une quantité importante d'informations. Le gène *mcr-1* a été découvert par séquençage entier du génome durant un programme de surveillance active du bétail en Chine (4). Un nombre conséquent d'études rétrospectives analysant des séquences génomiques précédemment enregistrées ont été menées depuis, montrant la dispersion mondiale du gène (45, 56, 60, 63, 129, 180, 181).

Les technologies utilisées pour le séquençage complet du génome bactérien sont Illumina (Illumina Inc., San Diego, CA, USA) qui produit des séquences courtes (300 pb) et nécessite plusieurs jours, et le séquençage d'une seule molécule en temps réel PacBio RS II (Pacific Bioscience, Manlo Park, CA, USA), produisant des séquences longues (60kb) en quelques heures (182). L'utilisation des séquenceurs Illumina n'est pas adaptée pour couvrir les génomes bactériens présentant de multiples éléments répétitifs car un trop grand nombre de morceaux de séquences est obtenu après assemblage, alors que PacBio RS II permet d'obtenir une seule séquence sans région manquante (55).

Les séquences obtenues sont assemblées, puis le génome est annoté et un mappage est effectué contre un plasmide de référence, en général pHNSHP45 pour *mcr-1* (4). Les séquences alignées sont ensuite comparées à l'une des bases de données de gènes de résistance : Antibiotic Resistance Gene-Annotation (ARG-ANNOT) (183), ResFinder (184), Comprehensive Antibiotic Resistance Database (185) et Antibiotic Resistance Genes Database (186), ou de génome de plasmide: GenEpid-J (187, 188) et PlasmidFinder qui a permis de découvrir le gène *mcr-4* (9).

Le séquençage du génome entier associé à de nouveaux outils bio-informatiques nous permet d'améliorer notre capacité à détecter plusieurs gènes de résistance en même temps (180, 182) mais présente les mêmes limites que la PCR : l'apparition de nouveaux gènes n'est pas reconnue par ces techniques qui nécessitent une mise à jour continue des bases de données (56, 189), qui devraient être fusionnées en une unique référence (189). En effet, la présence d'un gène de résistance dans le génome de la souche analysée ne signifie pas que l'isolat est résistant et certaines études ont identifié des bactéries porteuses du gène *mcr-1* mais de phénotype sensible aux polymyxines (41, 99, 194). Ces études *in silico* sont complémentaires des méthodes phénotypiques (190), il est donc important de les réaliser en parallèle.

La Table 6 résume les capacités des méthodes précédemment citées à détecter la résistance aux polymyxines, et plus particulièrement des gènes *mcr* et l'hétérorésistance, en reprenant leur principe et en indiquant la durée de chaque test et s'il donne la CMI.

Table 6. Comparaison des méthodes de détection de la résistance aux polymyxines.

Méthodes	Principe	Durée	M/A	Détection de :			
				ColR	<i>Mcr</i>	HR	CMI
Tests phénotypiques							
Milieux sélectifs	Croissance sélective	18-24h	M	+	-	+	-
Colispot	Inhibition de la croissance	18-24h	M	+	-	ND	-
Rapid NP polymyxin	Changement de pH	4h	M	+	-	-	-
Micromax	Détection de la lyse de la paroi par fluorescence	3h	M/A	+	-	-	-
MALDI-TOF MS	Détection de pics spécifiques	1h	A	+	+	-	-
Colistin MAC	Chélation par DPA	18-24h	M	+	+	±	+
Antibiogramme							
BMD (UMIC, Micronaut-MIC, Sensitest, Micronaut-S, Sensititre)	Inhibition de la croissance	18-24h	M/A	+	-	±	+
Diffusion en agar : Disques Bandelettes	Mesure de la zone d'inhibition de croissance	18-24h	M		-	-	+
Dilution en agar	Inhibition de la croissance	18-24h	M	-	-	+	+
Systèmes automatisés	Détection de la croissance:						
Microscan	Fluorimétrie	3.5-7h	A	+	-	-	-
Vitek 2	Algorithme	4-10h	A	+	-	-	-
Phoenix	Oxydoréduction	6-16h	A	+	-	-	-
Biologie moléculaire :							
PCR standard	Amplification	3h	A	+	+	-	-
PCR en temps réel	Amplification	1h	A	+	+	-	-
Eazyplex	Amplification	20 min	A	+	-	-	-
Puces à ADN	Hybridation d'ADN	6.5h	A	+	-	-	-
NGS	Séquençage de l'ADN						
Illumina	entier	4-56h	A	+	+	-	-
PacBio RS II		0.5-3h	A	+	+	-	-

ColR : Résistance à la colistine HR : Hétérorésistance (+) Oui (-) Non (±) Parfois

IV. OBJECTIFS

Mes travaux de thèse ont porté sur le développement, la mise au point et l'évaluation d'outils de diagnostic rapide adaptés à l'émergence de souches porteuses du gène *mcr-1*. J'ai dans un premier temps effectué une mise à jour des outils disponibles pour la détection de la résistance à la colistine, sous la forme d'une revue incluant également les nouveaux outils développés spécifiquement pour la détection des gènes de résistance plasmidiques, dont la première partie de ce manuscrit est extrait.

Mes travaux de recherche ont conduit au développement d'un milieu de culture spécifique, le LBJMR, permettant de répondre à deux problématiques actuelles concernant l'émergence de bactéries multi-résistantes en permettant l'isolement à la fois des bactéries à Gram négatifs résistants à la colistine et des bactéries à Gram positifs résistants à la vancomycine. Ainsi deux souches résistantes à la colistine, *Escherichia coli* et *Klebsiella pneumoniae*, et une souche d'*Enterococcus faecium* résistant à la vancomycine ont pu être isolées à partir d'un même prélèvement clinique. Ce projet a donné lieu à un dépôt de brevet.

Dans un deuxième temps, j'ai mené une évaluation comparative du kit UMIC Colistine développé afin de faciliter l'accès à la méthode d'antibiogramme de référence pour la colistine, à savoir la microdilution en milieu liquide, qui est fastidieuse à mettre en place en laboratoire de microbiologie clinique. Cette étude a montré l'efficacité de ce test et sa fiabilité pour être utilisé au laboratoire de l'hôpital de la Timone à Marseille. J'ai ensuite participé à son développement industriel au sein de l'entreprise Biocentric.

Enfin, au cours de mes travaux, j'ai pu isoler une *K. pneumoniae* hétérorésistante à la colistine à partir d'un échantillon clinique, et étudié son mécanisme de résistance. Une nouvelle mutation du gène *mgrB* a pu être décrite dans la sous-population résistante et les méthodes de typage ont démontré qu'il s'agissait d'une hétérorésistance.

En parallèle de mon travail sur la résistance à la colistine, j'ai participé à la description phénotypique et génomique d'une nouvelle espèce bactérienne, *Pseudomonas massiliensis*.

V. DÉVELOPPEMENT DE NOUVEAUX OUTILS DE DÉTECTION

1) Mise au point du milieu de culture LBJMR

LBJMR medium: a new polyvalent culture medium for isolating and selecting vancomycin and colistin-resistant bacteria. Lucie Bardet*, Stéphanie Le Page*, Thongpan Leangapichart, Jean-Marc Rolain# Soumis à BMC Microbiology (IF = 2.58)

Le dépistage au niveau mondial de la résistance à la colistine portée par un plasmide a mis en évidence le manque de sensibilité du dépistage actuel. La culture est la technique utilisée en premier lieu pour isoler des souches cliniques, or une revue de la littérature sur les milieux existant comportant des polymyxines a permis d'établir que les milieux commerciaux existant ne sont pas adaptés à la détection de la résistance à la colistine de bas niveau conférée par le gène *mcr-1*.

Le milieu LBJMR a été créé afin de répondre à cette problématique, et en même temps de permettre l'isolement d'autres souches d'intérêt clinique : les entérocoques résistants à la vancomycine. J'ai donc procédé au développement et à l'évaluation du milieu LBJMR sur un nombre conséquent de souches cliniques, incluant notre collection de souches porteuses du gène *mcr-1*. Sa capacité de détection de toutes les souches résistantes à la colistine a également été comparée au milieu SuperPolymyxin récemment développé dans ce but. La sensibilité des deux milieux était de 100% pour la détection des entérobactéries résistantes à la colistine, y compris celles portant le gène *mcr-1*. En revanche, le milieu LBJMR a montré une meilleure sensibilité sur les souches non-fermentaires généralement détectée dans les prélèvements de patients atteints de la mucoviscidose et permet l'isolement spécifique des souches à Gram positif résistantes à la vancomycine.

Dans un second temps, le milieu LBJMR a été évalué comme outil de dépistage sur des échantillons cliniques préalablement dépistés par RT-PCR pour le gène *mcr-1*, ce qui a permis de détecter une vingtaine de nouvelles souches parmi des échantillons de selles humaines issues de différentes régions du monde.

Le milieu LBJMR est donc un outil adapté aux problématiques actuelles et pourrait être utilisé en laboratoire de microbiologie clinique. Sa composition et son utilisation ont donné lieu à un dépôt de brevet.

LBJMR medium: a new polyvalent culture medium for isolating and selecting vancomycin and colistin-resistant bacteria

Running title: LBJMR medium for multi-drug resistant bacteria

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Abstract

Background:

Multi-drug resistant bacteria are a phenomenon which is on the increase around the world, particularly with the emergence of colistin-resistant *Enterobacteriaceae* and vancomycin-resistant enterococci strains. The recent discovery of a plasmid-mediated colistin resistance and the description of the transferable *mcr-1* gene, raised concerns about the need for an efficient detection method for these pathogens, to isolate infected patients as early as possible. The LBJMR medium was developed to screen for all polymyxin-resistant Gram-negative bacteria, including *mcr-1* positive isolates, and vancomycin-resistant Gram-positive bacteria.

Results:

The LBJMR medium was developed by adding colistin sulfate salt at a low concentration (4 µg/mL) and vancomycin (50 µg/mL), with glucose (7.5 g/L) as a fermentative substrate, to a Purple Agar Base (31 g/L). A total of 143 bacterial strains were used to evaluate this universal culture medium, and the sensitivity and specificity of detection were 100% for the growth of resistant strains. 68 stool samples were cultured on LBJMR, and both colistin-resistant Gram-negative and vancomycin-resistant Gram-positive strains were specifically detected.

Conclusions:

The LBJMR medium is a multipurpose selective medium which makes it possible to identify bacteria of interest from clinical samples and to isolate contaminated patients in hospital settings. This is a simple medium that could be easily used for screening in clinical microbiology laboratories.

Key-word: colistin, culture media, *mcr-1*, vancomycin-resistant enterococci, *Enterobacteriaceae*, multi-drug resistant, detection method, screening.

Background

The worldwide emergence of multidrug-resistant (MDR) bacteria represents a major public health issue. Controlling the spread of these bacteria relies upon both reducing the prescription of antibiotics and preventing transmission from carrier patients to others ¹. More specifically, this prevention targets the emerging carbapenemase-producing *Enterobacteriaceae* and vancomycin-resistant enterococci (VRE) strains, with the development of specific detection methods, mostly based on chromogenic and selective culture media ².

The increase in infections due to carbapenemase-producing *Enterobacteriaceae* has led to the revival of colistin as a last-resort treatment ³. Its widespread use, particularly illustrated with the livestock food in many countries ⁴, has inevitably led to the emergence of colistin-resistant strains over the past ten years ^{5,6}. Until recently, all colistin resistance mechanisms which had been described were attributed to chromosomal mutations ⁷. In China in 2015, Liu and colleagues were the first to report the plasmid-mediated colistin-resistance gene in animals and humans, which they named *mcr-1* ⁸. This was followed by descriptions of the variants *mcr-1.2* and *mcr-1.3* ^{9,10}, *mcr-2* gene ¹¹ and, more recently, the *mcr-3* gene ¹². A significant number of human cases (in the majority of cases asymptomatic carriers) was described ^{13,14}, as were cases of strains with a previously unknown colistin resistance mechanism that were found to harbor the *mcr-1* gene ¹⁵⁻¹⁷. This mobile colistin resistance element presents low resistance levels, with minimal inhibitory concentrations (MIC) of colistin around 4 µg/ml, which is close to the breakpoint of colistin resistance (> 2 µg/mL), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) ¹⁸. Those data raised concerns about the detection and isolation of these pathogens, since the presence of the colistin resistant *mcr-1* plasmid-mediated gene has been added to recommendations for clinical microbiology laboratories in France ¹⁹. While carbapenemase screening is perfectly controlled with phenotypic and molecular techniques ²⁰, there is a need for colistin-resistance screening tools ². Given the diversity of colistin-resistant mechanisms ²¹, phenotypic tests, such as the development of a chromogenic culture medium ^{2,22} are preferred for a rapid detection.

In addition to colistin-resistant *Enterobacteriaceae*, the detection of vancomycin-resistant enterococci isolates is of clinical concern. The prevalence of VRE strains is increasing in Europe, especially *Enterococcus faecium* ²³ and has led to nosocomial infections in the United States ²⁴, and their dissemination is associated with high mortality rates ²⁵. The development of an effective screening tool for the early detection of those multi-drug resistant

pathogens has become a priority, in order to adapt treatment and isolate patients who are either infected or carriers.

In this study, we developed a polyvalent selective culture medium which can isolate both colistin-resistant Gram-negative bacteria, including *Enterobacteriaceae* strains harboring the *mcr-1* gene, and vancomycin-resistant Gram-positive bacteria. This universal culture medium was named LBJMR, standing for Lucie Bardet-Jean-Marc Rolain, and has recently been patented ²⁶.

Materials and methods

Bacterial strains and samples cultured on LBJMR

A total of 143 bacterial strains were used in this study, of which 101 were *Enterobacteriaceae*: 75 with an acquired mechanism of resistance to colistin (including 32 strains harboring the *mcr-1* gene ^{6,17,27–35} and 43 to another resistance mechanism), six with an intrinsic colistin resistance mechanism ³⁶, and 20 colistin-susceptible strains (Table S1). This set also comprised 17 non-fermentative Gram-negative bacteria: ten were colistin-resistant strains ³⁷ including one *Pseudomonas aeruginosa*, one *Acinetobacter baumannii* ³⁷ and other pathogens that are frequently diagnosed in samples from patients with cystic fibrosis, and seven colistin-susceptible strains ^{38,39}. Finally, 25 Gram-positive strains, including eight vancomycin-resistant enterococci ⁴⁰, three intrinsically vancomycin-resistant genera and 14 vancomycin-susceptible strains ⁴¹ were also included in this study (Table S1).

The colistin-resistance genes which were potentially involved were previously screened for *Enterobacteriaceae* ^{6,42}, as were the vancomycin resistance genes *vanA* and *vanB* for the VRE strains ⁴³. MICs of colistin and vancomycin were determined using E-test[®] strips (BioMérieux, Marcy l'Étoile, France) for Gram-negative and Gram-positive strains, respectively, and MICs of daptomycin were determined for the four reference strains of *Enterococcus faecium* (DSM 17050, 25698, 25697 and 13590), and were evaluated according to the EUCAST guidelines ¹⁸.

In addition, 68 samples were cultivated on LBJMR medium, including 66 stool samples (56 from humans and ten from chickens) that were previously screened for *mcr-1* by RT-PCR (56 positive and ten negative) and two clinical rectal swabs obtained from patients in the La Timone hospital, from which a vancomycin-resistant *E. faecium* VRE strain was isolated. Colonies with different morphologies isolated by culture of samples on LBJMR were isolated by subculture on Trypticase Soy Agar (TSA, Becton-Dickinson, Heidelberg,

Germany), and the species were identified using Matrix-Assisted Laser Desorption-Ionization-Time Of Flight Mass Spectrometry (MALDI-TOF MS), as described previously ⁴⁴.

LBJMR development

In order to choose the most suitable medium for selecting colistin-resistant *Enterobacteriaceae*, including strains harboring the *mcr-1* gene, five commercial chromogenic media usually used for the detection of *Enterobacteriaceae* were supplemented with a range of concentration of colistin sulfate salt (MP Biomedicals, Illkirch, France) from 0 to 32 µg/mL: Drigalski Lactose Agar (Biokar diagnostics, France), BBL™ Eosin Methylene Blue Agar (EMB; Becton-Dickinson, Heidelberg, Germany), Difco™ Violet Red Bile Agar (Becton-Dickinson, Heidelberg, Germany), BBL™ MacConkey Agar II (Becton-Dickinson, Heidelberg, Germany), and Difco™ Purple Agar Base (Becton-Dickinson, Heidelberg, Germany) supplemented with 7.5 g /L of glucose (MP Biomedicals, Illkirch, France). Four *Klebsiella pneumoniae*, including two colistin-resistant, and four *Escherichia coli*, including two which were colistin-resistant with the *mcr-1* gene, were inoculated at 10⁶ CFU on each prepared agar ¹⁷.

The Purple Agar Base supplemented with glucose (7.5 g/L), colistin sulfate (0, 4 and 8 µg/mL) and vancomycin (0 and 50 µg/mL) (Sandoz, Levallois-Perret, France) was evaluated with 24 representative strains, including 20 *Enterobacteriaceae* (five intrinsic genera, eight with acquired resistance and seven colistin-susceptible strains as control) and four Gram-positive strains susceptible to vancomycin, which were inoculated at 10⁶ CFU. This experiment was also conducted by replacing glucose with lactose (Laboratoires Humeau, La Chapelle-sur-Erdre, France). The concentration of vancomycin was then determined using 20 Gram-positive strains representative of vancomycin resistance (three intrinsic genera and 17 enterococci, including seven VRE) and ten chicken stool samples from Algeria, five of which were positive for *mcr-1* by RT-PCR, and five of which were negative ^{17,42}.

LBJMR Evaluation

The sensitivity and specificity of the LBJMR medium to detect all colistin-resistant Gram-negative bacteria and all vancomycin-resistant Gram-positive bacteria were evaluated with all the strains listed in Table S1 and samples listed in Table S2, which were incubated on LBJMR in an aerobic atmosphere at 37°C for between 24 and 48 hours.

