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# Colistin resistance in ESBL- and Carbapenemase-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates in Cambodia

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## ABSTRACT

**Objectives:** Despite the critical importance of colistin as a last-resort antibiotic, limited studies have investigated colistin resistance in human infections in Cambodia. This study aimed to investigate the colistin resistance and its molecular determinants among Extended-spectrum beta-lactamase (ESBL)- and carbapenemase-producing (CP) *Klebsiella pneumoniae* (*K. pneumoniae*) and *Escherichia coli* (*E. coli*) isolated in Cambodia between 2016 and 2020.

**Methods:** *E. coli* ( $n = 223$ ) and *K. pneumoniae* ( $n = 39$ ) were tested for colistin minimum inhibitory concentration (MIC) by broth microdilution. Resistant isolates were subjected to polymerase chain reaction (PCR) for detection of mobile colistin resistance genes (*mcr*) and chromosomal mutations in the two-component system (TCS).

**Results:** Eighteen isolates (10 *K. pneumoniae* and 8 *E. coli*) revealed colistin resistance with a rate of 5.9% in *E. coli* and 34.8% in *K. pneumoniae* among ESBL isolates, and 1% in *E. coli* and 12.5% in *K. pneumoniae* among CP isolates. The resistance was associated with *mcr* variants (13/18 isolates, *mcr-1*, *mcr-3*, and *mcr-8.2*) and TCS mutations within *E. coli* and *K. pneumoniae*, with the first detection of *mcr-8.2* in Cambodia, the discovery of new mutations potentially associated to colistin resistance in the TCS of *E. coli* (PhoP I47V, PhoQ N352K, PmrB G19R, and PmrD G85R) and the co-occurrence of *mcr* genes and colistin resistance conferring TCS mutations in 11 of 18 isolates.

**Conclusions:** The findings highlight the presence of colistin resistance in ESBL- and CP- *Enterobacteriaceae* involved in human infections in Cambodia as well as chromosomal mutations in TCS and the emergence of *mcr-8.2* in *E. coli* and *K. pneumoniae*. It underscores the need for continuous surveillance, antimicrobial stewardship, and control measures to mitigate the spread of colistin resistance.

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## 1. Introduction

Colistin (known as polymyxin E) is a cationic antimicrobial peptide and belongs to the antimicrobial class designated polymyxins that originates from the bacteria *Paenibacillus polymyxa*. Colistin is considered a last-line antibiotic for the treatment of infections in humans caused by multidrug-resistant (MDR) Gram-negative

bacteria, but the emergence of colistin-resistant strains worldwide compromise its use [1]. In humans, colistin was previously discontinued because of its nephrotoxicity and neurotoxicity [2,3]. However, with the rise of MDR Gram-negative infections, which have become a global concern and the need for effective antimicrobials, the therapeutic potential of colistin is being reassessed. As the last resort antibiotic, the emergence of colistin resistance in Gram-negative bacteria poses a significant threat to public health, and understanding its prevalence and mechanisms is crucial for effective antimicrobial stewardship.

Colistin's main mechanism of action is the disruption of bacterial membranes leading to severe alterations involving their

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permeability. It has been shown that colistin increases permeability, not only by altering the bilayer structure but also by acting on membrane fluidity, having an effect on the reduction of efflux pump activities [2].

From a mechanistic point of view, the resistance to colistin could be due to both *mcr* genes and the two-component system (TCS) modifications. The dissemination of colistin resistance has received increased attention since a mobilized gene conferring resistance to colistin, *mcr-1*, was described in *Enterobacteriaceae* from humans and food-producing animals in China in 2015 [4]. Additional gene homologs have since been identified (*mcr-1* to *mcr-10*) [5–7]. The *mcr* genes encode phosphoethanolamine (PEtN) transferase enzyme that adds PEtN to lipid A, and consequently results in a more cationic lipopolysaccharide (LPS). Through horizontal and vertical transfers, *mcr* genes have spread widely into diverse environmental niches. These ecosystems constitute underestimated vast reservoirs (“phantom resistome”) of these *mcr* genes [5]. In addition, various chromosomal mutations associated with colistin resistance leading to modifications in PmrA/PmrB and PhoQ/PhoP proteins (TCS), and the inactivation of the MgrB protein decreases the affinity of colistin for the LPS [8]. The 144-bp *mgrB* gene encodes a small transmembrane protein that exerts negative feedback on the above-mentioned pathway by interaction with the sensor kinase PhoQ at the periplasmic level [9].

