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Spread of MCR-3 Colistin Resistance in China: An Epidemiological, Genomic and Mechanistic Study

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ABSTRACT

Background: Mobilized resistance to colistin is evolving rapidly and its global dissemination poses a severe threat to human health and safety. Transferable colistin resistance gene, *mcr-3*, first identified in Shandong, China, has already been found in several countries in multidrug-resistant human infections. Here we track the spread of *mcr-3* within 13 provinces in China and provide a complete characterization of its evolution, structure and function.

Methods: A total of 6497 non-duplicate samples were collected from thirteen provinces in China, from 2016 to 2017 and then screened for the presence of *mcr-3* gene by PCR amplification. *mcr-3*-positive isolates were analyzed for antibiotic resistance and by southern blot hybridization, transfer analysis and plasmid typing. We then examined the molecular evolution of MCR-3 through phylogenetic analysis. Furthermore, we also characterized the structure and function of MCR-3 through circular dichroism analyses, inductively coupled plasma mass spectrometry (ICP-MS), liquid chromatography mass spectrometry (LC/MS), confocal microscopy and chemical rescue tests.

Findings: 49 samples (49/6497 = 0.75%) were *mcr-3* positive, comprising 40 samples (40/4144 = 0.97%) from 2017 and 9 samples (9/2353 = 0.38%) from 2016. Overall, *mcr-3*-positive isolates were distributed in animals and humans in 8 of the 13 provinces. Three *mcr-3*-positive IncP-type and one *mcr-1*-bearing IncHI2-like plasmids were identified and characterized. MCR-3 clusters with PEA transferases from *Aeromonas* and other bacteria and forms a phylogenetic entity that is distinct from the MCR-1/2/P(M) family, the largest group of transferable colistin resistance determinants. Despite that the two domains of MCR-3 not being exchangeable with their counterparts in MCR-1/2, structure-guided functional mapping of MCR-3 defines a conserved PE-lipid recognizing cavity prerequisite for its enzymatic catalysis and its resultant phenotypic resistance to colistin. We therefore propose that MCR-3 uses a possible "ping-pong" mechanism to transfer the moiety of PEA from its donor PE to the 1(or 4′)-phosphate of lipid A via an adduct of MCR-3-bound PEA. Additionally, the expression of MCR-3 in *E. coli* prevents the colistin-triggered formation of reactive oxygen species (ROS) and interferes bacterial growth and viability.

Interpretation: Our results provide an evolutionary, structural and functional definition of MCR-3 and its epidemiology in China, paving the way for smarter policies, better surveillance and effective treatments.

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Research in Context

Evidence Before this Study

On July 18, 2018, we searched PubMed with the terms "*mcr-3* and China [21 from local epidemiology]", "MCR-3 and function [1 references]", "MCR-3 and evolution [no references]", for reports published between January 2000 and July 2018. We did not restrict our search by language of publication. Our search identified some group reported the sporadic cases of MCR-3 in very confined area and very limited in number in China. Evidently, comprehensive epidemiology of MCR-3 remains unclear in China. More importantly, we found no reports addressing mechanisms of MCR-3 action. Therefore, it is very necessary to elucidate its potential spread, evolution and functional aspects of MCR-3 polymyxin resistance.Added Value of this Study

Our results represent a first multi-province study on the dissemination of MCR-3 in China. Also, we report the origin and possible evolution of MCR-3. We have integrated multiple approaches to systematically address the biochemical mechanism and physiological roles of MCR-3 action.Implications of all the Available Evidence

Our data shows that 49(49/6497 = 0.75%) isolates from 13 provinces in China, comprising 40 samples (40/4144 = 0.97%) from 2017 and 9 samples (9/2353 = 0.38) from 2016, were *mcr-3* positive. This study suggests that the threat of *mcr-3* to public health should be assessed because of the potential prevalence of *mcr-3*. In addition, given that the resistance mechanism of MCR-3 is similar to *mcr-1*, we suggest that further studies are needed to clarify the evolutionary pattern of *mcr-3*.

1. Introduction

Antimicrobial resistance (AMR) has become a global public health priority. The accelerated development of multidrug resistance (MDR) is attributed in part (if not completely) to the massive and inappropriate use of antimicrobials in agricultural and clinical settings. Human infections caused by MDR pathogens result in over 70,000 deaths in the United States each year [1, 2]. In fact, a team led by Prof. Lord Jim O'Neil has estimated that AMR could result in 10 million deaths a year worldwide by 2050 [3]. Although the accuracy of this frightening prediction is uncertain, we acknowledge the enormous burden AMR causes at multiple levels (economic, social, clinical and public health) [4]. This highlights the importance and urgency of a coordinated international action to prevent and control the worldwide spread of AMR [4, 5].