Inocula of the strains were prepared at 0.5 McFarland (corresponding to 1,5x10⁸ CFU/mL according to the EUCAST expert system) and serial 10-fold dilutions were then

performed in Phosphate Buffer Saline. 10 µL of these suspensions were deposited on agars and simultaneously on TSA medium to determine the detection limits for each bacterium by assessing the viable concentration by colony count. Growth or inhibition of these microorganisms was observed after overnight incubation at 37°C in an aerobic atmosphere. Those experiments were performed in duplicate.

Finally, as the LBJMR medium was developed so as to avoid *Proteus* swarming, *Proteus mirabilis* and *Proteus vulgaris* strains were inoculated alone or mixed with *E. coli* P10 (*mcr-1*) at the same concentration to evaluate the isolation ability of the LBJMR medium. Cultures were incubated at 37°C for 72 hours.

Mcr-1 screening on stool samples

A total of 1,052 stool samples were used in this study, 899 from humans from different regions of the world: Marseille (n=212) and Angers (n=128) in France, Thailand (n=212), Laos (n=189), Nigeria (n=143), and Senegal (n=15). 153 samples were from animals, including chickens from Algeria (n=10), rodents from Cambodia (n=21), and rodents (n=60), pigs (n=16) and goats (n=46) from Laos.

The presence of the *mcr-1* gene in those stools was first screened using RT-PCR as previously described⁴². Briefly, the DNA of the stools was extracted, after pre-treatment by proteinase K and incubation at 95°C, using Biorobot EZ1 Advanced XL (Qiagen, Hilden, Germany). Positive DNA was confirmed using standard PCR. Samples with positive DNA were then cultured on LBJMR, after a prior enrichment step performed by inoculating approximately 1 g of the sample in tryptic soy broth (TSB) medium (BioMérieux, Marcy l'Étoile, France) and incubating for 24 hours at 37°C. 100 µL of these liquid media were then plated on agars that were also incubated at 37°C for 24 hours.

For each Gram-negative isolate identified by MALDI-TOF MS, colistin MIC was determined with a colistin E-test[®] strip. For each colistin-resistant isolate, the presence of *mcr-1* was investigated by RT-PCR, as previously described⁴². Susceptibility testing to 26 antibiotics was performed on *mcr-1*-positive strains using the disk diffusion method, and interpreted according to the EUCAST guidelines¹⁸.

Comparison of the LBJMR medium with other polymyxin-containing media

The sensitivity and specificity of the LBJMR medium was first compared to the BD[™] Cepacia Medium (Cepacia, Becton-Dickinson, Heidelberg, Germany) medium with all the colistin-resistant *Enterobacteriaceae* strains listed in Table S1. The LBJMR medium was then

compared to the SuperPolymyxin medium with 103 bacterial strains, including 61 of the *Enterobacteriaceae*, 17 non-fermentative Gram-negative, and 25 Gram-positive strains (Table S1). Other polymyxin-containing media were also compared concomitantly to the LBJMR medium, using EMB Agar (Sigma-Aldrich, Illkirch, Germany) as a control, with some of these strains as described below. Inocula were prepared following the same protocol as for the LBJMR evaluation.

Columbia Colistin Nalidixic Acid Agar + 5% sheep blood (CNA) (BioMérieux, Marcy l'Étoile, France) and three mixed culture media: EMB with colistin sulfate (4µg/mL) and vancomycin (50 µg/mL), LBJMR with Daptomycin (10 µg/ml, Novartis, Horsham, United Kingdom) in place of vancomycin and LBJMR with Amphotericin B (5 µg/ml, Bristol-Myers Squibb, Rueil-Malmaison, France) were concomitantly tested with 45 *Enterobacteriaceae* strains representative of colistin resistance (30 *mcr-1*, ten susceptible and five intrinsically resistant genera). LBJMR with Amphotericin B was also evaluated with the eight VRE strains. Finally, the LBJMR medium was compared to the SuperPolymyxin medium with 12 samples, including the two clinical rectal swabs from the La Timone hospital and ten human stools from Thailand, five of which had been identified as positive by culture for the detection of an isolate harboring the *mcr-1* gene, and five controls that were negative for *mcr-1* using RT-PCR.

Statistical analysis

The data were analyzed using a Chi-test to compare the Cepacia and LBJMR media and a Student-t test for a pairwise comparison of SuperPolymyxin and LBJMR selective media for the detection of non-fermentative Gram-negative colistin-resistant strains. Significance was assessed at $p < 0.05$.

Results

LBJMR development

Because colistin is a cationic molecule, we looked for a medium which was deprived of any ions in its original composition and which was also deprived of any electrolytes to avoid *Proteus* spp. swarming. In addition, the optimal conditions for LBJMR medium (growth of colistin- and vancomycin-resistant strains and inhibition of colistin- or vancomycin susceptible strains) were obtained on Purple Agar Base with glucose (Table S3), 4 µg/mL of colistin sulfate and 50 µg/ml of vancomycin (Table S4). The culture of ten chicken stools from Algeria enabled the detection of *E. coli* strains harboring the *mcr-1* gene

from three of the five samples which were positive for *mcr-1* by RT-PCR, including strain 235 which had previously been isolated on Cepacia medium¹⁷ and no Gram-negative strains were detected from the five negative samples for which (Table S2). A correlation was observed between the Ct-values that reflect the DNA concentration and the results of culture, depending on bacterial concentration (Table S2).

The final preparation of the LBJMR medium was as follows: 31 g/L of Purple Agar Base, 7.5 g/L of glucose, 4 µg/mL of colistin sulfate and 50 µg/mL of vancomycin. 7.5 g/L of lactose was used in place of glucose for the 24 strains initially tested and the same results were obtained. Glucose was selected because, combined with bromocresol purple, a pH indicator, it revealed the polyvalent capacity of the LBJMR medium with an easy and fast visualization of the species of interest in the clinical routine: both *Enterobacteriaceae* and enterococci gave yellow colonies, contrasting on the purple agar and exhibiting different sizes (2-3 mm and 0.1-1 mm, respectively).

LBJMR evaluation

All the polymyxin-resistant *Enterobacteriaceae* strains (n= 81) tested were able to grow on the LBJMR medium, with the lowest detection limit (10^1 CFU), including those with the *mcr-1* gene, as well as all the colistin-resistant non-fermentative Gram-negative (n= 10) and vancomycin-resistant Gram-positive strains (n= 11), with detection limits dependent on their MIC, as shown in **Table 1**. Meanwhile, all the bacteria susceptible to colistin or vancomycin were inhibited (not detected at 10^5 CFU), giving rise to 100% sensitivity and specificity for the growth of Gram-negative colistin-resistant and Gram-positive vancomycin-resistant strains (Table 1). The swarming of *Proteus* sp. strains was fully inhibited on LBJMR, even after 48 hours of incubation.

Of the 1,052 stools screened for *mcr-1* by RT-PCR, 68 were cultured on LBJMR, including 56 that were positive after 32 cycles of RT-PCR, as well as ten negative stool samples as controls. 16 cultures were positive on LBJMR medium, enabling the detection of 17 colistin-resistant *Enterobacteriaceae* strains harboring the *mcr-1* gene (colistin MICs ranging from 2 to 8 µg/mL): 15 were identified as *E. coli* and two as *K. pneumoniae* by MALDI-TOF MS (Table S2). Indeed, two bacterial species, *E. coli* and *K. pneumoniae*, were detected from the culture of the same sample (FHM128) (Table S2). Five isolates were already known from a previous study on Cepacia medium^{6,17}. All the *E. coli* isolates from France were Extended-Spectrum Beta-Lactamase-producing strains (data not shown). In conclusion, the LBJMR medium enabled the isolation of new colistin-resistant strains

harboring the *mcr-1* gene. The weak culture results compared to PCR could be explained by the length of storage of the tested samples (up to four years) at -80°C, possibly inducing the death of bacteria while their DNA was still detectable⁴⁵. However, it should be noted that, for some samples, we isolated another kind of strain, mostly Gram-positive bacteria which have an intrinsic resistance to vancomycin, largely *Pediococcus pentosaceus* and *Weissella cibaria*, as shown in Table S2.

Finally, the culture of two clinical samples from the hospital enabled the isolation of two vancomycin-resistant *E. faecium* isolates (VRE1 and VRE2). In addition, two colistin-resistant *Enterobacteriaceae* isolates were also detected in the VRE2 sample, identified as *K. pneumoniae* LB3 (MIC = 64 µg/mL) and *E. coli* LB4 (MIC= 16 µg/mL), both negative for the *mcr-1* gene (Figure 1).

Comparison between LBJMR and other selective polymyxin-containing media

The LBJMR medium was more sensitive than the Cepacia and CNA media for detecting the strains exhibiting the *mcr-1* gene (Table S5). Indeed, only four of those strains could grow on the CNA medium, because it also contains nalidixic acid and fewer than half of them grew on the Cepacia medium. Comparison of the sensitivities of the LBJMR (100%) and Cepacia (47 %) media using a Chi-square statistical test gave a significant *p*-value (<10⁻⁴).

LBJMR and SuperPolymyxin media presented the same sensitivity for *Enterobacteriaceae*, with the growth of all colistin-resistant strains (detection limit of 10¹ CFU), including the 30 *mcr-1* strains tested, and the inhibition of all susceptible strains (Table 1). In addition, the culture of ten human stools gave the same results: five were positive, with the detection of an *E. coli* positive for *mcr-1*, and the five controls remained negative (Table S2). In contrast, the LBJMR medium showed a significantly higher sensitivity than the SuperPolymyxin medium for the detection of colistin-resistant non-fermentative Gram-negative strains, with a significant *p*-value of 0.019 (<0.01) with a pairwise comparison using a Student-t test (Table 1). More specifically, the LBJMR medium was able to detect those screened in the cystic fibrosis samples, such as *Burkholderia cepacia*, even at low concentrations, and could replace specific media such as the Cepacia medium for their isolation. The addition of amphotericin B or the replacement of vancomycin by daptomycin in the LBJMR medium did not affect the growth of *Enterobacteriaceae* strains (Table S5). Finally, all the VRE strains were inhibited on the SuperPolymyxin medium because of the presence of daptomycin, while they were detected on the LBJMR medium (Table 1).

These results were confirmed by the culture of the two clinical samples from the hospital: vancomycin-resistant *E. faecium* could only grow on the LBJMR medium and not on the SuperPolymyxin medium for both samples, and the two *Enterobacteriaceae* from the VRE2 sample found on the LBJMR medium were also detected on the SuperPolymyxin medium.

Finally, the concomitant addition of colistin and vancomycin to EMB agar showed the systematic inhibition of *E. coli* strains harboring the *mcr-1* gene, corresponding to the tested strains with the lowest MIC.

Discussion

In accordance with recommendations to isolate patients carrying multi-drug resistant bacteria, it is becoming essential to detect colistin-resistant Gram-negative pathogens, particularly with the recent description of plasmid-mediated colistin-resistant genes among *Enterobacteriaceae* strains⁸⁻¹². We do not currently know the potential consequences of patient colonization by those resistant bacteria, as it has already been demonstrated that acquisition of resistance is associated with a decrease in virulence⁴⁶⁻⁴⁸, but it is necessary to implement efficient tools to prevent and monitor as closely as possible any epidemic outbreak and develop a rapid therapeutic strategy.

Currently, the only available method for detecting colistin resistance, according to joint EUCAST and Clinical Laboratory Standard Institute (CLSI) expert systems, is antibiotic susceptibility testing using broth microdilution, which is not suitable for daily screening in clinical microbiology laboratories. Current polymyxin-containing culture media are also not appropriate because of their high concentration of polymyxin. Indeed, they were not developed to isolate *Enterobacteriaceae* but to specifically detect bacteria that are intrinsically resistant to polymyxins, avoiding contaminants such as *Enterobacteriaceae* species.

Given the worldwide screening of low-level *mcr-1* and transferable colistin-resistance genes, the development of a reliable culture medium is necessary.

The significant number of strains tested on our selective medium showed that the LBJMR medium allows all colistin-resistant *Enterobacteriaceae* strains to grow, even those with a very low MIC for colistin and harboring the *mcr-1* gene, as well as all the vancomycin-resistant enterococci strains, when all the susceptible strains were inhibited. Furthermore, the screening conducted on stool samples that were positive for *mcr-1* by PCR, allowed the isolation of new *mcr-1* strains (Table S2). Finally, LBJMR could also detect

colistin-resistant non-fermentative Gram-negative bacteria, including pathogens which are often found in patients suffering from cystic fibrosis (Table 1). We thereby created a universal culture medium, able to replace specific culture media usually used in routine diagnosis.

The performances of the LBJMR medium are summarized in Table 2: samples can be cultured directly on the LBJMR selective medium, without a previous decontamination step, and colonies can be directly analyzed from the primary culture, without subcultures. Bacteria of interest are easily recognizable, as shown in Figure 1: both colistin-resistant *Enterobacteriaceae* and vancomycin-resistant enterococci exhibit yellow colonies (2-3mm and 0.1-1 mm respectively), on a purple agar base. Because LBJMR also permits the growth of intrinsic resistant bacteria, screening should be completed with a rapid identification using MALDI-TOF MS ⁴⁹, directly from the LBJMR medium. Antibiotic susceptibility testing and PCR screening can be performed in the same time.

Nordmann *et al.* developed a different selective medium called SuperPolymyxin, based on EMB agar and containing 3.5 µg/mL of colistin sulfate, 10 µg/mL of daptomycin and 5 µg/mL of amphotericin B ⁵⁰, associated with a complementary phenotypic test, the rapid polymyxin NP ⁵¹, to screen for colistin-resistant Gram-negative bacteria ⁵². These two media are chromogenic and are able to recognize bacterial species based on color. Sensitivities for colistin-resistant *Enterobacteriaceae* were the same for the SuperPolymyxin and LBJMR media, but only the LBJMR medium could detect VRE strains (Table 1). Indeed, the SuperPolymyxin medium is composed of daptomycin and amphotericin B, which prevents the growth of contaminants including bacteria of interest such as VRE strains, because they are mostly susceptible to daptomycin. In our study, we tested stool samples and obtained some contaminants, including Gram-positive bacteria which were intrinsically resistant to vancomycin (Table S2) or some yeasts.

The advantage of the LBJMR medium in terms of isolating vancomycin resistant enterococci can be also a disadvantage in a few cases, because Gram-positive bacteria which are intrinsically resistant to vancomycin bacteria, including *Pediococcus*, can also be isolated. In the LBJMR medium, Gram-positive bacteria appear with small colonies and a simple identification by MALDI-TOF can identify them. For the elimination of yeast in samples, amphotericin B can be added in our medium.

Conclusion

The LBJMR medium is an adequate screening tool for all colistin-resistant isolates from clinical samples, independently of their resistance level or mechanism. LBJMR could be used in routine laboratory work to detect colistin-resistant and vancomycin-resistant bacteria, allowing for the direct analysis of colonies and, thus, the early isolation of contaminated patients in hospital settings. This medium is currently being investigated as a routine medium in our institute to determine its usefulness in detecting such bacteria.

List of abbreviations

CNA: Colistin Nalidixic Acid

CLSI: Clinical Laboratory Standard Institute

EMB: Eosin-Methylene Blue

EUCAST: European Committee on Antimicrobial Susceptibility Testing

MALDI-TOF MS: Matrix-Assisted Laser Desorption-Ionization-Time Of Flight Mass Spectrometry

MIC: Minimum Inhibitory Concentration

TSA: Trypticase Soya Agar

VRE: Vancomycin-Resistant Enterococci

Declarations

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Consent for publication: Not applicable

Availability of data and material: Ok

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Authors' contributions: LB designed the study, performed the experiments and analyzed and interpreted the data. SLP was analyzed, interpreted the data and involved in revising the manuscript. TL contributed to the development and analysis of data. JMR was responsible for the conception and design of the study, and the analysis of data. All authors read and approved the final manuscript

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Table 1. Detection limits of targeted bacteria on LBJMR and SuperPolymyxin culture media. MIC of colistin (CT) and vancomycin (VCN) are given in $\mu\text{g/mL}$. *Enterobacteriaceae* with *mcr-1* gene are in bold font.