Worldwide, colistin is still widely used in livestock as treatment, metaphylaxis, prophylaxis, and growth promotion [10] and the indiscriminate use of colistin has led to the development of resistance. In Cambodia, the circulation of colistin resistance in the human population is still poorly studied and colistin is (i) one of the most commonly used antibiotics in small-scale urban pig farms and (ii) in poultry farms in a packaged amoxicillin-colistin mixture for respiratory and gastrointestinal infections [11–14]. From a mechanistic point of view, up to now, few mechanisms

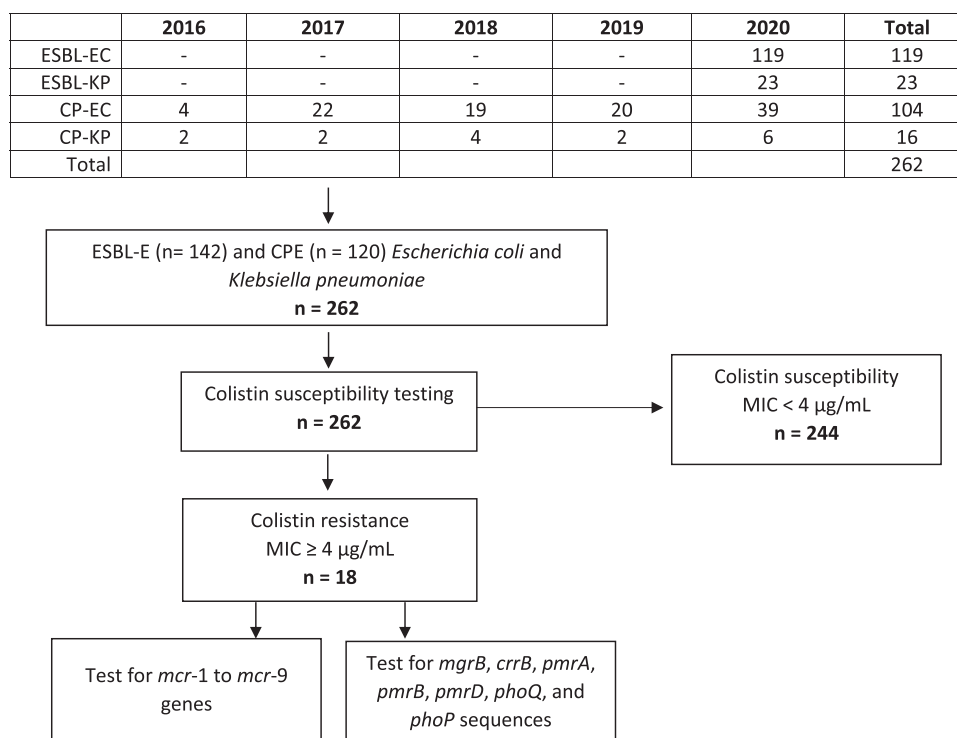
of colistin resistance were reported in Cambodia, only *mcr-1* and *mcr-3* have been identified from MDR *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*) isolated from humans, poultry, and pigs, and no data are available concerning TCS [14,15].

In this context, we aim to fill a knowledge gap by exploring colistin resistance and the molecular determinants contributing to colistin resistance among a collection of Extended Spectrum beta-lactamase (ESBL) and Carbapenemase producing (CP) *E. coli* and *K. pneumoniae* isolates from the Medical Biology Laboratory of the Institut Pasteur du Cambodge (MBL-IPC), Cambodia between 2016 and 2020.

## 2. Materials and methods

### 2.1. Bacterial isolates

A panel of 262 ESBL and/or CP isolates of *E. coli* and *K. pneumoniae* was isolated at the MBL-IPC from patients who presented at various public and private health care facilities in Cambodia. ESBL-*E. coli* ( $n = 119$ ), ESBL-*K. pneumoniae* ( $n = 23$ ), CP-*E. coli* ( $n = 104$ ), and CP-*K. pneumoniae* ( $n = 16$ ) isolates represent all the ESBL-*E. coli*/*K. pneumoniae* strains isolated at MBL-IPC in 2020, and all the CP-*E. coli*/*K. pneumoniae* strains isolated in 2016 to 2020 (Fig. 1). The isolates were grown from various sample types including urine, blood culture, sputum, and pus and identified by matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker Daltonics). Phenotypic Antibiotic Susceptibility Testing was performed by the disc diffusion method in accordance with the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for the following antibiotics (Bio-Rad): amoxicillin (20  $\mu\text{g}$ ), amoxicillin-clavulanate (20–10  $\mu\text{g}$ ), ticarcillin (75  $\mu\text{g}$ ), mecillinam (10  $\mu\text{g}$ ), cefotaxime (5  $\mu\text{g}$ ), ceftazidime (10  $\mu\text{g}$ ),



**Fig. 1.** Samples collection and experimental design. All the ESBL-E and CPE isolates pertaining to *E. coli* ( $n = 223$ ) or *K. pneumoniae* ( $n = 39$ ) species were submitted to colistin susceptibility testing using MIC and DNA of the 18 colistin resistant isolates were tested for both *mcr* variants and TCS genes. Abbreviations: MBL-IPC, Medical Biology Laboratory of the Institut Pasteur du Cambodge; ESBL-E, Extended Spectrum beta-lactamase producing *Enterobacteriaceae*; CPE, carbapenemase producing *Enterobacteriaceae*; EC, *Escherichia coli*; KP, *Klebsiella pneumoniae*; MIC, minimum inhibitory concentration; *mcr*, mobile colistin resistance; TCS, two-component system.