Polymyxins refer to an array of non-ribosomally-synthesized, cationic antimicrobial cyclic-peptides (CAMP) [6]. Among the five known subtypes, polymyxin B and polymyxin E (Colistin) are extensively used in agricultural production and clinical therapies [6–8]. Historically, the primary target of colistin is thought to be the negatively charged lipid A moiety of lipopolysaccharides (LPS) on the outer-leaflet of the bacterial outer membrane [9]. Despite its potential nephrotoxicity and neurotoxicity [6, 10–12], colistin is still used for treatment as an ultimate line of defense against critical infections caused by MDR pathogens (esp. carbapenemase-producing *Enterobacteriaceae*) [7, 13, 14]. However, an acquired resistance to polymyxin has been frequently found in certain species of bacterial pathogens like *Klebsiella pneumoniae* (*K. pneumoniae*) [15] and *Salmonella enterica* (*S. enterica*) [16, 17]. The chemical mechanism underlying the colistin resistance consistently involves bacterial lipid A-centered surface remodeling, including i) The addition of 4-amino-4-deoxy-L-arabinose in *S. enterica* [16, 17] and *Pseudomonas aeruginosa* [18]; ii) The attachment of phosphoethanolamine (PEA) in *Neisseria* [19], *Acinetobacter baumannii* [20] and *Campylobacter jejuni* [21]; and iii) Glycine/diglycine modification in the pandemic *Vibrio cholerae* biotype EI Tor [22–25]. Intrinsic resistance to polymyxin is limited to the originally-resistant population. However, the recent emergence and global discovery of plasmid-borne mobilized colistin resistance determinants (*mcr-1*) potentially threatens the clinical effectiveness of colistin as a last-resort antibiotic against carbapenem-resistant superbugs [26].

The *mcr-1* gene product, MCR-1, is a PEA lipid A transferase, belonging to the "YhjW/YjdB/YijP" alkaline phosphatase super-family [26, 27]. MCR-1 catalyzes the transfer of the PEA group from its physiological donor phosphatidylethanolamine (PE) to the 1(4′)-phosphate position of lipid A glucosamine (GlcN) moieties [19, 28, 29]. Structure-guided functional studies have determined this mechanism and demonstrated that the enzymatic activity of MCR-1 renders the recipient strains resistant to polymyxin [27, 30–35]. Intriguingly, the determinants of transferable colistin resistance have extended beyond MCR-1, to a number of new MCR-like members [36] (namely MCR-2 [37–39], MCR-3 [40], MCR-4 [41], MCR-5 [42], MCR-6 [Genbank no.: ASK49942] (Indeed, it is a MCR-1/2 progenitor from *Moraxella* sp. MSG47-C17 [43], and exhibits high level of homology to ICR-Mo of *M. osloensis* [44]. Thus, it is supposed to be renamed as ICR-M), MCR-7 [45] and MCR-8 [46]), as well as over a dozen of new heterogeneous MCR-1 variants (e.g., MCR-1.2 [47] and MCR-1.6 [48]). Unlike the predominant MCR-1 which is distributed world-wide [49], both MCR-2 (81% identity to MCR-1 and originally found in Belgium [37, 38], and very recently detected in pigs/poultries [39] and human vaginal swabs [50] from China) and MCR-5 (only detected in Germany [42]) are thought to be two rare members of the MCR-like protein family. This is slowly changing with the discovery of MCR-2 and its variants in countries like China [39]. As for MCR-4, it has been detected in a pig isolate of *S. enterica* in Italy 2013 [41], swine isolates of *E. coli* from Spain and Belgium in 2015–2016 [41], and clinical isolates of carbapenemase-producing *Enterobacter cloacae* from Singapore in 2017 [51]. In terms of epidemiological/geographic distribution, MCR-3 seems to be second only to MCR-1. Phylogenetic analysis indicates that MCR-3 is evolutionarily distinct from MCR-1 and closely clustered with chromosomally-encoded MCR-like proteins in certain species of *Aeromonas* (Fig. 3) [52, 53]. To the best of our knowledge, the new *mcr-3* gene has been discovered in 3 of 7 continents, namely Asia (China [40, 54], Singapore [51], Japan [55], Thailand [40] and Malaysia [40]), Europe (Denmark [56, 57], France [58] and Spain [59]) and North America (the United States [40]). Given that i) In Europe, colistin is used to treat bacterial infections of livestock (such as pigs, cows, and goats) [60]; and ii) Colistin is heavily supplemented as a growth promoter of livestock (pigs and poultries) in Asian countries (e.g., China, Japan, and Vietnam) [61], it is possible that indiscriminate antibiotic use has selected for the emergence of new colistin resistance determinants like *mcr-3*.

In fact, the growing body of *mcr-3* variants includes *mcr-3.2* [D295E] [40], *mcr-3.3* [G373V] [40], *mcr-3.4* [M23V], *mcr-3.5* [T488I] [57, 58], and *mcr-3.7* [M23V, A457E, T488I] [54]. Most of them are located on IncHI2-type plasmids, like pWJ-1 (~260 kb), IncP-like plasmid (~50 kb), F46: A-: B20 IncF plasmid [58], respectively. The range of *mcr-3*-harboring host bacteria includes *E. coli* [40, 54, 57–59], *K. pneumoniae* [40] and *S. typhimurium* [40, 56]. Of note, *mcr-3*-bearing bacteria in Europe are frequently prevalent in either human infections [40, 56] (even bloodstream infections [57]) or epidemic MDR lineages of *E. coli* (ST744 [58] and ST131 [57]). To our surprise, *mcr-3* has been found to coexist with *mcr-1* [56, 59] (or *mcr-4* [51]) in a single cell, in MDR pathogens [56,

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