Bacterial strains		Detection limits on culture media (CFU):		
<i>Gram-negative</i>		CT MIC	LBJMR	SuperPolymyxin
<i>Colistin-resistant Enterobacteriaceae</i>	<i>E. coli</i> SE65	4	10^1	10^1
	<i>E. coli</i> 117R	4	10^1	10^1
	<i>E. coli</i> 1R	4	10^1	10^1
	<i>E. coli</i> 1R 2104	4	10^1	10^1
	<i>E. coli</i> 44A	4	10^1	10^1
	<i>E. coli</i> 6R	4	10^1	10^1
	<i>E. coli</i> 85R	4	10^1	10^1
	<i>E. coli</i> 95R	4	10^1	10^1
	<i>E. coli</i> 96R	4	10^1	10^1
	<i>E. coli</i> 134R	3	10^1	10^1
	<i>E. coli</i> 143R	3	10^1	10^1
	<i>E. coli</i> LH121	16	10^1	10^1
	<i>E. coli</i> LH140 ¹	12	10^1	10^1
	<i>E. coli</i> LH257	12	10^1	10^1
	<i>E. coli</i> LH57 ¹	8	10^1	10^1
	<i>E. coli</i> LH1	6	10^1	10^1
	<i>E. coli</i> LH30	6	10^1	10^1
	<i>E. coli</i> TH214	6	10^1	10^1
	<i>E. coli</i> TH99	4	10^1	10^1
	<i>E. coli</i> 235	4	10^1	10^1
	<i>E. coli</i> P6	6	10^1	10^1
	<i>E. coli</i> P10	4	10^1	10^1
	<i>E. coli</i> P17	4	10^1	10^1
	<i>E. coli</i> FHA102 ²	12	10^1	10^1
	<i>E. coli</i> FHM19 ³	12	10^1	10^1
	<i>E. coli</i> FHA113 ⁴	12	10^1	10^1
	<i>E. coli</i> NH94 ⁵	12	10^1	10^1
	<i>E. coli</i> TH176	6	10^1	10^1
	<i>K. pneumoniae</i> FHA60	8	10^1	10^1
	<i>K. pneumoniae</i> FHM128	4	10^1	10^1
	<i>K. pneumoniae</i> 119R	3	10^1	10^1
	<i>K. pneumoniae</i> LH131 ⁶	32	10^1	10^1
<i>K. pneumoniae</i> LH61 ⁷	24	10^1	10^1	
<i>K. pneumoniae</i> LH17	12	10^1	10^1	

	<i>K. pneumoniae</i> LH92	12	10 ¹	10 ¹
	<i>K. pneumoniae</i> LB1 ⁶	32	10 ¹	10 ¹
	<i>P. mirabilis</i> FH112	>256	10 ¹	10 ¹
	<i>P. vulgaris</i> PV148	>256	10 ¹	10 ¹
	<i>P. alcalifaciens</i> TH44	>256	10 ¹	10 ¹
	<i>M. morgani</i> FM102	>256	10 ¹	10 ¹
	<i>S. marcescens</i> E13	128	10 ¹	10 ¹
Others colistin-resistant	<i>B. cepacia</i> FHM-BC1	64	10 ³	10 ⁵
	<i>B. cepacia</i> FHM-BC2	>256	10 ¹	10 ¹
	<i>A. xylooxidans</i> FHM-AX	3	10 ¹	>10 ⁵
	<i>S. maltophilia</i> FHM-SM	12	10 ³	>10 ⁵
	<i>I. limosus</i> FHM-IL	>256	10 ⁴	10 ⁴
	<i>P. pulmonicola</i> FHM-PP	>256	10 ¹	10 ³
	<i>S. putrefaciens</i> FHM-SP	NC	10 ³	>10 ⁵
	<i>O. anthropi</i> FHM-OA	NC	10 ²	>10 ⁵
	<i>P. aeruginosa</i> FHM-PACOLR1	>256	10 ¹	10 ¹
	<i>A. baumannii</i> ABIsac_ColiR	8	10 ²	10 ¹
Colistin-susceptible	<i>E. asburiae</i> P113	0,19	>10 ⁵	>10 ⁵
	<i>E. cloacae</i> NH151	0,50	>10 ⁵	>10 ⁵
	<i>E. cloacae</i> NH74	0,38	>10 ⁵	>10 ⁵
	<i>K. pneumoniae</i> CIP 82.91	0,125	>10 ⁵	>10 ⁵
	<i>K. pneumoniae</i> LB2	0,128	>10 ⁵	>10 ⁵
	<i>K. pneumoniae</i> TH20S	0,125	>10 ⁵	>10 ⁵
	<i>K. pneumoniae</i> TH28S	0,125	>10 ⁵	>10 ⁵
	<i>Proteus vulgaris</i> P100	0,94	>10 ⁵	>10 ⁵
	<i>S. enterica</i> 108R	1	>10 ⁵	>10 ⁵
	<i>S. enterica</i> 122R	0,5	>10 ⁵	>10 ⁵
	<i>E. coli</i> ATCC 25922 CIP 76.24	0,094	>10 ⁵	>10 ⁵
	<i>E. coli</i> 161	0,094	>10 ⁵	>10 ⁵
	<i>E. coli</i> 169	0,094	>10 ⁵	>10 ⁵
	<i>E. coli</i> FHM88S	0,125	>10 ⁵	>10 ⁵
	<i>E. coli</i> TH134S	0,094	>10 ⁵	>10 ⁵
	<i>E. coli</i> LH53S	0,094	>10 ⁵	>10 ⁵
	<i>E. coli</i> LH165S	0,074	>10 ⁵	>10 ⁵
	<i>E. coli</i> TH77S	0,064	>10 ⁵	>10 ⁵
	<i>E. coli</i> 282S	0,074	>10 ⁵	>10 ⁵
	<i>E. coli</i> FHM19S	0,125	>10 ⁵	>10 ⁵
	<i>P. aeruginosa</i> FHM-PA4	2	>10 ⁵	>10 ⁵
	<i>P. aeruginosa</i> FHM-PA5	0,50	>10 ⁵	>10 ⁵
<i>P. aeruginosa</i> FHM-PA6	0,38	>10 ⁵	>10 ⁵	

	<i>S. xianemensis</i> 111P	0,125	>10 ⁵	>10 ⁵
	<i>S. xianemensis</i> 111A	0,094	>10 ⁵	>10 ⁵
	<i>A. nosocomialis</i> ABG13S	0,064	>10 ⁵	>10 ⁵
	<i>A. pittii</i> MK	0,125	>10 ⁵	>10 ⁵
	Gram-positive	VCN MIC		
Vancomycin-resistant	<i>E. faecium</i> DSM17050 ^a	>256	10 ¹	>10 ⁵
	<i>E. faecium</i> DSM13590 ^a	>256	10 ¹	>10 ⁵
	<i>E. faecium</i> DSM25698 ^a	>256	10 ²	>10 ⁵
	<i>E. faecium</i> DSM25697 ^a	>256	10 ¹	>10 ⁵
	<i>E. faecium</i> FHMVRE1 ^a	>256	10 ¹	>10 ⁵
	<i>E. faecium</i> FHMVRE2 ^a	>256	10 ¹	>10 ⁵
	<i>E. faecium</i> VRE	24	10 ³	>10 ⁵
	<i>E. faecalis</i> JH2-2: Tn 1549 ^b	64	10 ²	>10 ⁵
	<i>W. cibaria</i> P18A	>256	10 ¹	>10 ⁵
	<i>W. cibaria</i> P18B	>256	10 ¹	>10 ⁵
	<i>L. lactis</i> P18C	>256	10 ¹	>10 ⁵
Vancomycin-susceptible	<i>S. aureus</i> CF_Marseille	1	>10 ⁵	>10 ⁵
	<i>E. faecium</i> TH26	0,75	>10 ⁵	>10 ⁵
	<i>E. faecium</i> 349	0,75	>10 ⁵	>10 ⁵
	<i>E. faecium</i> TH12	2	>10 ⁵	>10 ⁵
	<i>E. faecium</i> LH165	0,75	>10 ⁵	>10 ⁵
	<i>E. faecium</i> A17	0,75	>10 ⁵	>10 ⁵
	<i>E. faecium</i> TH43	0,75	>10 ⁵	>10 ⁵
	<i>E. faecium</i> TH95	0,75	>10 ⁵	>10 ⁵
	<i>E. faecium</i> 282	0,75	>10 ⁵	>10 ⁵
	<i>E. faecalis</i> JH2-2: C2	1,5	>10 ⁵	>10 ⁵
	<i>E. faecalis</i> JH2-2S	1,5	>10 ⁵	>10 ⁵
	<i>E. gallinarum</i> SE3	1,5	>10 ⁵	>10 ⁵
	<i>E. casseliflavus</i> SE3	1,5	>10 ⁵	>10 ⁵
	<i>E. hirae</i> LH111	1	>10 ⁵	>10 ⁵

Colistin-resistant strains with a mutation on another gene are indicated as follow: ¹*phoP* E375K, ²*pmrB* A159V, ³*pmrB* P7-Q12(del 6aa), ⁴*pmrB* T156K, ⁵*pmrB* I92 insertion, ⁶*mgrB* Stop, ⁷*mgrB* Sub, ⁸*pmrB* T157P. *Enterococci* strains with vancomycin-resistant genes are indicated as follows: ^a*vanA* and ^b*vanB*.

Table 2. Performance of LBJMR medium

Criteria	LBJMR medium
Isolates screened	Colistin-resistant Gram-negatives: <ul style="list-style-type: none">- <i>Enterobacteriaceae</i>, including those harboring the <i>mcr-1</i> gene- Non-fermentative Gram-negative colistin-resistant strains, including those involves in cystic fibrosis samples Vancomycin-resistant Gram positives, including Enterococci
Aspect of colonies	Yellow on purple agar: 2-3 mm for <i>Enterobacteriaceae</i> , 0.1-1 mm for Enterococci
Incubation	Aerobic atmosphere, 37°C, 24H (sterile at 48H)
Culture of samples	Direct on LBJMR, no previous decontamination
Isolates analysis	Colonies can be picked directly from primary cultures on LBJMR for analysis: <ul style="list-style-type: none">- MALDI-TOF identification- Antibiotic Susceptibility testing- PCR screening for resistance genes.
Avoid contamination	<ul style="list-style-type: none">- Inhibition of <i>Proteus</i> swarming- Inhibition of yeast possible by adding amphotericin B

Figure legend

Figure 1. Aspect of the different types of colonies on LBJMR after culture of a clinical sample. *Enterobacteriaceae* exhibited yellow colonies that are larger (2-3 mm) than enterococci (0.1-1 mm).

Supplementary data

Table S1. List of the studied strains. The MIC of colistin (CT) or vancomycin (VCN) are indicated in $\mu\text{g/mL}$.

Table S2. List of stools used and Gram-negative isolates detected on LBJMR. Positive isolates for *mcr-1* and their corresponding samples are in bold font. New isolates harboring *mcr-1* are underlined. Their MICs of colistin are indicated in $\mu\text{g/mL}$. (-) Negative (ND) refers to Not Done.

Table S3. Comparison of different agar bases supplemented with different concentrations of colistin. Genes involved in colistin resistance are indicated in bold and colistin MICs are indicated in $\mu\text{g/mL}$.

Table S4. Development of the selective medium for the detection of colistin-resistant strains.

Table S5. Comparison of different polymyxin-containing media. Numbers indicate the total number of strains that grew on each culture media.

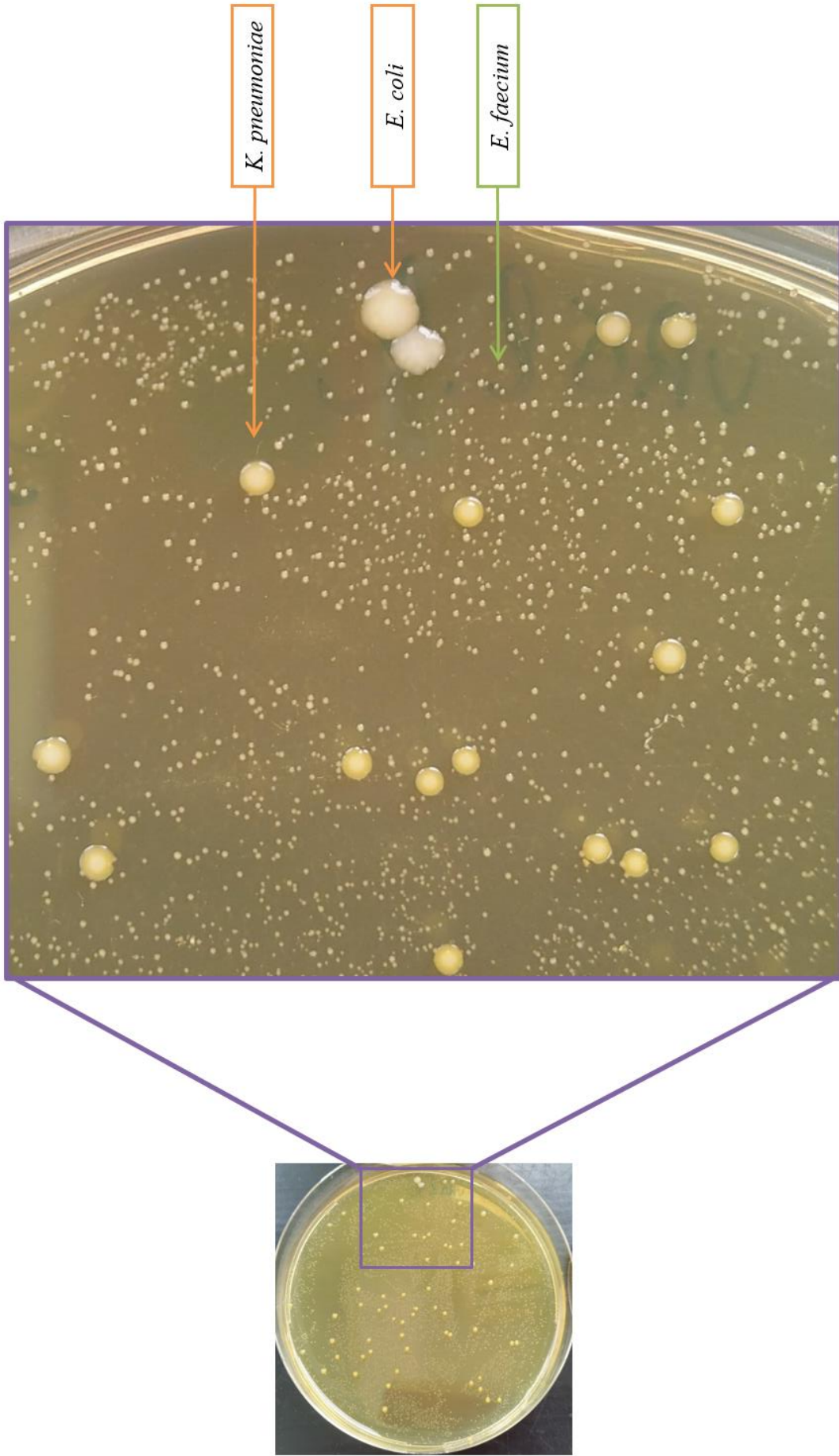


Figure 1. Aspect of the different types of colonies on LBJMR after culture of a clinical sample. *Enterobacteriaceae* exhibited yellow colonies that are larger (2-3 mm) than enterococci (0.1-1 mm).

2) Évaluation comparative du kit UMIC Colistine

Les recommandations de 2017 préconisent que la CMI soit évaluée pour les polymyxines, or la méthode de référence est difficile à mettre en place en routine. Afin de permettre aux laboratoires de microbiologie clinique de répondre à ces exigences, le kit UMIC a été développé, proposant un système de microdilution en milieu liquide prêt à l'emploi et ne nécessitant que du matériel courant pour fonctionner.

Ce test a été évalué comparativement à la méthode de référence en accord avec la norme ISO 20776 et les recommandations des systèmes expert EUCAST et CLSI. Un nombre représentatif de souches a été utilisé, comprenant différentes espèces résistantes à la colistine par différents mécanismes. Les résultats ont montré une sensibilité de 100% du kit Umic Colistine pour la détection des souches *mcr-1* de l'étude. Le kit Umic Colistine est donc un outil fiable pour la détection d'isolats cliniques résistants.

1 **Comparative evaluation of the UMIC Colistine kit to assess MIC of colistin of Gram-**
2 **negative rods.**

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19 **Words: 1690**

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22 **Figures: 3**

23 **Tables: 3**

24 **Abstract**

25 **Background**

26 The recent description of the first plasmid-mediated colistin-resistant gene *mcr-1*, conferring a
27 transferable and low-level resistance to colistin, raised concern about the necessity to
28 implement a rapid and reliable screening method to detect colistin-resistant clinical isolates.
29 The only available method to assess the MIC of colistin is the Broth Microdilution reference
30 method according to joint EUCAST and CLSI expert systems. The UMIC Colistine is a
31 commercial kit of broth microdilution developed to easily assess colistin MIC by proposing a
32 ready-to-use system. Here, we evaluate the UMIC Colistine system on 92 Gram-negative rods
33 (76 *Enterobacteriaceae* including 31 harboring the *mcr-1* gene and 16 non-fermentative), by
34 comparison to reference broth microdilution, following the ISO 20776 standard.

35 **Results**

36 Categorical agreement was of 100% with 70.2% (n=66) of colistin-resistant strains, and 29.8%
37 (n=28) of colistin-susceptible strains. Essential agreement was 91.5%, with 68.1% (n=64) of
38 identical values, and 100% for colistin-resistant strains, including all the strains harbouring the
39 *mcr-1* gene. 8 of strains classified as susceptible presented a difference between MIC values
40 of 2 dilutions. No very major error or major error was reported. Reproducibility of the UMIC
41 Colistine system was 100% with quality control strains by performing the 15-replicates plan
42 described in CLSI guidelines. Correlation between UMIC Colistine and reference method was
43 0.99.

44 **Conclusions**

45 The UMIC Colistin kit is easy-to-perform and showed good results compared to reference
46 method. The UMIC Colistine system is a rapid and reliable broth microdilution method to
47 assess the colistin MIC of clinical isolates in clinical microbiology laboratories.

48

49 **Background**

50 The emergence of multi-drug resistant Gram-negative bacteria is a worldwide
51 phenomenon. Particularly, carbapenemase-producing *Enterobacteriaceae* are of clinical
52 concern and have led to the revival of old antibiotics as last resort treatment, such as polymyxins
53 (1). Until recently, all the described polymyxin-resistant genes were chromosomally encoded,
54 including those coding for the two-component systems PmrA/PmrB, PhoP/PhoQ (2), and their
55 negative regulator MgrB in *Klebsiella pneumoniae* species (3). The recent description of the
56 first plasmid-encoded resistance gene, named *mcr-1* (4), was followed by its screening in
57 samples from all over the world and from different origin, human or animal (5), and by the
58 description of *mcr-1.2* (6) and *mcr-1.3* (7) variants and *mcr-2* (8), *mcr-3* (9) and *mcr-4* (10)
59 genes. This transferable resistance is characterized by low levels of resistance, with Minimal
60 Inhibitory Concentration (MIC) of colistin around 4 µg/mL (4), which is closed to the clinical
61 breakpoint established by European Committee of Antibiotic Susceptibility Testing (EUCAST)
62 or epidemiological cut-off values (ECV) recommended by the Clinical and Laboratory
63 Standards Institute (CLSI) (Resistant > 2 µg/mL and susceptible ≤ 2 µg/mL)(11). This
64 phenomenon has raised the global clinical concern about the capacity of laboratories to detect
65 it (12).

66 Currently, the only available Antibiotic Susceptibility Testing (AST) method for colistin
67 according to the conjoint EUCAST/CLSI Expert system is the reference Broth Microdilution
68 (BMD) method, supported by a consequent number of comparative studies that concluded on
69 the unreliability of currently used techniques such as MIC strips like E-test (Biomérieux, Marcy
70 l'Étoile, France) or automated techniques like Vitek2 (Biomérieux, Marcy l'Étoile, France) or
71 Phoenix (Becton-Dickinson, Sparks, MD) (13–20). As BMD is a time-consuming technique, it
72 is not suitable for diagnosis laboratory in public hospitals, and the establishment of a rapid and
73 reliable method for colistin MIC determination is obviously warranted to control the spread of
74 this mobile colistin resistance (12, 21).