imipenem (10 µg), ertapenem (10 µg), amikacin (30 µg), gentamicin (10 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), co-trimoxazole 1,25-23,75 µg), nitrofurantoin (100 µg), and fosfomycin (200 µg) [16]. In case of resistance to cefotaxime and/or ceftazidime, both of them pertaining to third-generation cephalosporin (3GC), a second-line antibiotic panel was performed including ticarcillin-clavulanate (75-10 µg), piperacillin-tazobactam (30-6 µg), aztreonam (30 µg), cefepime (30 µg), and tigecycline (15 µg) (Bio-Rad). The phenotypic detection of ESBL was performed by double-disc synergy test: discs containing 3GC (including ceftaxime 5 µg and ceftazidime 10 µg,) and cefepime 30 µg were applied to Mueller-Hinton plates 30 mm next to an amoxicillin-clavulanic acid disc (20-10 µg). A positive result is indicated when the inhibition zones around any of the cephalosporin discs are augmented in the direction of the disc containing clavulanic acid. The same protocol is applied on Mueller-Hinton agar supplemented with 250 mg/L cloxacillin (cephalosporinase inhibitor) to facilitate the detection of ESBL in case of hyper-production of cephalosporinase. All the strains resistant to ertapenem and/or imipenem were previously tested by polymerase chain reaction (PCR) for the presence of genes encoding for carbapenemases (OXA-48-like, NDM, and KPC; Table 1) [17]. All isolates were maintained for prolonged storage at -80 °C in Difco skim milk (Becton Dickinson) medium and glycerol and subcultured for colistin susceptibility testing and DNA extraction.

## 2.2. Colistin susceptibility testing

The minimum inhibitory concentration (MIC) of colistin was determined using broth microdilution according to Clinical Laboratories Standards Institute guidelines [18]. Colistin sulphate powder from Sigma-Aldrich was resuspended at 100 mg/mL and bacterial inoculums were grown at  $5 \times 10^5$  CFU/mL in Mueller-Hinton broth 2 (Sigma, reference item 90922) for 24 h with different colistin concentrations (ranging from 0 to 0.25, 0.5, 1, 2, 4, 8, 16 and 32 µg/mL) [19,20]. Bacterial growth was determined by measuring the optical density of the cultures at 600 nm in Multiskan FC Microplate Photometer (ThermoFisher). *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 25922 were used as controls.

## 2.3. Molecular determinants

To identify the mechanisms of resistance, chromosomal mutations on *mgrB*, *crrB* (only for *K. pneumoniae*), *pmrA*, *pmrB*, *pmrD* (only for *E. coli*), *phoQ*, and *phoP* genes (TCS), and the presence of *mcr* genes, *mcr-1* to *mcr-9* were investigated by PCR and sequencing for isolates exhibiting MICs superior or equal to the epidemiological cutoff values of 4 µg/mL for colistin. DNA extraction was performed on bacterial colonies by the direct boiling method. The colony was diluted in 100 microliters of distilled water in a microtube, incubated at 95 °C for 10 min in a water bath and centrifuged at 10 000 g for 10 min. The DNA was collected by pipetting the supernatant. DNA concentration was estimated by spectrophotometry using a NanoDrop™ 2000 (Thermo Scientific) and stored at -20 °C. *mcr-1* to *mcr-5* were amplified according to Rebelo et al. (2018) and *mcr-6* to *mcr-9* according to Borowiak et al. (2020) [7,21]. Concerning chromosome-mediated colistin resistance (*pmrA*, *pmrB*, *phoP*, *phoQ*, *mgrB*, *pmrD*, and *crrB*), genes were amplified in simplex PCR using the primers described in the Supplementary File S1 [22–25]. Positive PCR obtained in this study were used as positive controls PCR for all the amplifications. PCR products of *mcr* genes and TCS genes were sent to Macrogen, Korea, for capillary electrophoresis sequencing in order to identify *mcr* variants and modifications on the TCS. Sequence data were analysed using MegaX software and submitted to the GenBank database [26].

## 2.4. Statistical analysis

Comparison of categorical variables was performed using a Chi-squared test. A *P* value < 0.05 was considered significant.

## 3. Results

Among the 262 isolates, 223 were identified as *E. coli*, whereas 39 were identified as *K. pneumoniae*. There were 44.4% of the patients who were men, and the average age was 36 years old (ranging from 0 to 104 y). Patients came from 21 different provinces out of a total of 25 (Phnom Penh being the most represented [46.8%]), and presented at 20 different health care facilities. There were 71.4% of the strains that were isolated from urine samples, and 48.7% of the patients have received antibiotic therapy in the last 3 months, including 30.8% of them receiving 3GC and 15.4% receiving carbapenems, whereas 13.5% of them reported travel outside of Cambodia in the last 6 months.

### 3.1. Colistin susceptibility testing

Among the ESBL isolates, 7 isolates of *E. coli* (5.9%) and 8 isolates of *K. pneumoniae* (34.8%) had an MIC superior or equal to 4 µg/mL for colistin, whereas in the CP isolates, colistin-resistance represents 1.0% ( $n = 1$ , *bla*<sub>OXA48</sub>) for *E. coli* and 12.5% ( $n = 2$ , *bla*<sub>NDM</sub>) for *K. pneumoniae* (Table 2). In total, 18 isolates showed a colistin-resistant phenotype (8 *E. coli* and 10 *K. pneumoniae*), mainly associated with the ESBL phenotype ( $P = 0.02$ ) and the *K. pneumoniae* species ( $P < 0.001$ ). MICs of colistin within our samples ranged from 4 to > 32 µg/mL with higher MICs for *K. pneumoniae* compared to *E. coli*.