75 The UMIC Colistine kit (Biocentric, Bandol, France) was develop to assess BMD
76 method in clinical laboratory by proposing a ready-to-use system based on the reference
77 method. The UMIC Colistin kit consists of unitary test containing 12-wells strips with 11 wells
78 filled by a range of lyophilized colistin concentrations (from 0.06 to 64 µg/mL, with 2-fold
79 dilutions between 2 consecutive wells), and 2 mL bottles of Mueller-Hinton Broth (MH)
80 medium.

81 Here, we comparatively evaluated the UMIC Colistine strips with the reference method
82 prepared accordingly to CLSI recommendations, following the International Organization for
83 Standardization (ISO) 20776-1 standard.

84

85 **Methods**

86 *Bacterial strains*

87 A total of 92 bacterial strains were used in this study, including 76 *Enterobacteriaceae*
88 (3, 22–31) and 16 non-fermentatives (32–36), presented in Table 1. Seven of them were clinical
89 isolates when the 85 others were well-characterized from previous studies: colistin MIC were
90 then assessed by E-test method and colistin-resistance genes were screened by PCR as
91 previously described (3, 37). Among them, 62 were colistin-resistant, including 31 harbouring
92 the *mcr-1* gene with MIC ranging from 2 to 32 µg/mL (22–25, 29), and 25 colistin-susceptible
93 (Table 1).

94

95 *Broth microdilution/reference method preparation*

96 The reference method was prepared accordingly to the ISO 20776-1 standard, with a
97 stock solution of colistin prepared from colistin sulphate salt (MP Biomedicals, Illkirch, France)
98 and adjusted accordingly to the Table 4 of the CLSI 2017 M100-S27 guideline. The BBL™
99 Mueller-Hinton II Broth (MH2) (Becton-Dickinson, Heidelberg, Germany) was prepared
100 following manufacturer's instructions. The stock solution of colistin was diluted in the MH2
101 medium in order to fill the 96-wells plates following the same scheme of UMIC Colistine strips
102 (0.06 to 64 µg/mL of colistin), with a growth control well containing only MH2 medium. Stock
103 solution and plates were prepared extemporaneously each day of test.

104

105 *Comparison*

106 Bacterial suspension of 0.5 MacFarland were prepared for each tested strain and
107 inoculated within the 15 minutes in both systems in a way to obtain the same final inoculum of
108 5×10^5 CFU/mL (Colony Forming Unit / mL) or 5×10^4 CFU/well for both tested methods, as
109 recommended by CLSI guidelines. For the UMIC Colistin strip: 10 µL were directly added in
110 a 2 mL MH bottles of which 100 µL were added in each well of the strip. For the reference
111 method, the same 200-fold dilution was performed in freshly prepared plates, in two steps: a
112 first 20-fold dilution in MH, then a 10-fold dilution when filling the colistin-containing wells
113 of the prepared plate.

114 Plates were incubated in aerobic atmosphere at 37°C for 18H. Results were read directly
115 or after adding 50 µL of a prepared Iodonitrotetrazolium chloride solution (5 mg/mL) (Sigma-
116 Aldrich, Illkirch, France), and could also be analyzed using the ELX808 Ultra Microplate
117 Readers (Biotek Instruments, Winooski, USA).

118

119 *Quality control and reproducibility*

120 Quality controls *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 35851
121 and *E. coli* NCTC 18553 were used to assess the reproducibility of the UMIC Colistine system,
122 as well as two *E. coli* strains harboring the *mcr-1* gene (clinical MIC breakpoint of 4 µg/mL),
123 and an intrinsically colistin-resistant *Proteus mirabilis* as control. The 15-replicates (3 x 5 days)
124 plan was performed according to the CLSI guidelines (M07-A10): 3 different inocula were
125 tested per day for five consecutive test days. The plan is successful when 0 or 1 values are out
126 of range (38).

127 Subcultures of each day were performed from the same primary culture as
128 recommended. The reproducibility of the test is acceptable if $\geq 95\%$ of the MIC values are
129 within one twofold dilution of the end point. Quality controls were used in each series of test,
130 and series were repeated when giving unacceptable results.

131

132 *Results analysis*

133 Data were analyzed with the recommendation of the CLSI 2017 and the ISO standard
134 20776-2. A categorical agreement was obtained when the MIC value classified the strain in the
135 same clinical category (resistant or susceptible) than reference method. An essential agreement
136 was obtained when the value was identic or within one twofold dilution from the reference
137 value. A major error corresponded to a result classified as resistant when it is susceptible by
138 reference method (false resistant), and a very major error, when the result was classified as
139 susceptible when it is resistant by reference method (false susceptible). Correlation between the
140 two systems was calculated using Pearson method (value 128 was retained when MIC was $>$
141 64 µg/mL) Tests presenting more than one skipped wells was deleted from the study, if not
142 performed again.

143 **Results and discussion**

144 *MIC results*

145 Categorical agreement was of 100%, with 71.7% (n = 66) colistin-resistant strains and
146 28.3% (n = 26) colistin-susceptible strains obtained with both methods. Essential agreement
147 was of 91.3% (n = 84), with 67.4% (n = 62) of identical MIC values (Figure 1). More precisely,
148 the essential agreement was of 100% for colistin-resistant strains, including all the tested strains
149 harboring the *mcr-1* gene, as shown in Table 2. On the other hand, it was only of 69.2% for
150 susceptible strains, with a shift of 2 dilutions for 8 of them, which represent a homogenous
151 repartition among the different species (Tables 1 and 2). There was no major error, nor very
152 major error. The UMIC Colistine kit showed good results on a representative amount of
153 bacterial strains of different species. Its sensitivity was excellent, as it could detect all the strains
154 with the *mcr-1* gene as colistin-resistant strains, and also was its specificity. Indeed, even if
155 susceptible strains have shown more differences in results, they remained classified as
156 susceptible. All the results are shown in Table 1.

157

158 *Reliability of UMIC Colistine test*

159 The reproducibility of the UMIC Colistine system was of 100% with all the values
160 within twofold dilution from the mean value, as shown in Table 3. Correlation between UMIC
161 Colistine and reference method was 99.5% (Pearson's $r = 0.995$) and is represented in Figure
162 2. Then plan was successful for *P. aeruginosa* ATCC 27853 with quality controls within the
163 expected ranges. For *E. coli* NCTC 18553 all values were at 2 in the UMIC Colistine, which is
164 supposed to be an acceptable value only occasionally. The plan was unsuccessful for UMIC
165 Colistine with *E. coli* ATCC 25922 with 3 values out of range (Table 3). Indeed, as for
166 susceptible strains, we could observe a lower MIC value in UMIC Colistine than in reference
167 method. Several explanations are considered. First, the *E. coli* strain was not a first subculture
168 and could have changed its MIC value. Second, the UMIC Colistine system could
169 underestimate the values, but that was not the general behaviour for MIC over 1 $\mu\text{g/mL}$. Third,
170 the preparation of BMD plates was not precise enough, and wells with low and close
171 concentrations of colistin could be underestimated, giving overestimated MIC values.

172 *Comparison of both tests*

173 Manual preparation of BMD plates was time-consuming (30 minutes to 1 hour per day),
174 need a large amount of sterile material (sterilized MH medium, plates, colistin solution...) and
175 is source of errors, as we supposed it was the case for our susceptible strains. It included several

176 manipulations that could lead to a consequent number of errors and variations and as susceptible
177 values are much closed under 1 µg/mL, we could speculate that the preparation was not precise
178 enough, supported by the fact that 4 of susceptible strains with a MIC of 1 µg/mL were from
179 the same prepared plate. Finally, the untreated polystyrene microplate used could explain those
180 differences, leading to an underestimated concentration, and overestimated MIC for susceptible
181 strains (39).

182 Despite of these results, the essential agreement of the UMIC Colistin kit was acceptable
183 ($\geq 90\%$). The UMIC Colistine kit provides a complete assay that only requires classic laboratory
184 material. It is rapid and easy to use, and the skipped wells are mostly avoided as the principle
185 is to add 100 µL in empty wells. The results sheet provided is easy to use and keep results safe,
186 as shown in Figure 3.

187

188 **Conclusion**

189 The UMIC Colistine kit consists in an easy to realize technique that gives reliable
190 results. UMIC Colistine is a reliable method to perform Broth Microdilution and assess the
191 colistin MIC of clinical isolates in clinical microbiology laboratories.

192

193 **Key-words**

194 Colistin, *Enterobacteriaceaes*, *mcr-1*, resistance, broth microdilution, antibiotic susceptibility
195 testing, detection method, screening.

196 **List of abbreviations**

197 AST: Antibiotic Susceptibility Testing

198 ATCC: American Type Cultures Collection

199 BMD: Broth Microdilution MIC: Minimal Inhibitory Concentration

200 CLSI: Clinical and Laboratory Standards Institute

201 CFU: Colony Forming Unit

202 ECV: Epidemiological Cut-off Values

203 EUCAST: European Committee of Antibiotic Susceptibility Testing

204 ISO: International Organization for Standardization

205 McF: MacFarland

206 MH: Mueller-Hinton

207 NCTC: National Collection of Type Cultures

208

209 **Competing interests**

210 UMIC Colistine kits were provided by Biocentric Company.

211 **Authors' contributions**

212 LB conducted the experiments, analyzed the data and wrote the manuscript. LO participated to
213 experiments and writing. SB participated to the experiments and analysis of results. SLP gave
214 support for the design of the study. JMR conceived the design of the study and corrected the
215 manuscript. All authors read and approved the manuscript.

216 **Authors' information?**

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- 353

354 **Tables**

355 **Table 1. Colistin MICs obtained by broth microdilution comparing UMIC Colistine to the**
356 **reference method ($\mu\text{g/mL}$).** Strains harboring the *mcr-1* gene are indicated in bold, and the
357 other colistin-resistance genes are indicated as follow: ¹*phoQ* ²*pmrB* ³*mgrB* ⁴*pmrA*. References
358 of strains within published studies are indicated into square brackets. Non acceptable values are
359 in red.

360 **Table 2. Essential agreements (identical ± 1 dilution from the reference MIC), presented**
361 **by bacterial species, then by category.**

362 **Table 3. Quality control and reproducibility.** The table indicates the number of values for
363 each MIC and QC. QC target values are highlighted in grey. Values that are out of range are in
364 bold.

365

366 **Figures**

367 **Figure 1. Categorical agreements for colistin MIC values between UMIC Colistine and**
368 **reference method:** Grey shades highlight values within essential agreement. Breakpoints are
369 indicated with lines, according to CLSI recommendations ($R > 2 \mu\text{g/mL}$ $S \leq 2 \mu\text{g/mL}$). Values
370 out of range are underlined.

371 **Figure 2. Correlation between commercial UMIC Colistin kit and reference method for**
372 **Broth Microdilution.** MICs are in $\mu\text{g/mL}$. The size of the marker is proportional to the quantity
373 of results obtained at the expected values.

374 **Figure 3. MIC results reading with UMIC Colistine kit.** The UMIC strip is put over the
375 result sheet, and the MIC is easy to read as the well where growth occurred hide the number.
376 The first readable result corresponds to the MIC, indicated by the arrow: $0.25 \mu\text{g/mL}$. This
377 strain is susceptible to colistin.

378 **Table 1. Colistin MICs obtained by broth microdilution comparing UMIC Colistine to the**
 379 **reference method (µg/mL).** Strains harboring the *mcr-1* gene are indicated in bold, and the other colistin-
 380 resistance genes are indicated as follow: ¹*phoQ* ²*pmrB* ³*mgrB* ⁴*pmrA*. References of strains within published
 381 studies are indicated into square brackets. #Heteroresistance

Bacterial species	Isolates	Samples origins	Colistin MIC (µg/mL)	
			Reference	UMIC
<i>Escherichia coli</i>	SE65	Human, Algeria (22)	4	4
	117R	Human, Saudi arabia (23)	4	4
	1R2013	Human, Saudi arabia (23)	8	8
	1R 2104	Human, Saudi arabia (23)	8	4
	44A	Human, Saudi arabia (23)	4	4
	6R	Human, Saudi arabia (23)	4	4
	85R	Human, Saudi arabia (23, 31)	4	8
	95R	Human, Saudi arabia (23)	4	8
	96R	Human, Saudi arabia (23)	8	8
	134R	Human, Saudi arabia (23)	4	4
	143R	Human, Saudi arabia (23)	8	8
	LH121	Human, Laos (24)	4	4
	LH140¹	Human, Laos (24)	8	8
	LH257	Human, Laos (24)	16	8
	LH57¹	Human, Laos (24)	4	4
	LH1	Human, Laos (24)	4	4
	LH30	Human, Laos (24)	4	4
	TH214	Human, Thailand (24)	4	8
	TH99	Human, Thailand (24)	16	16
	FHA102 ²	Human, France (24)	16	16
	FHM19 ²	Human, France (24)	16	8
	FHA113 ²	Human, France (24)	8	8
	NH94 ²	Human, Nigeria (24)	16	16
	TH176	Human, Thailand (24)	8	8
	LB4	Human, France	8	8
	235	Chicken, Algeria (24)	4	4
	P6	Pig, Laos (24)	4	4
	P10	Pig, Laos (24)	4	8
	P17	Pig, Laos (24)	8	4
	P7	Pig, Laos (24)	4	4
LH165S	Human, Laos	1	0.25	
TH77S	Human, Thailand	0.5	0.25	
282S	Chicken, Algeria	1	1	
<i>MAR</i>	Human, France	0.5	0.125	
<i>Klebsiella pneumoniae</i>	FHA60	Human, France (3, 25)	16	16
	FHM128	Human, France (3, 25)	16	8
	119R	Human, Saudi arabia (23)	8	8
	LH131³	Human, Laos (25)	32	32
	LH61³	Human, Laos (25)	32	32
	LH17²	Human, Laos (25)	32	32
	LH92	Human, Laos (25)	16	16
	LB1 ³	Human, France (26)	64	64
	FHM169 ³	Human, France (3)	16	16

	LH12 ³	Human, Laos (3)	32	32
	TH28 ³	Human, Thailand (3)	32	32
	TH54 ²	Human, Thailand (3)	16	16
	TH224 ²	Human, Thailand (3)	8	16
	TH205 ³	Human, Thailand (3)	8	8
	FHM120 ³	Human, France (3)	32	32
	FHA105 ³	Human, France (3)	64	64
	FHM77	Human, France (3)	16	16
	LH140	Human, Laos (3)	64	64
	LH94	Human, Laos (3)	32	32
	TH68	Human, Thailand (3)	64	64
	LB3	Human, France	>64	>64
	SF	Human, France	>64	>64
	CIP 82.91		0.25	0.25
	LB2	Human, France (26)	1	0.25
	TH28S	Human, Thailand	0.5	0.25
<i>Klebsiella oxytoca</i>	FHA41 ³	Human, France (3, 27)	32	64
	FHA124	Human, France (3)	16	32
	TH44	Human, Thailand	0.5	0.125
<i>Enterobacter aerogenes</i>	EA1509E ⁴	Human, France (28)	>64	>64
	BEN	Human, France	32	16
<i>Enterobacter asburiae</i>	LH74	Human, Laos	>64	>64
	1502	Human, France	0.5	0.25
	1503	Human, France	0.5	0.25
<i>Enterobacter cloacae</i>	SB1	Human, France (29)	4	4
	NH131	Human, Nigeria	>64	>64
	NH132	Human, Nigeria	>64	>64
	NH52	Human, Nigeria	>64	>64
	RAL	Human, France	>64	>64
	BAT	Human, France	1	0.25
	NH151	Human, Nigeria	0.5	0.25
	NH74	Human, Nigeria	0.5	0.25
<i>Proteus mirabilis</i>	NDM-1	Human, Israel (30)	>64	>64
<i>Stenotrophomonas maltophilia</i>	BA	Human, France	>64	>64
<i>Pseudomonas aeruginosa</i>	PA_ColiR	Human, France	>64	>64
	FHM-122	Human, France	2	2
	FHM-140	Human, France	2	2
	FHM-122'	Human, France	1	1
	FHM-134	Human, France	1	1
	FHM-122''	Human, France	1	1
	FHM-131	Human, France	1	1
	VIN	Human, France	0.5	0.25
<i>Acinetobacter baumannii</i>	ABIsac_ColiR	Human, France (32)	64	64
	CR17	Human, Spain (33)	0.5	0.5
	Big	Human, Iran	1	0.25
	Small	Human, Iran	1	0.5
	NDM-1	Human, Syria (34)	1	0.25
<i>Acinetobacter nosocomialis</i>	ABG13S	Human, Spain (35)	1	0.5
<i>Acinetobacter pittii</i>	G867	Human, France (36)	1	0.25

382 **Table 2. Essential agreements (identical ± 1 dilution from the reference MIC), presented**
 383 **by bacterial species, then by category.**

	MIC ranges	Identic	Essential agreement
<i>Acinetobacter baumannii</i> (n = 5)	0.5 - 64	2 (40%)	3 (60%)
<i>Enterobacter</i> sp. (n = 13)	0.5 - > 64	7 (53.8%)	12 (92.3%)
<i>Escherichia coli</i> (n = 34)	0.5 - 16	23 (65.7%)	32 (94.1%)
<i>Klebsiella pneumoniae</i> (n = 25)	0.25 - > 64	21 (84%)	24 (96%)
<i>Pseudomonas aeruginosa</i> (n = 8)	0.5 - > 64	7 (87.5%)	8 (100%)
Autres :			
- <i>P. mirabilis</i> (1)	> 64	1 (100%)	1 (100%)
- <i>K. oxytoca</i> (3)	1-64	0	2 (66%)
- <i>A. nosocomialis</i> (n = 1)	1	0	1 (100%)
- <i>A. pittii</i> (n = 1)	1	0	0
- <i>S. maltophilia</i> (n = 1)	64	1 (100%)	1 (100%)
<hr/>			
All (n = 92)	0.5 - > 64	62 (69.6%)	84 (92.3%)
<hr/>			
Resistant strains (n = 66) :			
- <i>mcr-1</i> (n = 31)	4 - 64	23 (74.2%)	31 (100%)
- other mechanism (n = 35)	4 - > 64	30 (85.7%)	35 (100%)
Susceptible strains (n = 26) :			
	0.25 - 1	9 (34.6%)	18 (69.2%)

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386 **Table 3. Quality control and reproducibility.** The table indicates the number of values for
 387 each MIC and QC. QC target values are highlighted in grey. Values that are out of range are in
 388 bold.

Quality control (QC)	<i>E. coli</i> ATCC 25922					<i>P. aeruginosa</i> ATCC 27853				<i>E. coli</i> NCTC 18553*		
MIC (µg/mL)	0.125	0.25	0.5	1	2	0.5	1	2	4	2	4	8
UMIC	3	7	5			5	10			15		
Reference				13	2		5	10			9	6

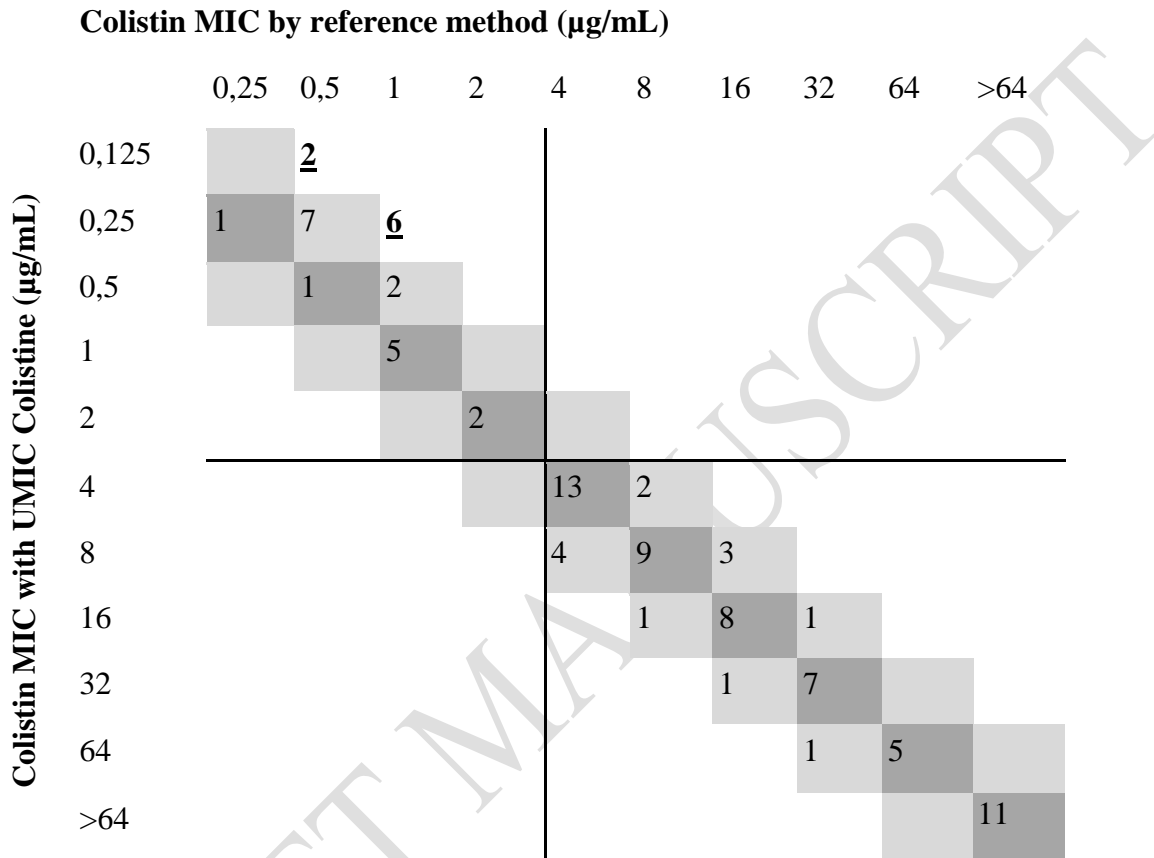
389 *Quality control harboring *mcr-1* gene: MIC should be 4 µg/mL, 2 and 8 are occasionally accepted

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391 **Figure 1. Categorical agreements for colistin MIC values between UMIC Colistine and**
 392 **reference method:** Grey shades highlight values within essential agreement. Breakpoints are
 393 indicated with lines, according to CLSI recommendations ($R > 2 \mu\text{g/mL}$ $S \leq 2 \mu\text{g/mL}$). Values
 394 out of range are underlined.

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403 **Figure 3. MIC results reading with UMIC Colistine kit.** The UMIC strip is put over the
404 result sheet, and the MIC is easy to read as the well where growth occurred hide the number.
405 The first readable result corresponds to the MIC, indicated by the arrow: 0.25 µg/mL. This
406 strain is susceptible to colistin.

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VI. DÉVELOPPEMENT INDUSTRIEL

Dans la poursuite de mes travaux sur l'évaluation du kit UMIC Colistine, j'ai effectué mon dernier semestre d'internat au sein de l'entreprise Biocentric, dans le but de participer au développement des kits UMIC. Actuellement il existe 6 kits UMIC : Colistine, Vancomycine-Teicoplanine, Pipéracilline/Tazobactam, Méropénème, Ertapénème et Ceftobiprole.

Une partie des étapes de production est pour l'instant sous-traitée, et l'objectif à court terme est de pouvoir réaliser l'ensemble de la production au sein de l'entreprise, ainsi que la partie bactériologique du contrôle qualité, ce qui nécessite de nouveaux équipements.

Un autre objectif de l'entreprise est l'amélioration des kits en proposant notamment des barrettes conditionnées à l'unité, leur identification par impression afin d'améliorer leur traçabilité, et enfin améliorer la lecture, d'une part avec l'incorporation d'un indicateur coloré, et d'autre part avec l'automatisation de la lecture à l'aide d'un lecteur.

En me basant sur mon expérience de laboratoire, j'ai dans un premier temps participé à l'établissement des protocoles de fabrication, puis à la rédaction du protocole de validation et d'évaluation des nouvelles barrettes UMIC.

Ma première mission a ainsi été de mettre en place les contrôles qualité en interne, en accord avec la norme ISO 20776-1. La Table 7 reprend les différents contrôles qualité recommandés pour l'évaluation de chaque molécule ainsi que les valeurs de CMI attendues.

Table 7. Contrôles qualité et valeurs de CMI attendues pour chaque antibiotique. ($\mu\text{g/mL}$)

Antibiotique	<i>S. aureus</i> ATCC 29213	<i>E. faecalis</i> ATCC 29212	<i>E. coli</i> ATCC 25922	<i>P. aeruginosa</i> ATCC 27853	Souches résistantes spécifiques*
Ceftobiprole	0,125-1	0,06-0,5	0,03-0,125	16-64	
Colistine			0,25-2	0,5-4	<i>E. coli</i> NCTC 13846 ^a : 2-8
Ertapénème	0,06-0,25	4-16	0,004-0,016	2-8	
Méropénème	0,03-0,12	2-8	0,008-0,06	0,12-1	
Pipéracilline/Tazobactam	0,25/4-2/4	1/4-4/4	1/4-4/4	1/4-8/4	<i>E. coli</i> ATCC 35218 ^b : 0,5-2 <i>K. pneumoniae</i> ^c ATCC 700603 ^c :8.32
Vancomycine	0,5-2	1-4			
Teicoplanine	0,25-1	0,25-1			

*Les gènes de résistance de ces souches contrôles sont : ^a*mcr-1*, ^b*bla_{TEM-1}*, ^c*bla_{SHV-18}*

Ainsi j'ai participé à l'élaboration d'un plan de production nécessitant la création d'un nouveau laboratoire de bactériologie, dans le respect ses exigences réglementaires et normatives en vigueur.

VII. AUTRES TRAVAUX SCIENTIFIQUES

1) Analyse d'une *K. pneumoniae* hétérorésistante à la colistine

Deciphering heteroresistance to colistin in a *Klebsiella pneumoniae* isolate from Marseille, France. Bardet L, Baron S, Leangapichart T, Okdah L, Diene SM, Rolain J-M. 2017. *Antimicrob Agents Chemother* 61:e00356-17. (IF = 4.301)

Au cours de mes travaux sur des milieux de culture sélectifs, j'ai pu isoler une souche de *Klebsiella pneumoniae* hétérorésistante à la colistine à partir d'un échantillon de selle d'un patient de l'hôpital de la Timone. La sous-population résistante présentait une mutation non-sens dans le gène *mgrB* : l'insertion d'une cytosine induisant un codon Stop et donc une protéine tronquée. Des études comparatives ont confirmé qu'il s'agissait d'une hétérorésistance, incluant l'antibiogramme complet des deux sous-populations isolées, ainsi que leur typage par les méthodes MultiLocus Sequence Typing (MLST) et Pulse-Field Gel Electrophoresis (PFGE).



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*

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,

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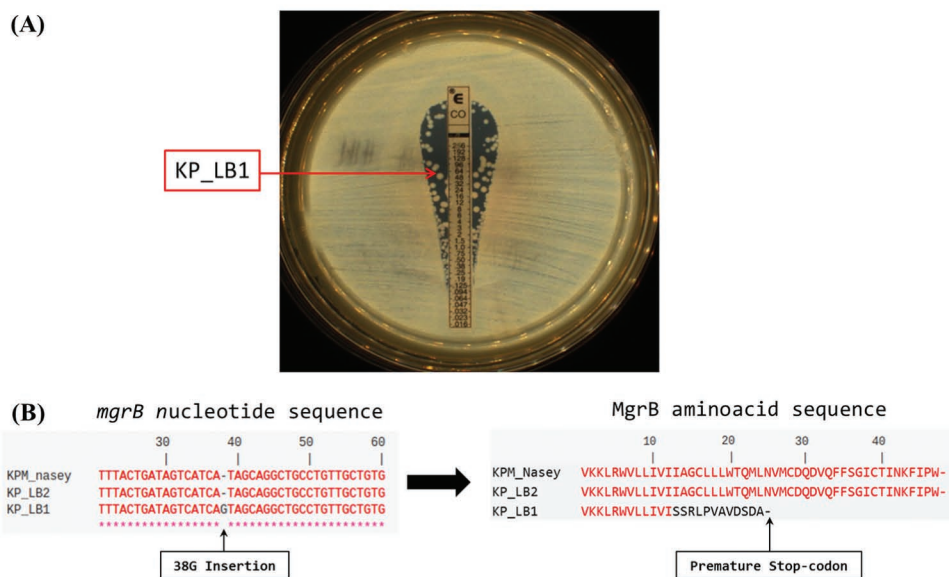


FIG 1 (A) Colistin MIC determination using a colistin Etest strip. The arrow indicates the colistin-resistant subpopulation LB1. (B) Alignments of *mgrB* nucleotide sequences and amino acid MgrB protein sequences of the two subpopulations of colistin-heteroresistant *Klebsiella pneumoniae* with KPM_Nasey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

had a colistin MIC of 128 $\mu\text{g}/\text{ml}$ and exhibited gray and plate colonies on COS medium, while the colistin-susceptible subpopulation, named LB2, had a colistin MIC of 1 $\mu\text{g}/\text{ml}$ 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://bigsdab.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)

^aBoth 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), ceftazidime (FOX), aztreonam (ATM), imipenem (IPM), ertapenem (ETP), nalidixic acid (NAL), ciprofloxacin (CIP), ofloxacin (OFL), amikacin (AMK), gentamicin (GEN), tobramycin (TOB), and fosfomycin (FOF). LB1 was resistant to colistin (CST), while LB2 was susceptible.

^bComité 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

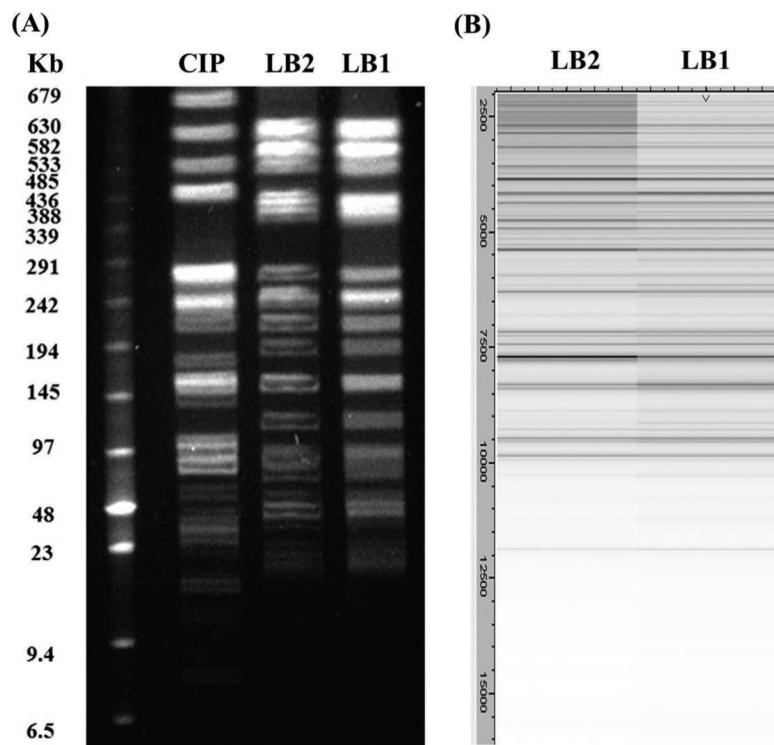


FIG 2 (A) PFGE profiles of XbaI-digested genomic DNAs showing genomic relatedness among the CIP 82.91, LB2, and LB1 *K. pneumoniae* strains. DNA sizes of the low-range pulsed-field gel marker (Biolabs, New England) are indicated in kilobases on the left. (B) MALDI-TOF MS spectra from LB1 and LB2 strain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

Microbial Culturomics Application for Global Health: Noncontiguous Finished Genome Sequence and Description of *Pseudomonas massiliensis* Strain CB-1(T) sp. nov. in Brazil.

Bardet L, Cimmino T, Buffet C, Michelle C, Rathored J, Tandina F, Lagier J-C, Khelaifia S, Abrahão J, Raoult D, Rolain J-M. (2017). OMICS. omi.2017.0027. (IF = 2.723)

L'IHU (Institut Hospitalo-Universitaire) Méditerranée Infection mène depuis 2013 une étude de culturomique visant à diversifier les milieux et les conditions de culture afin d'élargir les connaissances sur le microbiote humain.

Ce projet permet ainsi la découverte de nouvelles espèces bactériennes qui sont caractérisées par taxono-génomique, une stratégie consistant en la comparaison systématique des caractéristiques ces nouveaux isolats avec les espèces les plus proches.

Mon travail a consisté à décrire *Pseudomonas massiliensis* sp. nov., avec l'ensemble de ses caractéristiques phénotypiques et génomiques.

Microbial Culturomics Application for Global Health: Noncontiguous Finished Genome Sequence and Description of *Pseudomonas massiliensis* Strain CB-1^T sp. nov. in Brazil

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Abstract

Culturomics is a new postgenomics field that explores the microbial diversity of the human gut coupled with taxono-genomic strategy. Culturomics, and the microbiome science more generally, are anticipated to transform global health diagnostics and inform the ways in which gut microbial diversity contributes to human health and disease, and by extension, to personalized medicine. Using culturomics, we report in this study the description of strain CB1^T (=CSUR P1334=DSM 29075), a new species isolated from a stool specimen from a 37-year-old Brazilian woman. This description includes phenotypic characteristics and complete genome sequence and annotation. Strain CB1^T is a gram-negative aerobic and motile bacillus, exhibits neither catalase nor oxidase activities, and presents a 98.3% 16S rRNA sequence similarity with *Pseudomonas putida*. The 4,723,534 bp long genome contains 4239 protein-coding genes and 74 RNA genes, including 15 rRNA genes (5 16S rRNA, 4 23S rRNA, and 6 5S rRNA) and 59 tRNA genes. Strain CB1^T was named *Pseudomonas massiliensis* sp. nov. and classified into the family *Pseudomonadaceae*. This study demonstrates the usefulness of microbial culturomics in exploration of human microbiota in diverse geographies and offers new promise for incorporating new omics technologies for innovation in diagnostic medicine and global health.

Keywords: global health, microbial culturomics, *Pseudomonas massiliensis*, system diagnostics, taxono-genomics

Introduction

OMICS TECHNIQUES REPRESENT A NEW APPROACH to the understanding of microbial diversity. Particularly, metagenomics enabled the identification of a great diversity of bacterial species, but a consequent number of them did not correspond to an identified organism (Lagier et al., 2012a). Culturomics was introduced to decipher the human gut microbiota, combining high-throughput culture, by the diversification of culture conditions, and rapid identification of the growing colonies by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or 16S rRNA sequencing (Lagier et al., 2015).

Bacterial taxonomy and the identification of a new species were usually delineated by phenotypic, chemotaxonomic, and genetic parameters such as the 16S rRNA gene sequence

identity and phylogeny (Stackebrandt and Ebers, 2006; Tindall et al., 2010), genomic G+C content diversity, and DNA-DNA hybridization (DDH) (Rosselló-Mora, 2006; Wayne et al., 1987). However, those tools exhibited limitations, such as the variation in the cutoff values between species or genera and the lack of reproducibility (Welker and Moore, 2010).

The emergence of high-throughput sequencing enabled access to the genomic data of many bacterial species, and we recently designed a new method, known as taxono-genomics, using these available sequences, to describe new bacterial species (Ramassamy et al., 2014). Taxono-genomics consists of a systematic comparison of phenotypic properties, including proteomic information obtained by MALDI-TOF-generated spectrum (Seng et al., 2009), and genome analysis (Fournier et al., 2015).