### 3.2. Characteristics of colistin-resistant isolates

The 18 colistin-resistant isolates were collected within 4 years (from 2017 to 2020) from patients aged 1 y to 73 y and living in 10 provinces (Table 1). Regarding antibiotic treatment, information was available for 15 of the 18 resistant isolates, and 9 patients (60%) had received antibiotic treatment prior to bacterial isolation. Unfortunately, the specific antibiotic classes and names were not available. Concerning travel history, only 1 of the 15 patients (6.7%) reported traveling outside of Cambodia in the last 6 months. Travel information was missing for three patients. Among the colistin-resistant isolates, amikacin was the only antibiotic that remained constantly active on both *E. coli* and *K. pneumoniae*, whereas tigecycline, fosfomycin, and nitrofurantoin remained active on *E. coli* (EUCAST breakpoints for *K. pneumoniae* are currently not available for these 3 drugs; Table 1). Concerning beta-lactam antibiotics among ESBL isolates, only amoxicillin – clavulanate and mecillinam remained inconsistently active (46.7% of resistance for amoxicillin – clavulanate, 36.4% for mecillinam). All the colistin-resistant isolates were also resistant to cefepime, aztreonam (except one CP-*E. coli*) and trimethoprim/sulfamethoxazole.

### 3.3. Molecular determinants

We identified molecular determinants associated with colistin-resistance among the 18 isolates (Table 3). All sequences have been submitted to the GenBank database, and the accession numbers are listed in Supplementary File S2. Our results allowed identifying *mcr*-like genes for 7 among 8 *E. coli* (87.5%) resistant isolates and 6 among 10 *K. pneumoniae* (60%) with the presence of *mcr-1* (*E. coli* + *K. pneumoniae*), *mcr-3* (*E. coli*), and *mcr-8.2* (*E. coli* + *K. pneumoniae*) variants. Among the 6 *mcr*-like genes detected for *K. pneumoniae*, we have been unable to identify 2 of them (ESBL-KP4 and ESBL-KP5) because the sequencing of their multiplex *mcr-6/9*

**Table 1**  
Epidemiological data and antibiotic susceptibility testing of the 18 colistin resistant isolates.

Isolate	MIC µg/mL	Year	Sample	Sex	Age	Residence	Clinic	Health centre	Bacterial species	RM	AMX	AMC	TIC	TCC	PTZ	MEC	CTZ	CTX	FEP	ATM	IPM	ETP	AN	GM	CIP	LVX	SXT	NIF	FOS	TGC
ESBL-KP8	16	2020	Urine	M	8 y	Kandal	UTI	2	KP	ESBL	R	S	R	R	R	S	R	R	R	R	S	S	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP7	16	2020	Urine	M	13 y	Pursat	Leukaemia	2	KP	ESBL	R	S	R	R	R	R	R	R	R	R	S	S	S	S	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP6	8	2020	Urine	M	10 y	Kampot	Leukaemia	2	KP	ESBL	R	R	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP5	4	2020	Urine	M	10 y	Kampot	Leukaemia	2	KP	ESBL	R	S	R	R	R	R	R	R	R	R	S	R	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP4	8	2020	Urine	M	1 y	Kampong Speu	UTI	2	KP	ESBL	R	S	R	R	R	S	R	R	R	R	S	S	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP3	>32	2020	Urine	F	1 y	Siem Reap	Hydronephrosis	3	KP	ESBL	R	R	R	R	R	S	R	R	R	R	S	S	S	R	S	S	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP2	>32	2020	Sputum	M	48 y	Kampong Cham	Pneumonia	1	KP	ESBL	R	R	R	R	R	ND <sup>1</sup>	R	R	R	R	S	S	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-KP1	8	2020	Sputum	F	48 y	Phnom Penh	Pneumonia	1	KP	ESBL	R	R	R	R	R	ND <sup>1</sup>	R	R	R	R	S	S	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
ESBL-EC7	16	2020	Urine	M	4	Phnom Penh	Encephalopathy	2	EC	ESBL	R	S	R	R	R	S	R	R	R	R	S	S	S	R	R	R	R	S	S	S
ESBL-EC6	8	2020	Urine	F	1	Prey Veng	Icterus	2	EC	ESBL	R	S	R	S	S	S	S	R	R	R	S	S	S	S	R	R	R	S	S	S
ESBL-EC5	8	2020	Urine	F	1 y	Prey Veng	Teratoma	2	EC	ESBL	R	R	R	R	R	S	R	R	R	R	S	S	S	R	R	R	R	S	S	S
ESBL-EC4	4	2020	Urine	F	2 y	Kampong Thom	UTI	3	EC	ESBL	R	R	R	R	R	R	R	R	R	R	S	S	S	R	R	R	R	S	S	S
ESBL-EC3	8	2020	Urine	F	18 y	Siem Reap	UTI	3	EC	ESBL	R	S	R	R	R	S	R	R	R	R	S	S	S	R	R	R	R	S	S	S
ESBL-EC2	4	2020	Blood	F	42 y	Phnom Penh	Sepsis	1	EC	ESBL	R	R	R	R	R	ND <sup>1</sup>	R	R	R	R	S	S	S	S	R	R	R	ND <sup>1</sup>	S	S
ESBL-EC1	8	2020	Pus	F	68 y	Phnom Penh	Abscess	1	EC	ESBL	R	S	R	R	S	ND <sup>1</sup>	R	R	R	R	S	S	S	S	R	R	R	ND <sup>1</sup>	S	S
CP-KP2	8	2020	Urine	F	4 y	Svay Rieng	Acute renal failure	2	KP	CPE (NDM)	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
CP-KP1	>32	2017	Urine	M	73 y	Phnom Penh	UTI	1	KP	CPE (NDM)	R	R	R	R	R	R	R	R	R	R	R	R	S	R	R	R	R	NA <sup>2</sup>	NA <sup>2</sup>	NA <sup>2</sup>
CP-EC1	8	2017	Urine	F	36 y	Phnom Penh	UTI	1	EC	CPE (OXA-48)	R	R	R	R	R	R	R	R	R	R	S	S	R	S	R	R	R	S	S	ND

Abbreviations: AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; AN, amikacin; ATM, aztreonam; CIP, ciprofloxacin; CP, carbapenemase producing *Enterobacteriaceae*; CTX, cefotaxime; CTZ, ceftazidime; EC, *Escherichia coli*; ESBL, extended-spectrum beta-lactamase; ETP, ertapenem; F, female; FEP, cefepime; FOS, fosfomycin; GM, gentamicin; IPM, imipenem; KP, *Klebsiella pneumoniae*; LVX, levofloxacin; M, male; MEC, mecillinam; MIC, minimum inhibitory concentration; NA, not applicable; ND, no data; NIF, nitrofurantoin; PTZ, piperacillin-tazobactam; R, resistant; RM, resistance mechanism; S, susceptible; SXT, trimethoprim-sulfamethoxazole; TCC, ticarcillin-clavulanic acid; TIC, ticarcillin; TGC, tigecycline; UTI, urinary tract infection.

ND<sup>1</sup>, mecillinam, and nitrofurantoin have only been tested on strains isolated from urine samples.  
NA<sup>2</sup>, TGC, NIF, and FOS EUCAST breakpoints for *Klebsiella pneumoniae* are currently not available.

**Table 2**  
Rates of colistin resistance within 262 ESBL and CPE isolated at Institut Pasteur du Cambodge (Medical Biology Laboratory).

	<i>Escherichia coli</i> (n = 223)		<i>Klebsiella pneumoniae</i> (n = 39)		Total resistance (n = 262)
	Susceptible	Resistant	Susceptible	Resistant	
ESBL (n = 142)	112	7	15	8	15
%	94.1	5.9	65.2	34.8	10.6
CPE (n = 120)	103	1	14	2	3
%	99.0	1.0	87.5	12.5	2.5
Total (n = 262)	215	8	29	10	18
%	96.4	3.6	74.4	25.6	6.9

Abbreviations: CPE, carbapenemase producing *Enterobacteriaceae*; ESBL, Extended Spectrum beta-lactamase.

products gave a mixed sequence making it impossible to analyse [7].

Investigations on TCS modifications revealed several chromosome-mediated colistin resistances (Table 3). For *E. coli*, six among eight colistin-resistant isolates carried non-synonymous mutations within five among the six studied loci already described in previous studies, leading to amino acid alterations in MgrB, PmrA, PmrB, PmrD, and PhoQ [24,27–31]. The two remaining isolates were only *mcr-1* (ESBL-EC6) and *mcr-8.2* (ESBL-EC7) positive. In addition, we found four novel non-synonymous mutations leading to amino acid alterations in PhoP (I47V), PhoQ (N352K), PmrB (G19R), and PmrD (G85R) that might potentially be related to colistin resistance of *E. coli*. For *K. pneumoniae*, all the colistin-resistant isolates (n = 10) revealed insertion or non-synonymous mutations leading to amino acid alterations already described in previous studies within MgrB, PmrA, PmrB, and CrrB but none within *phoP* and *phoQ* [25,32–39]. It is important to note the co-occurrence of both *mcr* genes and colistin resistance conferring TCS mutations in 5 of 8 *E. coli* and 6 of 10 *K. pneumoniae*.

#### 4. Discussion

The aim of this study was to explore the colistin resistance within ESBL- and carbapenemase-producing *E. coli* and *K. pneumoniae* isolated at the Medical Biology Laboratory of the Institut Pasteur du Cambodge (MBL-IPC), Cambodia, between 2016 and 2020 and to characterize the molecular basis of colistin resistance by exploring both *mcr* variants and TCS genes. Among 262 *E. coli* and *K. pneumoniae* isolates, 18 (6.87%) were colistin-resistant, 13 of which carried *mcr*-like genes. Even though some isolates may not be clinically relevant, as they were obtained from the urine of patients hospitalized for non-infectious reasons, it is noteworthy that these enterobacteria are being carried, sometimes by very young patients.