Using our strategy that combines culturomics and taxono-genomics on the stool sample of a 37-year-old Brazilian

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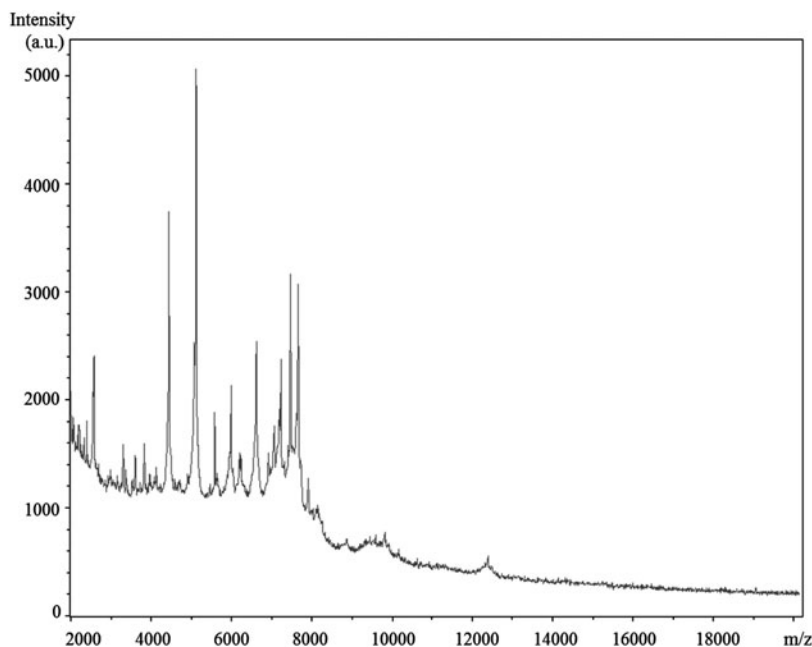


FIG. 1. Reference mass spectrum of *Pseudomonas massiliensis* strain CB1^T. This reference spectrum was generated by comparing 12 individual colonies.

woman, we could isolate and describe strain CB1^T, a new species that we named *Pseudomonas massiliensis*. In this study, we characterize and report the novel isolate *P. massiliensis* sp. nov. strain CB1^T (= CSUR P1334 = DSM 29075), including the description of its whole genome sequence and annotation. These characteristics support the circumscription of the species *P. massiliensis*.

Material and Methods

Sample

A stool sample was consensually collected from an obese 37-year-old Brazilian patient. The patient gave signed, in-

formed consent and the agreement of the local ethics committee of the Institut Fédératif de Recherche 48 (Marseille, France) was obtained under Agreement 09–022. The patient was not taking any antibiotics at the time of sampling and the fecal sample was frozen at -80°C after collection and was shipped to Marseille, France.

Strain isolation

Culturomics was used, as previously described, to decipher the microbial diversity of the gut microbiota (Lagier et al., 2012a). Briefly, 1 g of the stool sample was preincubated at 37°C in a blood culture bottle and this liquid medium was

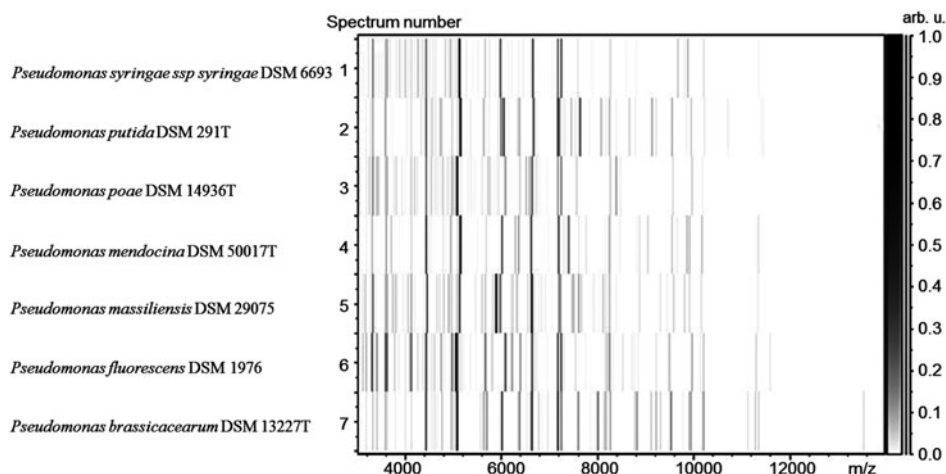


FIG. 2. MALDI-TOF MS-based virtual gel view comparing *P. massiliensis* strain CB1^T to other members of the family *Pseudomonadaceae* by their generated protein expression profiles, or spectra. The x -axis records the m/z value and the y -axis indicates the spectrum number, with corresponding bacterial species indicated on the left. The peak intensity is expressed by a gray scale displayed on the right (arbitrary units). MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

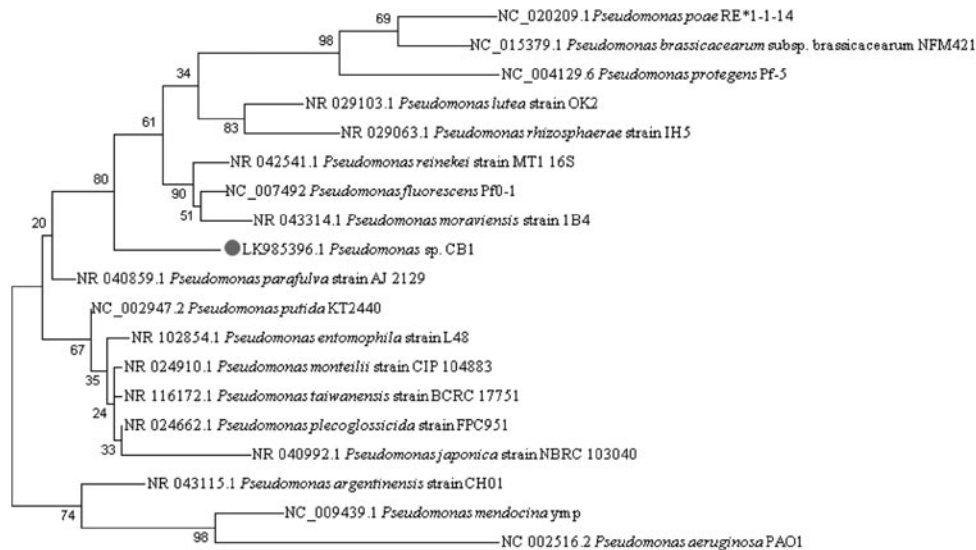


FIG. 3. Phylogenetic tree highlighting the position of *P. massiliensis* sp. nov. strain CB1^T relative to other type strains within the *Pseudomonadaceae*. Neighbor joining tree based on nearly complete 16S rRNA gene sequences of *Pseudomonas* sp. CB1 sp. nov and related species of genus *Pseudomonas sensu stricto*. The significance of each branch is indicated by a bootstrap value (%) calculated for 1000 subset.

then seeded on agar plates following different culture conditions and after different time of preincubation.

Strain identification

All types of colonies that have grown were identified using MALDI-TOF-MS as previously described (Seng et al., 2009), using a Microflex spectrometer (Bruker Daltonics, Leipzig, Germany). The spectra obtained were compared to the spectra of the continuously incremented Bruker database. Colonies exhibiting a spectrum with a MALDI-TOF-MS score <1.9 were further characterized by the 16S rRNA gene sequence comparison to the GenBank database provided by the National Center for Biotechnology Information (NCBI) (Benson et al., 2017).

Amplification of the 16S rRNA gene was done by standard polymerase chain reaction, using the universal primers FD1 and RP2 and HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), and sequencing was performed using the Big Dye Terminator Sequencing Kit (Thermo Fischer, Vantaa, Finland) and the following internal primers: 536F, 536R, 800F, 800R, 1050F, 1050R, 357F, and 357R, as previously described (Weisburg et al., 1991). Strains that exhibited a 16S rRNA sequence similarity value of less than 98.7% with their phylogenetically closest species in nomenclature were considered representatives of putative new species (Stackebrandt and Ebers, 2006).

A 16S rRNA phylogenetic tree was realized for the new species with the closest sequences recovered using a

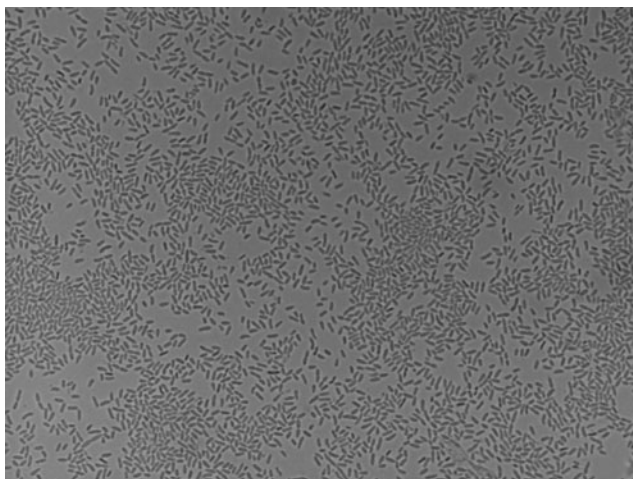


FIG. 4. Gram staining of *P. massiliensis* strain CB1^T, using a DM100 photonic microscope (Leica Microsystems, Nanterre, France) at 100 \times .

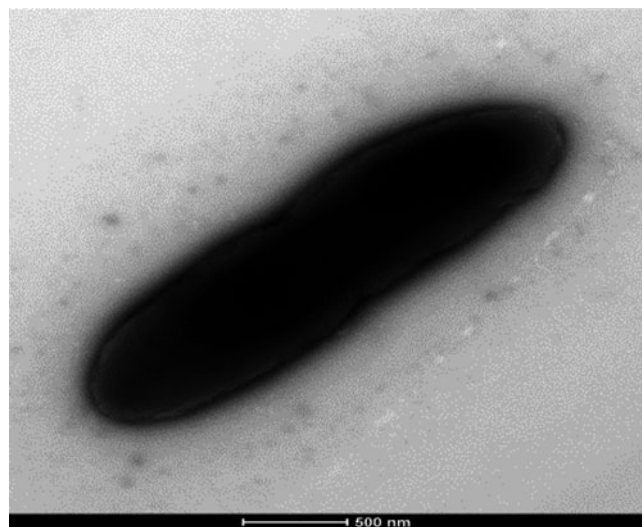


FIG. 5. Transmission electron microscopy of *P. massiliensis* strain CB1, using Morgani 268D (Philips) at an operating voltage of 60 kV. The scale bar represents 500 nm.

TABLE 1. DIFFERENTIAL CHARACTERISTICS OF *PSEUDOMONAS MASSILIENSIS* STRAIN CB1^T (DATA FROM THIS STUDY), *PSEUDOMONAS PUTIDA*, *PSEUDOMONAS FLUORESCENS*, AND *PSEUDOMONAS AERUGINOSA* (XIAO ET AL., 2009)

Properties	<i>P. massiliensis</i>	<i>P. putida</i>	<i>P. fluorescens</i>	<i>P. aeruginosa</i>
Cell diameter (μm)				
Oxygen requirement	aerobic	aerobic	aerobic	aerobic
Gram stain	–	–	–	–
Growth with 5% NaCl	+	+	+	+
Growth at 4°C	–	+	+	–
Growth at 42°C	+	–	–	+
Motility	+	+	+	+
Endospore formation	–			
Production of				
Catalase	–	+	+	+
Oxidase	–	+	+	+
Urease	–	–	–	–
β -galactosidase	–	–	–	–
β -glucosidase	–	–	–	–
indole	–	–	–	–
Acid produced from				
D-glucose	+	+	+	+
Utilization of				
L-Arabinose	–	+ (–)	–	–
Mannose	+	–	+	–
Mannitol	+	–	+	+
D-glucose	+	+	+	+
D-maltose	+	–	–	–

nucleotide blast against the 16S rRNA Database in Silva's "The All-Species Living Tree" Project (LTPs119) and the NCBI database (<http://blast.ncbi.nlm.gov/gate1.inist.fr/Blast.cgi>). Filtered sequences (over 1450 nt) were aligned using Muscle, and phylogenetic inferences were obtained using the Maximum Likelihood Tree. Phylogenetic inferences were analyzed using the likelihood method based on

the Kimura 2 parameter model and MEGA 6 software (Tamura et al., 2013).

Growth conditions

The optimal growth conditions of the strain were tested using aerobic incubation for 2 days on COS medium under

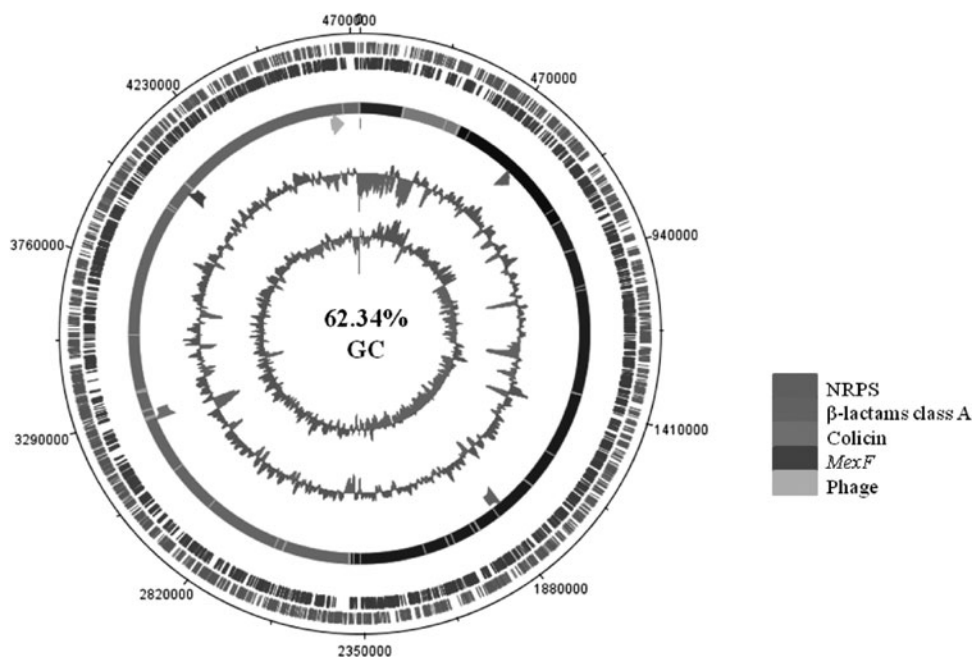


FIG. 6. Graphical circular map of genome. From the outside to center: forward (red) and reverse (blue) strand, contigs (color circle), feature genes indicated in legend, GC plot, GC skew.

TABLE 2. NUCLEOTIDE CONTENT AND GENE COUNT LEVELS OF THE GENOME

Attribute	Genome (total)	
	Value	% of total ^a
Size (bp)	4,723,534	100
G+C content (bp)	2,944,883	62.34
Coding region (bp)	4,262,037	90.23
Total genes	4313	100
RNA genes	74	1.72
Protein-coding genes	4239	100
Genes with function prediction	3312	78.13
Genes assigned to COGs	3141	74.10
Genes with peptide signals	874	20.62
Genes with transmembrane helices	517	11.90
CRISPRs Repeats	0	0

^aThe total is based on either the size of the genome in base pairs or the total number of protein coding genes in the annotated genome.

COG, Clusters of Orthologous group.

different temperatures (25°C, 30°C, 37°C, 45°C, and 55°C). Other atmospheres were tested: aerobic with 5% CO₂ and anaerobic and microaerophilic conditions using GENbag anaer and GENbag microaer systems, respectively (BioMérieux, Marcy l'Étoile, France). To determine the optimal pH for growth and NaCl tolerance, ranges of pH (5.4, 7.3, 8.5, and 9.1) and NaCl concentrations (0%, 2%, 5%, and 10% v/w) were tested by inoculation on modified Colombia agar (Sigma-Aldrich, St. Louis, MO).

Phenotypic characterization

The colonies were observed on COS medium after an overnight incubation at 37°C. Strain sporulation was tested by applying a 60°C thermo-shock for 20 min to the colonies and its motility was determined by observing a fresh colony between the slides using a DM100 photonic microscope (Leica Microsystems, Nanterre, France) at 100×. Gram staining was performed using the Gram 2-F kit (BioMérieux) and cell morphology was determined using transmission electron microscopy after negative staining.

Biochemical characteristics were defined using API 20NE, API 50CH, and API ZYM strips (BioMérieux), in line with the manufacturer's instructions. Catalase and oxidase activities were determined using commercially available kits: ID Color Catalase (BioMérieux) and BD BBL Taxo (Becton-Dickinson, Franklin Lakes, NJ), respectively.

Antibiotic susceptibility testing was performed using the disk diffusion method on Mueller Hinton Agar (Becton-Dickinson) and was interpreted using the European Committee on Antimicrobial Susceptibility Testing guidelines (www.eucast.org). The 16 antibiotics tested were as follows: ticarcillin, ticarcillin/clavulanic acid, piperacillin/tazobactam, ceftazidime, cefepime, imipenem, ertapenem, aztreonam, gentamicin, tobramycin, nalidixic acid, ciprofloxacin, trimethoprim/sulfamethoxazole, rifampicin, fosfomycin, and colistin.

Genomic sequencing and assembly

The DNA of the colonies obtained was extracted using the BioRobot EZ1 Advanced XL (Qiagen) after a pre-lysis treatment with lysozyme as previously described (Hadjadj

TABLE 3. NUMBER OF GENES ASSOCIATED WITH THE 25 GENERAL COG FUNCTIONAL CATEGORIES

Code	Value	% of total ^a	Description
J	180	4.25	Translation
A	1	0.02	RNA processing and modification
K	299	7.05	Transcription
L	148	3.49	Replication, recombination and repair
B	2	0.05	Chromatin structure and dynamics
D	38	0.90	Cell cycle control, mitosis and meiosis
Y	0	0	Nuclear structure
V	37	0.87	Defense mechanisms
T	218	5.14	Signal transduction mechanisms
M	211	4.98	Cell wall/membrane biogenesis
N	105	2.48	Cell motility
Z	0	0	Cytoskeleton
W	0	0	Extracellular structures
U	94	2.22	Intracellular trafficking and secretion
O	126	2.97	Posttranslational modification, protein turnover, chaperones
C	192	4.53	Energy production and conversion
G	219	5.17	Carbohydrate transport and metabolism
E	431	10.17	Amino acid transport and metabolism
F	69	1.63	Nucleotide transport and metabolism
H	149	3.51	Coenzyme transport and metabolism
I	131	3.09	Lipid transport and metabolism
P	256	6.04	Inorganic ion transport and metabolism
Q	102	2.41	Secondary metabolites biosynthesis, transport, and catabolism
R	502	11.84	General function prediction only
S	278	6.56	Function unknown
-	451	10.64	Not in COGs

^aThe total is based on the total number of protein coding genes in the annotated genome.

TABLE 4. LIST OF THE GENES ASSOCIATED WITH ANTIBIOTIC RESISTANCE IN *P. MASSILIENSIS* STRAIN CB1^T

ORF	Gene name	GC%	Size AA	Function	Best blast hit in GenBank	% aa coverage	% aa identity
1659	(Bla)Beta-lactamase_ class-A	65.6	364	Zn-dependent hydrolase	<i>Pseudomonas entomophila</i> L48	88	72
3698	(Flq)OqxBgb	62.5	1061	Multidrug efflux transporter <i>MexF</i>	<i>Pseudomonas rhizosphaerae</i>	100	90

et al., 2016), obtaining a 50 μ L DNA extract. Genomic DNA of *P. massiliensis* was sequenced using MiSeq Technology (Illumina, Inc., San Diego, CA) and two applications: paired end and mate pair as previously described (Hadjadj et al., 2016). The reads of both applications were trimmed and the optimal assembly was obtained through SPAdes software (Bankevich et al., 2012).

Sequence analysis and genome annotation

The genome annotation was performed by Rapid Annotation using the Subsystem Technology server (Aziz et al., 2008) and the predicted bacterial protein sequences were compared using BLASTP against the GenBank and the Clusters of Orthologous Groups (COGs) databases (Evalue $1e^{-03}$, coverage 0, 7, and identity percent 30%). tRNA and rRNA genes were searched using tRNAScanSE (Lowe and Eddy, 1997) and RNAmmer (Lagesen et al., 2007), respectively. The HHMscan was used to find the PFAM-conserved domains (PFAM-A and PFAM-B domains) (Eddy et al., 2011). The number of transmembrane helices and the lipoprotein signal peptides were predicted using Phobius (Käll et al., 2004).

The resistome was analyzed by the Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT) database (Gupta et al., 2014). The presence of putative bacteriocins was determined using the bacteriocins of the URMITE (BUR) database (<http://drissifatima.wix.com/bacteriocins>) (Drissi et al., 2015). The presence of polyketide synthases and nonribosomal peptide synthetase (NRPS) was also determined using a homemade

database, and the predicted proteins were examined using antiSMASH (Weber et al., 2015). Identification of phage sequences was performed using PHAge Search Tool (Zhou et al., 2011). The identified sequences were compared using BLASTP against the GenBank database.

Finally, Artemis (Rutherford et al., 2000), DNA Plotter (Carver et al., 2009), and Mauve alignment tool (Darling et al., 2004) were used for data management, visualization of genomic features, and multiple genomic sequence alignment, respectively.

Comparative genome analysis

The new species was compared with the bacteria exhibiting the closest 16S rRNA sequences and with a complete genome sequence recoverable in the NCBI database. An annotation of all proteomes was carried out to define the distribution of functional classes of predicted genes according to the COGs of proteins (using the same method as for genome annotation). Core genes are defined as genes with an orthologue in each genome used for comparative analysis. All comparison processes were performed in the Multi-Agent Software System DAGOBAN (Gouret et al., 2011), including Figenix (Gouret et al., 2005) libraries, which provides pipeline analysis, and Phylopattern (Gouret et al., 2009) for tree manipulation.

To evaluate the genomic similarity among the studied strains, we identified two parameters: dDDH, which exhibits a high correlation with DDH (Auch et al., 2010; Meier-

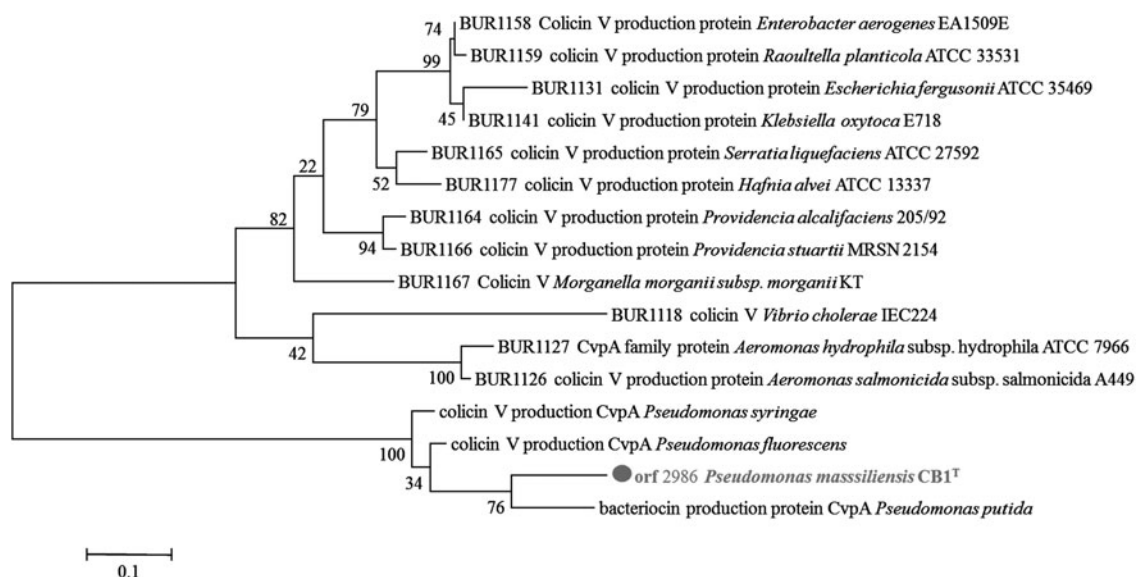


FIG. 7. Phylogenetic tree of the bacteriocin colicin V (WP_040262223.1) in *P. massiliensis*.

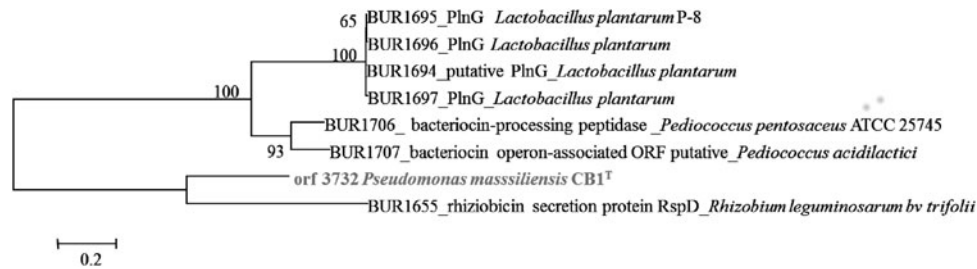


FIG. 8. Phylogenetic tree highlighting the position of the bacteriocin peptidase (WP_040263026.1) in *P. massiliensis*.

Kolthoff et al., 2013), and the average genomic identity of orthologous gene sequences (AGIOS) (Ramasamy et al., 2014), which was designed to be independent of DDH.

In this study, we compared the genome sequences of *P. massiliensis* CB1^T (GenBank accession number CCYK01000000) with those of *Pseudomonas putida* KT2440 (NC_002947.2), *Pseudomonas fluorescens* Pf0-1 (NC_007492.2), *Pseudomonas protegens* Pf-5 (NC_004129.6), *Pseudomonas syringae* pv. phaseolicola 1448A (CP000058), *Pseudomonas brassicacearum* subsp. *brassicacearum* NFM421 (NC_015379.1), *Pseudomonas mendocina* ymp (NC_009439.1), and *Pseudomonas poae* RE*1-1-14 (NC_020209.1).

Results

Strain identification and phylogenetic analysis

The *P. massiliensis* strain CB1^T was isolated in June 2014 by cultivation on Colombia agar with 5% sheep blood agar (COS) (BioMérieux) in aerobic conditions after 14 days of

preincubation. The spectrum obtained from MALDI-TOF MS for strain CB1^T did not obtain a significant score, suggesting that our isolate could be a new species. The spectrum was added to our database (Fig. 1). The gel view showed spectral differences with other members of the family *Pseudomonadaceae* (Fig. 2).

Strain CB1^T exhibited a 98.2% 16S rRNA sequence identity with *Pseudomonas parafulva* strain AJ 2129 (NR_040859.1) (Fig. 3), which is under the threshold recommended for delineating a new species (Stackebrandt and Ebers, 2006). Consequently, strain CB1^T was considered a new strain and named *P. massiliensis* sp. nov. and its 16S rRNA sequence was deposited in GenBank under number LK985396.

Phenotypic characteristics

Colonies were small, circular, smooth, yellowish, opaque, and α -hemolytic. Cells were gram negative, motile short rod (Fig. 4) with a width of 0.5 μ m and a length of 2.6 μ m (Fig. 5), and did not exhibit sporulation after thermo-shock. Strain

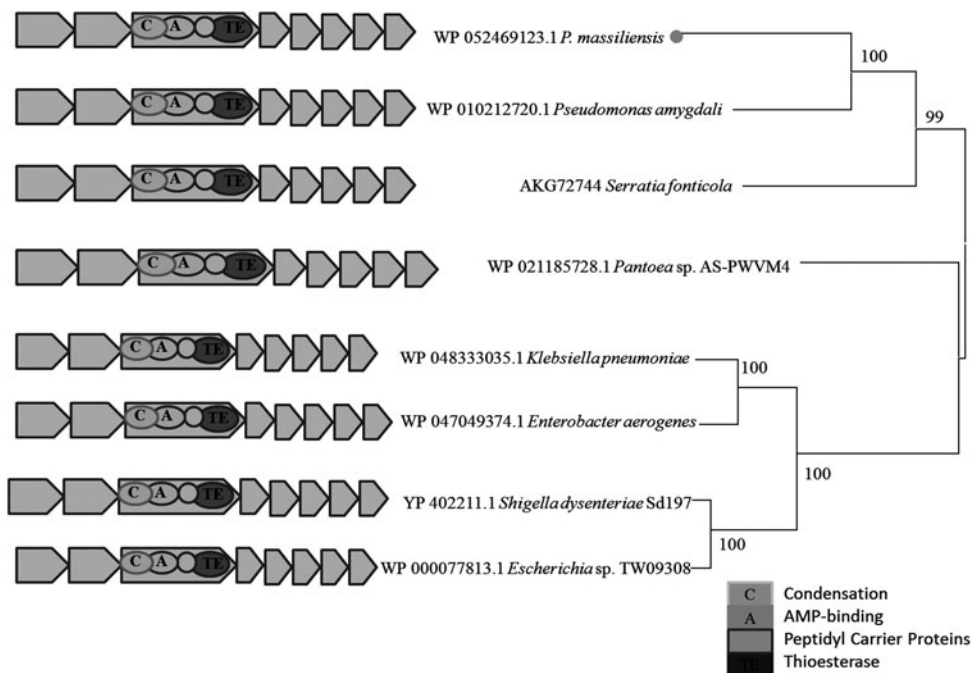


FIG. 9. Comparison of nonribosomal peptide synthetase (NRPS) of enterobactin of *P. massiliensis* with enterobactin cluster in other species.

CB1^T was mesophilic with a growth between 25°C and 45°C, aerobic, and grew in the presence of 5% CO₂ and in a microaerophilic atmosphere, but not anaerobically. Growth did not require NaCl, with a tolerance at 2%. The colonies could grow within a pH range from 5.3 to 8.4. Optimal growth was observed at 37°C in aerobic conditions at a pH of 7.5.

Strain CB1^T exhibited neither catalase nor oxidase activities. Using API 20 NE, positive reactions were observed for nitrate reduction, esculine, D-mannose, D-mannitol, potassium gluconate, malate, and trisodium citrate. An acid production assay using an API 50 CH strip gave positive reactions for glycerol, glucose, fructose, inositol, mannitol, esculine, cellobiose, maltose, lactose, and trehalose. An API ZYM strip showed the presence of alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. Negative reactions were obtained for other constituents. These characteristics are compared with other members of *Pseudomonadaceae* in Table 1 (Xiao et al., 2009).

Strain CB1^T was resistant to ticarcillin, ticarcillin/clavulanic acid, cefepime, trimethoprim/sulfamethoxazole, rifampicin, and fosfomycin, with an intermediate status for aztreonam, and was susceptible to the other antibiotics tested.

Genomic properties

The genome (Genbank accession number CCYK0100000) is 4,723,534 bp long with a 62.34% G+C content (Fig. 6). Of the 4313 predicted genes, 4239 are protein-coding genes and 74 are RNAs, including 15 rRNA genes (5 are 16S rRNA, 4 are 23S rRNA, and 6 are 5S rRNA) and 59 tRNA genes. The properties and statistics of the genome are summarized in Table 2. A total of 3312 genes (78.13%) were assigned to a putative function and 278 (6.56%) were identified as having an unknown function. The distribution of genes into COG functional categories is presented in Table 3.

The resistome analysis of *P. massiliensis* CB1^T by ARG-ANNOT identified a beta-lactamase class-A gene (1094 bp) and the multidrug efflux transporter *MexF* gene (Table 4). Two bacteriocins were detected through the BUR database: colicinV (WP_040262223.1) and peptidase (WP_040263026.1), whose phylogenetic trees are represented in Figures 7 and 8, respectively.

Moreover, 22 toxins and antitoxins that are potentially attributed to bacterial dormancy and persistence, and could enhance drug tolerance and biofilm formation were identified, as an intact prophage sequence of 47.7 kb (GC%: 60.54).

A secondary metabolite biosynthetic gene cluster (NRPS) was found and identified as an enterobactin with a size of 14.7 kb. The component F of this synthetase showed 68%, 63%, 62%, and 57% similarity with the respective sequences of *Pseudomonas amygdali* (WP_010212720.1), *Serratia fonticola* (WP_024485228.1), *Pantoea* sp. AS-PWVM4 (WP_052469123.1), and *Shigella dysenteriae* Sd197 (YP_402211.1), respectively (Fig. 9).

Comparison with genomes from other members of the family *Pseudomonadaceae*

Of the compared species, the draft genome of *P. massiliensis* CB1^T (4.72 Mb) is the smallest, with those of *P. protegens*, *P. syringae*, *P. brassicacearum*, *P. fluorescens*, *P. putida*, *P. mendocina*, and *P. poae* being 7.07, 6.11, 6.84,

TABLE 5. THE NUMBERS OF ORTHOLOGOUS PROTEIN SHARED BETWEEN GENOMES (UPPER RIGHT)

Species	<i>Pseudomonas massiliensis</i>	<i>Pseudomonas putida</i>	<i>Pseudomonas fluorescens</i>	<i>Pseudomonas brassicacearum</i>	<i>Pseudomonas protegens</i>	<i>Pseudomonas poae</i>	<i>Pseudomonas mendocina</i>	<i>Pseudomonas syringae</i>
<i>Pseudomonas massiliensis</i>	4239	2541	2742	2691	2663	2596	2195	2514
<i>Pseudomonas putida</i>	75,38	5350	3209	3153	390	2857	2551	2670
<i>Pseudomonas fluorescens</i>	74,84	77,33	5722	3714	3727	3283	2756	3015
<i>Pseudomonas brassicacearum</i>	75,19	77,33	82,93	6095	3650	3271	2783	3024
<i>Pseudomonas protegens</i>	75,82	78,19	82,1	81,87	6108	3246	2721	2936
<i>Pseudomonas poae</i>	75,07	77,52	81,39	81,19	81,67	4796	2503	2915
<i>Pseudomonas mendocina</i>	74,97	76,34	76,36	76,24	77,27	76,25	4594	2391
<i>Pseudomonas syringae</i>	74,02	75,67	77,42	77,29	77,55	77,29	74,85	5172

Average percentage similarity of nucleotides corresponding to orthologous protein shared between genomes (lower left) and numbers of proteins per genome (bold).

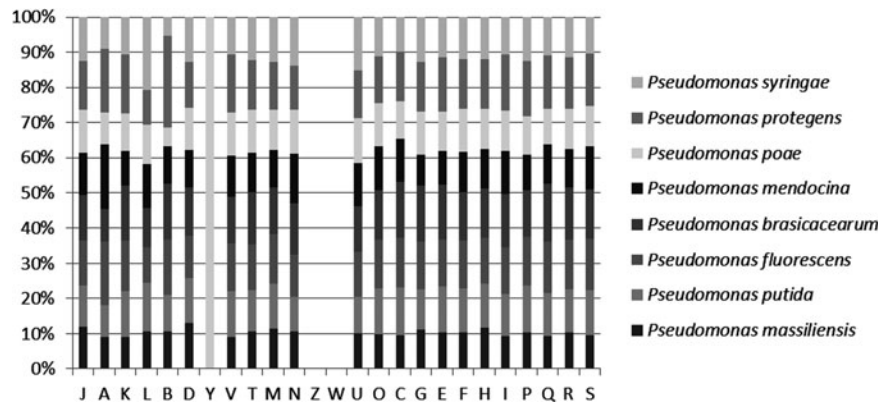


FIG. 10. Distribution of genes into COGs functional categories of every compared genome. COGs, Clusters of Orthologous groups.

6.44, 6.18, 5.07, and 5.51 Mb, respectively. The G+C content of *P. massiliensis* CB1^T (62.34%) is lower compared with *P. protegens* (63.30%) and *P. mendocina*, (64.68%), but higher compared with *P. syringae* (57.91%), *P. brassicacearum* (60.79%), *P. fluorescens* (60.52%), *P. putida* (61.52%), and *P. poae* (60.85%).

The gene content of *P. massiliensis* CB1^T (4239 genes) is smaller than those of the compared species, ranging from 4594 to 6108 genes (Table 5) and *P. massiliensis* CB1^T shared a maximum of 2691 orthologous genes with *P. brassicacearum* (63.5%). The distribution of genes into COG functional categories of every compared genome is presented in Figure 10.

The AGIOS values for *P. massiliensis* strain CB1^T and studied *Pseudomonas* species are summarized in Table 5 and ranged from 74.02% to 82.93%, confirming its new species status. The dDDH values of those species are represented in Table 6 and range from 17.4% ± 2.54% to 22.8% ± 2.97% among all the studied species, and from 18.3% ± 2.54% (*Pseudomonas caeni*) to 21.1% ± 2.73% (*P. alcaligenes*) between *P. massiliensis* CB1^T and the others.

Discussion

The development of high-throughput sequencing gave access to a large quantity of information, but it also generates gaps with genomes corresponding to previously uncultivable bacteria (Marx, 2016). The return to culture-based methods was necessary to isolate such bacterial species and study their properties and behavior (Browne et al., 2016). Culturomics was developed to explore the diversity of human microbiota and has the capacity to detect viable bacteria and minority populations (Lagier et al., 2015).