##### 4.1. Frequency of colistin-resistant isolates

Based on the available patient data, resistant isolates are distributed across various Cambodian provinces and cannot be linked to specific antibiotic treatments or travel activities. Additionally, information regarding patient occupation and specific locations are not available. In the ESBL isolates, 5.9% of *E. coli* and 34.8% of *K. pneumoniae* had an MIC superior or equal to 4 µg/mL for colistin, whereas in the carbapenemase producing *Enterobacteriaceae* (CPE), colistin-resistance represents 1% for *E. coli* and 12.5% for *K. pneumoniae*. We detected one CPE-*E. coli* (*bla*<sub>OXA48</sub>) and 2 CPE-*K. pneumoniae* (*bla*<sub>NDM</sub>) (Table 2) resistant to colistin representing an emerging threat for clinical management and infection control. Colistin resistance in carbapenem-resistant Enterobacterales has been showed in Southeast Asia [39]. Other authors showed that, for *E. coli*, the resistance rate is lower than for *K. pneumoniae* and the most common carbapenemase was NDM metallo-β-lactamases and OXA β-lactamases (OXA-48) [14]. The predom-

inance of *K. pneumoniae* colistin resistance in our Cambodian samples compared to *E. coli* colistin resistance is in agreement with studies in other countries, such as China, Iran, and India [40–42]. According to Atterby et al., *E. coli* and *K. pneumoniae* harbouring ESBL genes are common in rural Cambodian communities, especially in women, small children, poultry, and pigs [43]. In their review focused on Southeast Asia, Malchione et al., showed that the rate of carbapenem and colistin resistance among *E. coli* is low in Cambodia (< 1%) whereas no estimation could have been made for *K. pneumoniae* due to lack of data in this country [14]. Our study has helped to fill the gap and revealed that, in our sample, colistin resistance was ca. 3.6% among *E. coli* and 25.6% among *K. pneumoniae*. In India, authors estimated, respectively, 3.5% and 18.5% were the percentages of colistin-resistant *E. coli* and *K. pneumoniae* within MDR samples [41].

##### 4.2. Susceptibility profiles

Amikacin is the only antibiotic for which both *K. pneumoniae* and *E. coli* colistin-resistant isolates remained fully susceptible. *E. coli* colistin-resistant isolates remained fully susceptible to tigecycline, fosfomicin, and nitrofurantoin. However, the use of these antibiotics is limited as current guidelines recommend that amikacin should be used in combination with other antibiotics for severe infections [44], whereas nitrofurantoin can be used in monotherapy for the treatment of resistant bacteria but limited to uncomplicated lower urinary tract infections [45]. Fosfomicin is an effective antibiotic and is recommended by intravenous use to treat severe infections when other antibiotic treatments are not suitable, and by oral use for uncomplicated urinary tract infections. However, its use to treat severe infections should be limited in order to delay the development of resistance [44,45].

##### 4.3. Molecular determinants

All the 18 of 262 *Enterobacteriaceae* resistant to colistin harboured chromosomal mutations in the TCS and/or *mcr* genes (*mcr-1* and its variants). The co-occurrence of both *mcr* genes and mutations in the TCS observed in 11 out of 18 colistin-resistant isolates aligns with the findings of Premnath et al. [46]. They reported the presence of *mcr* genes alongside alterations in *mgrB* in colistin-resistant enterobacteria isolated from chicken meat in India.

##### 4.3.1. *mcr* variants

We identified 13 *mcr* among the 18 colistin-resistant isolates, corresponding to variants *mcr-1*, *mcr-3*, and *mcr-8.2*. The localization of *mcr* variants on plasmid and/or chromosome will be determined in a further study. Despite sparse data, polymyxin resistance was documented in 8 of 11 Southeast Asian nations, with *mcr-1* being the predominant genotype (including Cambodia) [14]. From a mechanistic point of view, according to these authors, the frequency of colistin resistance in Cambodia is low and only *mcr-1* and *mcr-3* have been identified before our study. In Cambodia, the

**Table 3**  
Mcr variants and mutations in TCS genes leading to amino acid changes in the 18 colistin resistant isolates.