This high-throughput culture method, combined to taxonomics strategy and applied to human samples from various regions of the world, enabled the identification of 1057 different bacterial species from the human gut microbiota, including 187 that had never previously been found in human samples and 197 putative new species (Lagier et al., 2016), of which 47 have officially validated names, such as *Enterococcus massiliensis* (Le Page et al., 2016), *Bacillus testis* (Cimmino et al., 2016), *Kallipyga gabonensis* (Hugon et al., 2013), *Enterobacter massiliensis* (Lagier et al., 2013) or *Clostridium ihumii* (Merhej et al., 2015), and also new genera

Drancourtella massiliensis (Durand et al., 2016) or *Beduini massiliensis* (Mourembou et al., 2015).

In this study, we report the identification and description of *P. massiliensis* sp. nov., represented by the strain CB1^T, isolated from the fecal flora of an obese 37-year-old Brazilian woman using this strategy. *P. massiliensis* (ma.si.li.e'n.sis. L. gen. masc. n. *massiliensis*, of Massilia, the Latin name of Marseille where *P. massiliensis* was first isolated) was deposited in two different collections of microorganisms, the Deutsche Sammlung von Mikroorganismen und Zellkulturen and the Collection de Souches de l'Unité des Rickettsies (CSUR) under numbers DSM 29075 and CSUR P1334, respectively. The 16S rRNA and genome sequences were deposited in GenBank under accession numbers LK985396 and CCYK01000000, respectively. *P. massiliensis* was then isolated in *Anopheles gambiae* Giles and *Culex quinquefasciatus* mosquitoes collected in Mali (Tandina et al., 2016).

The genus *Pseudomonas* was first proposed by Migula 1894, belongs to the family of *Pseudomonadaceae*, and is one of the most ubiquitous bacterial genera in the world (Peix et al., 2009), characterized by gram negative, motile, and aerobic species. Few species of the genus *Pseudomonas* are human opportunistic pathogens, mostly represented by *Pseudomonas aeruginosa* that causes wound infections or severe infections in immunocompromised patients, mainly those with cystic fibrosis, with its biofilm formation (Lee and Yoon, 2017).

This study attests the usefulness of the culturomics approach in the characterization of microbial communities. This work intend to extend the knowledge about the repertoire of microorganisms that colonize the human gut to better understand the composition of its microflora and link it to human health (Hugon et al., 2015, 2017; Lagier et al., 2012b). For such purposes, culturomics and metagenomics are complementary techniques to explore the human microbiota (Marx, 2016).

Conclusions and Outlook

Culturomics, and the microbiome science more generally, are anticipated to transform global health diagnostics and inform the ways in which gut microbial diversity contributes to human health and disease, and by extension, to personalized medicine.

TABLE 6. PAIR-WISE COMPARISON OF *P. MASSILIENSIS* WITH SIX OTHER SPECIES USING GGDC, FORMULA 2 (DDH ESTIMATES BASED ON IDENTITIES/HSP LENGTH)

	Pseudomonas massiliensis	Pseudomonas agarici	Pseudomonas alcaligenes	Pseudomonas balearica	Pseudomonas caeni	Pseudomonas aeruginosa	Clostridium butyricum
<i>Pseudomonas massiliensis</i>	100% ± 00%	20.9% ± 2.72%	21.1% ± 2.73%	20.9% ± 2.67%	18.3% ± 2.54%	20.9% ± 2.70%	19.9% ± 2.52%
<i>Pseudomonas agarici</i>		100% ± 00%	21.1% ± 2.76%	20.4% ± 2.68%	18.3% ± 2.54%	20.7% ± 2.5%	19.7% ± 2.52%
<i>Pseudomonas alcaligenes</i>			100% ± 00%	21.7% ± 2.83%	17.7% ± 2.54%	22.8% ± 2.97%	19.7% ± 2.52%
<i>Pseudomonas balearica</i>				100% ± 00%	17.4% ± 2.54%	21.3% ± 2.83%	19.3% ± 2.52%
<i>Pseudomonas caeni</i>					100% ± 00%	18.2% ± 2.53%	18.3% ± 2.52%
<i>Pseudomonas aeruginosa</i>						100% ± 00%	19.3% ± 2.52%
<i>Clostridium butyricum</i>							100% ± 00%

The confidence intervals indicate the inherent uncertainty in estimating DDH values from intergenomic distances based on models derived from empirical test data sets (which are always limited in size). These results are in accordance with the 16S rRNA (Fig. 1) and phylogenomic analyses, as well as the GGDC results. DDH, DNA-DNA hybridization.

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Author Disclosure Statement

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Abbreviations Used

AGIOS = average genomic identity of
orthologous gene sequences
ARG-ANNOT = Antibiotic Resistance
Gene-ANNOTation
BUR = Bacteriocins of the URMITE
COG = Clusters of Orthologous groups
CSUR = Collection de Souches de l'Unité
des Rickettsies
DDH = DNA–DNA hybridization

DSM = Deutsche Sammlung von
Mikroorganismen
MALDI-TOF MS = matrix-assisted laser desorption/
ionization time-of-flight mass
spectrometry
NCBI = National Center for Biotechnology
Information
NRPS = nonribosomal peptide
synthetase
PKS = polyketide synthases

CONCLUSION

La description récente des gènes de résistance à la colistine portés par un plasmide a soulevé des inquiétudes parmi la communauté scientifique internationale concernant le manque de nouveaux antibiotiques. Le dépistage mondial de souches porteuses de ces gènes plasmidiques témoigne de la haute capacité de transférabilité de la résistance. Il est ainsi temps de mettre en place un protocole adapté pour détecter efficacement les souches résistantes à la colistine en laboratoire de microbiologie clinique.

Les méthodes phénotypiques indiquent au microbiologiste la présence de souches résistantes aux polymyxines, mais ne définissent pas le mécanisme impliqué et le risque de transmission. Les méthodes moléculaires sont rapides et plus sensibles mais sont spécifiques des gènes de résistance examinés, or les mécanismes moléculaires conférant la résistance aux polymyxines sont nombreux. Enfin, l'analyse génomique permet le dépistage complet des gènes de résistance présents dans les bactéries génétiquement identifiées à partir d'échantillons cliniques, mais constitue une étude *in silico*, permettant de prédire et non d'observer la résistance. Ainsi, les méthodes phénotypiques et moléculaires sont complémentaires dans le dépistage des pathogènes résistants à la colistine afin d'analyser le comportement de l'isolat clinique.

Le milieu de culture LBJMR et le kit UMIC Colistine sont des outils de détection efficaces et pourraient s'inscrire dans un protocole de dépistage de souches d'EPC résistantes à la colistine. Les échantillons peuvent être mis en culture directement sur le milieu LBJMR, et les isolats obtenus peuvent être directement identifiés par MALDI-TOF. Leur CMI peut être rapidement déterminée avec le kit UMIC Colistine. En parallèle, la recherche des gènes *mcr* pourrait s'effectuer par qPCR ou par séquençage du génome. Ces méthodes devraient être évaluées sur des souches hétérorésistantes afin de connaître leur sensibilité pour la détection de toutes les souches résistantes aux polymyxines.

En conclusion, ces nouvelles techniques doivent être combinées pour évaluer une connaissance complète de la résistance à la colistine, en particulier pour les souches de MCR, afin d'aider le clinicien à adapter le traitement ou à isoler les patients porteurs.

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ANNEXE : AUTRE PUBLICATION SCIENTIFIQUE

***mcr-1* plasmid-mediated colistin resistance gene detection in an *Enterobacter cloacae* clinical isolate in France.** Baron S, Bardet L, Dubourg G, Fichaux M, Rolain J-M. 2017. J Glob Antimicrob Resist **10**:35–36. (IF = 1.087)



Letter to the Editor

***mcr-1* plasmid-mediated colistin resistance gene detection in an *Enterobacter cloacae* clinical isolate in France**


Keywords:

mcr-1 gene

Colistin

Antimicrobial resistance

Sir,

The plasmid-mediated colistin resistance gene *mcr-1* and its variant *mcr-2* (sharing 76.5% homology) were recently described but already appeared to have spread worldwide [1], modifying our understanding of colistin resistance. In University Hospital La Timone (Marseille, France), a diagnostic strategy was developed for antibiotic susceptibility testing of *Enterobacteriaceae* consisting of testing a panel of 12 antibiotics that enable clinicians to treat most infections. However, when a strain is susceptible to less than three antibiotics in the panel, last-line antibiotics and old antibiotics are tested, including tigecycline, minocycline, fosfomycin and colistin. This was the case for an *Enterobacter cloacae* isolated from an abdominal drain of a 50-year-old woman from Algeria hospitalised in Marseille, France, who had breast cancer with metastases, peritoneal carcinomatosis and ascites. During her hospitalisation she received ceftriaxone, metronidazole, clindamycin and trimethoprim/sulfamethoxazole for an intra-abdominal abscess due to *Staphylococcus aureus*. An abdominal drain was inserted but subcutaneous fluid was collected, from which *E. cloacae* was isolated. However, because the patient's condition did not deteriorate, antibiotic treatment remained unchanged. The *E. cloacae* strain had an extended-spectrum β -lactamase (ESBL) profile and was highly resistant to antibiotics from the first panel, with the exception of imipenem and amikacin (Table 1). Further analysis showed that it was also resistant to colistin [minimum inhibitory concentration (MIC)=2 mg/L by Etest and 4 mg/L by broth microdilution]. Given this atypical ESBL profile and the low colistin resistance MIC, the diagnostic strategy was to screen for the presence of the *mcr-1* gene using a new real-time PCR assay as well as standard PCR and sequencing [3]. These tests were positive. The presence of a *bla*_{TEM-1} and *bla*_{CTX-M-15} gene confirmed the ESBL profile. The search for carbapenemases with the Carba NP test and real-time quantitative PCR (qPCR) for *bla*_{OXA-48}, *bla*_{NDM} and *bla*_{KPC} genes was negative, suggesting that resistance to ertapenem and meropenem was linked to loss of porins. The multilocus sequence typing (MLST) profile (<http://pubmlst.org/ecloacae/>) showed that this strain belonged to the ST114 clone. Conjugation tests showed that the *mcr-1* gene was transferable to *Escherichia coli* J53RAZ. No faecal samples were available for investigation of carriage, and no known contact with animals was found. To the best of our

knowledge, *mcr-1* in *E. cloacae* clinical isolates has only been described in Asia [1,4,5].

In France, *mcr-1*-positive strains have only been reported to date among *E. coli* in animals and food [1] but as well as in clinical strains of *Klebsiella pneumoniae* [1]. Although the patient in the current study was from Algeria where *mcr-1* has already been described in *E. coli* [1], she had not travelled to this country for several years. This shows that there is a need to establish an *mcr-1* and *mcr-2* gene screening strategy. This report shows that its prevalence may be underestimated, as routine screening does not exist and colistin susceptibility is not tested in most European countries. Moreover, the *in vivo* impact of *mcr-1*-mediated colistin resistance is still unknown. It would seem appropriate to establish a screening strategy for patients carrying multidrug-resistant (MDR) bacteria for whom therapeutic strategies are already limited. In such cases, appropriate diagnostics tools should be used. Development of a universal agar plate to screen for polymyxin resistance in Gram-negative bacteria is a first solution [6]. This tool continues, however, to present some limitations as it has only been tested directly on strains and spiked stools inoculated with known strains and has not yet been tested routinely. In our laboratory, we developed a medium that presents good results with fresh stools and strains (unpublished data) that will be soon tested routinely. The combination of colistin MIC determination and *mcr-1* real-time qPCR [3] could confirm the diagnosis of plasmid-mediated colistin resistance, following the same strategy already established for resistance to carbapenems. However, given the frequent low level of MIC to colistin and the consequent risk of a false-negative result, direct molecular screening may be more appropriate. This screening could take place in a point-of-care laboratory using an 'MDR kit' to establish, as early as possible, the necessary isolation measures to be taken. Routine detection of plasmid-mediated colistin resistance raises questions regarding the management of patients who carry *mcr-1*-positive strains. Plasmid-mediated resistance involves a risk of transmission to patients. Plasmid-mediated *mcr-1* colistin resistance was first described in animals and it has been shown that this resistance is transferable to humans [1]. We also know that strains can be transferred from animals to humans [1]. Some countries widely use colistin for animal growth-promotion purposes [1] and this consumption may have an impact on colistin resistance in humans. Isolation of patients carrying *mcr-1*-positive strains should be the rule in order to prevent the spread of colistin resistance, further limiting therapeutic options for MDR bacterial infections.

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Table 1
Summary of *mcr-1*-positive *Enterobacter* spp. strains, patient characteristics and antibiotic susceptibility.

Strain	Country	Sample	Patient age (years)	Underlying conditions	Clinical symptoms	Colistin MIC (mg/L)	AST (susceptible antibiotics)	Reference
<i>E. cloacae</i> complex	Hong Kong (China)	Stool sample	55	Acute myeloid leukaemia	Asymptomatic colonisation	64	Carbapenem ^a	[5]
<i>E. aerogenes</i>	Guangzhou (China)	Vaginal secretions	37	Cervical cerclage	Vaginal infection	16	Carbapenems ^b	[2]
<i>E. cloacae</i>	Guangzhou (China)	Urine	70	–	UTI	>32	– ^b	[2]
<i>E. cloacae</i>	Zhejiang/Guangdong (China)	Unknown	NS	NS	Infection	NS	NS	[4]
<i>E. aerogenes</i>	Zhejiang/Guangdong (China)	Unknown	NS	NS	Infection	NS	NS	[4]
<i>E. cloacae</i>	Zhejiang/Guangdong (China)	Unknown	NS	NS	Colonisation	NS	NS	[4]
<i>E. cloacae</i>	Marseilles (France)	Subcutaneous fluid of abdominal drain	50	Breast cancer	Subcutaneous abdominal fluid collection	2	Imipenem, amikacin, nitrofurantoin, tigecycline ^c	This study

MIC, minimum inhibitory concentration; AST, antibiotic susceptibility testing; UTI, urinary tract infection; NS, not specified.

^a Only this antibiotic was cited in the article.

^b Susceptible antibiotics among the following antibiotics tested: ampicillin; amoxicillin/clavulanic acid; cefotaxime; ceftazidime; cefepime; carbapenems; gentamicin; ciprofloxacin; polymyxin B; and colistin.

^c Susceptible antibiotics among the following antibiotics tested: amoxicillin; amoxicillin/clavulanic acid; ticarcillin/clavulanic acid; piperacillin/clavulanic acid; ceftiofur; oxacillin; ceftriaxone; cefotaxime; cefepime; imipenem; ertapenem; meropenem; aztreonam; amikacin; tobramycin; gentamicin; trimethoprim/sulfamethoxazole; nalidixic acid; ciprofloxacin; nitrofurantoin; fosfomicin; rifampicin; doxycycline; minocycline; tigecycline; colistin; streptomycin; spectinomycin; and sulfadiazine.

Competing interests

None declared.

Ethical approval

Not required.

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SERMENT DE GALIEN

Je jure, en présence de mes maîtres de la Faculté, des conseillers de l'Ordre des pharmaciens et de mes condisciples :

- ❖ D'honorer ceux qui m'ont instruit dans les préceptes de mon art et de leur témoigner ma reconnaissance en restant fidèle à leur enseignement.*
- ❖ D'exercer, dans l'intérêt de la santé publique, ma profession avec conscience et de respecter non seulement la législation en vigueur, mais aussi les règles de l'honneur, de la probité et du désintéressement.*
- ❖ De ne jamais oublier ma responsabilité et mes devoirs envers le malade et sa dignité humaine, de respecter le secret professionnel.*
- ❖ En aucun cas, je ne consentirai à utiliser mes connaissances et mon état pour corrompre les mœurs et favoriser des actes criminels.*

Que les hommes m'accordent leur estime si je suis fidèle à mes promesses.

Que je sois couvert d'opprobre, méprisé de mes confrères, si j'y manque.

RÉSUMÉ

La découverte récente de *mcr-1*, premier gène de résistance à la colistine porté par un plasmide est cliniquement préoccupante en raison de sa grande capacité de transfert entre bactéries ainsi que des niveaux bas de résistance qu'il confère. Cette découverte met ainsi en évidence le besoin urgent d'améliorer la détection des isolats résistants à la colistine dans les laboratoires de microbiologie clinique, afin de pouvoir rapidement isoler les patients porteurs.

L'objectif de ma thèse a ainsi été de répondre à cette problématique par le développement et l'évaluation d'outils spécifiques afin de permettre la détection de toutes les bactéries résistantes à la colistine, y compris celles porteuses des gènes plasmidiques *mcr*. Une revue a été rédigée, visant à résumer les nouveaux outils développés pour détecter la résistance aux polymyxines, y compris la présentation des méthodes actuelles phénotypiques, antibiogrammes et génotypiques.

Le milieu de culture LBJMR a été développé pour détecter toutes les bactéries à Gram négatif résistantes à la colistine et également les souches d'entérocoques résistants à la vancomycine, qui représentent un autre problème majeur de santé publique. Le milieu LBJMR a été évalué avec un échantillon représentatif de souches bactériennes puis en procédant au dépistage de souches porteuses du gène *mcr-1* dans des échantillons de selles d'origine humaine. Le milieu LBJMR est polyvalent et a permis de dépister différentes 3 bactéries d'intérêt clinique à partir d'un même échantillon: *E. coli* et *K. pneumoniae* résistantes à la colistine, et *E. faecium* résistant à la vancomycine, et a donné lieu à un dépôt de brevet.

La méthode de référence pour déterminer la CMI est difficile à mettre en œuvre dans les laboratoires de routine. Le kit UMIC Colistine est un dispositif commercial prêt-à-l'emploi basé sur la méthode de référence. Il a été évalué conformément à la norme ISO 20776, sa sensibilité était excellente pour la détection des souches porteuses du gène *mcr-1*. Le système UMIC Colistine constitue ainsi une méthode rapide et fiable pour évaluer la CMI à la colistine des isolats cliniques dans les laboratoires de microbiologie clinique.

Enfin, mes travaux de thèse m'ont également amenés à analyser une souche de *K. pneumoniae* hétérorésistante à la colistine et à décrire une nouvelle espèce bactérienne : *Pseudomonas massiliensis*.