Isolate	Year	Resistance	Species	MIC µg/mL	<i>mcr</i>		MgrB		PhoP		PhoQ		PmrA		PmrB		PmrD		CrrB	
					Variant	Ref	Mutation	Ref	Mutation	Ref	Mutation	Ref	Mutation	Ref	Mutation	Ref	Mutation	Ref	Mutation	Ref
<b>ESBL-KP8</b>	2020	ESBL	KP	16	<i>mcr-8.2</i>	[49]	-	-	-	-	-	-	D86E	[38]	G345E	[36]	ND	-	-	-
<b>ESBL-KP7</b>	2020	ESBL	KP	16	<i>mcr-8.2</i>	[49]	-	-	-	-	-	-	-	-	G345E	[36]	ND	-	-	-
<b>ESBL-KP6</b>	2020	ESBL	KP	8	-	-	-	-	-	-	-	-	-	-	R256G	[34]	ND	-	-	-
<b>ESBL-KP5</b>	2020	ESBL	KP	4	<i>mcr-6-9</i>	[7]	-	-	-	-	-	-	G53R	[33]	R256G	[34]	ND	-	-	-
<b>ESBL-KP4</b>	2020	ESBL	KP	8	<i>mcr-6-9</i>	[7]	-	-	-	-	-	-	G53R	[33]	A246T	[25]	ND	-	DELETION	[25]
<b>ESBL-KP3</b>	2020	ESBL	KP	> 32	-	-	IS5/IS1182	[32]	-	-	-	-	-	-	R256G	[34]	ND	-	DELETION	[25,39]
<b>ESBL-KP2</b>	2020	ESBL	KP	> 32	-	-	-	-	-	-	-	-	A41T	[25]	L213M	[25]	ND	-	166V	-
<b>ESBL-KP1</b>	2020	ESBL	KP	8	<i>mcr-8.2</i>	[49]	-	-	-	-	-	-	-	-	T157P	[25,35]	ND	-	Q239H	[25,39]
<b>ESBL-EC7</b>	2020	ESBL	EC	16	<i>mcr-8.2</i>	[49]	-	-	-	-	-	-	-	-	A246T	-	-	-	T276A	-
<b>ESBL-EC6</b>	2020	ESBL	EC	8	<i>mcr-1</i>	[4]	-	-	-	-	-	-	-	-	-	-	-	-	ND	[25]
<b>ESBL-EC5</b>	2020	ESBL	EC	8	<i>mcr-8.2</i>	[49]	-	-	-	-	-	-	-	-	-	-	N11D	-	ND	-
<b>ESBL-EC4</b>	2020	ESBL	EC	4	<i>mcr-1</i>	[4]	-	-	147V*	This study	E464D	[24,27]	-	-	-	-	M20K	[24]	ND	-
<b>ESBL-EC3</b>	2020	ESBL	EC	8	<i>mcr-3</i>	[59]	-	-	-	-	A482T	[24,27]	-	-	-	-	A27T	[24]	ND	-
<b>ESBL-EC2</b>	2020	ESBL	EC	4	<i>mcr-1</i>	[4]	V8A	[28]	-	-	N352K*	This study	-	-	-	-	G85R*	This study	ND	-
<b>ESBL-EC1</b>	2020	ESBL	EC	8	<i>mcr-1</i>	[4]	V8A	[28]	-	-	-	-	T31S	[30]	E123D	[28-30]	D14N	[31]	ND	-
<b>CP-KP2</b>	2020	CPE	KP	8	<i>mcr-1</i>	[4]	-	-	-	-	-	-	T31S	[30]	V351I	[28-30]	S71C	[31]	ND	-
<b>CP-KP1</b>	2017	CPE	KP	> 32	-	-	-	-	-	-	-	-	-	-	-	-	V83A	[31]	ND	-
<b>CP-EC1</b>	2017	CPE	EC	8	-	-	-	-	-	-	N346K	[29]	-	-	E123D	[28-30]	D14N	[31]	ND	-
															V351I	[28-30]	V83A	[31]	ND	-
															ND	[28-30]	V83A	[31]	DELETION	[25]
															R256G	[34]	ND	-	-	-
															G19R*	This study	-	-	ND	[25]

Abbreviations: CPE, carbapenemase producing *Enterobacteriaceae*; EC, *Escherichia coli*; ESBL, extended spectrum beta-lactamase; KP, *Klebsiella pneumoniae*; *mcr*, mobile colistin resistance; MIC, minimum inhibitory concentration; ND, not done; TCS, two-component system; -, no detected mutations.

\* : novel mutations revealed in this study.

presence of *mcr-1* has been first identified in faeces from a hospitalized child [47]. A few colistin resistance genes (*mcr-1* and *mcr-3*) have also been detected from both MDR *E. coli* and *K. pneumoniae* isolated from humans, poultry, and pigs. In 2019, both *E. coli* and *K. pneumoniae* resistant isolates to colistin (*mcr* like genes) have been identified in the Kampong Cham Province [42]. Our study also confirmed the presence of *mcr-1* and *mcr-3* and interestingly allowed the first detection of *mcr-8.2* in both *E. coli* / *K. pneumoniae* isolated in the human population in Cambodia. Concerning *mcr-8*, this variant has been first detected in China in 2018 in both poultry and faecal carriage [48]. One year later, *mcr-8.2* was identified in pig stool samples in China, soon after being detected in various clinical and environmental samples and recently in paediatric sepsis [25,49,50]. According to the NCBI database, *mcr-8.2* has been recently detected in Southeast Asia, especially in Vietnam (*K. pneumoniae* from patients in intensive care unit, Ho Chi Minh City – BioSample: SAMN15541446). To our knowledge, *mcr-8.2* has not been detected in *E. coli* or in Cambodia outside the present study.

In our study, *mcr-1* and 8.2 were detected in both *E. coli* and *K. pneumoniae* whereas *mcr-3* was only present in *E. coli*. In total, 87.5% colistin-resistant *E. coli* and 60% of colistin-resistant *K. pneumoniae* contained *mcr* genes. This is in agreement with other studies showing that the presence of *mcr* mainly concerns *E. coli* [42]. Among the six *mcr*-like genes detected for *K. pneumoniae*, two of them could not be sequenced, even after several trials [7]. The next step to identify these two variants could be to perform molecular cloning for these mixed sequences. This could be due to the presence of several variants of *mcr* genes in a single isolate, as already shown by other authors [51]. Last, it should be noted that the presence of *mcr-10*, identified in 2020, was not explored in this study [6].

#### 4.3.2. TCS modifications

In *E. coli*, we identified mutations already described in previous studies within *mgrB*, *pmrA*, *pmrB*, *pmrD*, and *phoQ* [24,27–31] as well as four novel non-synonymous mutations in *phoP* (PhoP147V), *phoQ* (PhoQ N352K), *pmrB* (PmrB G19R), and *pmrD* (PmrD G85R) that might be related to colistin resistance. *PhoP* does not seem to be involved in colistin resistance for our sample because no known mutations have been identified. However, we can notice that we detected a mutation leading to an amino acid substitution (PhoP I47V – ESBL-EC4) at a position previously identified within *Enterobacter cloacae* (I47F) but leading to another amino acid change [52]. Concerning the substitution G19R on PmrB, the amino acid 19 has been associated with colistin resistance in *E. coli* by other authors but the mutation led to a different amino acid (G19E) [53].

For *K. pneumoniae*, we detected mutations within *mgrB*, *pmrA*, *pmrB*, and *crrB*. These mutations are single-nucleotide polymorphisms or insertion and deletion and have been previously associated with colistin resistance of *K. pneumoniae* [25,32–39]. Nine among 10 *K. pneumoniae* resistant isolates revealed mutations at different locations on *pmrB*. Contrarily to *E. coli* sample, all *K. pneumoniae* resistant isolates carried at least one chromosomal mutation associated with colistin resistance. To our knowledge, this is the first description of TCS modifications in *E. coli* / *K. pneumoniae* isolates from patients in Cambodia because only *mcr-1* and *mcr-3* have been identified in this country [42].

#### 4.4. Colistin use in Cambodia

In Cambodia, interviews with farmers, veterinarians, and animal feed retailers and field observations identified a broad range of antibiotics used in food animal farms including beta-lactam, fluoroquinolones, tetracycline, colistin, and lincosamides [12]. The antimicrobials most commonly mentioned or kept in small-scale urban pig farming were amoxicillin, tylosin, gentamicin, and colistin.

A previous study showed that within 261 *E. coli* isolated from pigs, 20% of phenotypic resistance to colistin were associated with *mcr-1* and *mcr-3*-like genes [11]. Another study examined colistin resistance in *E. coli* from pigs, pig carcasses, and pork in Thailand, Lao PDR, and Cambodia border provinces and the highest resistance rate was found in *E. coli* (17.8%) from Cambodia with the presence of *mcr-1*, 3, and 5 [54]. Our data revealed a lower percentage of colistin resistant isolates in humans (3.6%) but considering only ESBL/CP- *E. coli*. Dissemination of *mcr* in *Enterobacteriales* among humans, animals, and the environment is a public health issue and this study clearly demonstrates the increase of colistin resistance in the Cambodian human population infected by MDR *Enterobacteriaceae*. Since in Cambodia, there is an uncontrolled use of colistin in veterinary medicine contrarily to human health, the transfer of *mcr* genes, or bacteria carrying *mcr* genes from animals (or contaminated environment) to humans are the most likely. Two recent studies on chicken meat in Vietnam revealed that 49.7% to 52% have colistin-resistant *E. coli* (mostly associated with *mcr-1*) [55,56]. The authors suggested direct transmission of colistin resistance genes between farm animals and humans through environmental factors, such as flies or birds [56]. In China, the spread of *mcr-10*-carrying plasmids was observed among chickens, slaughterhouse workers, and healthy residents [57].

Putting together with evidence that colistin resistance circulates within humans, food, wild animals, environment, flies, and wastewater and that already 10 different *mcr* isoforms have been detected since 2015 in addition to mutations in TCS, there is a huge need to reconsider the use of this antimicrobial in medical veterinary use and to mitigate the international trade of this molecule listed on the World Health Organization Critically Important Antimicrobials for Human Medicine [10]. Efforts to monitor and control the spread of colistin resistance in Cambodia and neighbouring countries are crucial to preserving the effectiveness of antibiotics.

#### 4.5. Limitations

Our study has certain limitations, as our sample is not exhaustive, and because it is based on a retrospective study of bacteria isolated at the MBL-IPC, with a small number of CPE. To better describe colistin-resistant bacteria circulating in Cambodia, it would be interesting to complete this study by including enterobacteria from various compartments (humans, animals, and environment), identify plasmid type carrying *mcr*, explore *mcr-10*, and additional chromosomal loci involved in colistin resistance such as genes of the AcrAB-TolC efflux systems, *crrA*, *qseB/qseC*, *yciM*, and *lpxM*. A global genomic approach could help as well to explore virulence factors including aerobactin, salmochelin, and yersiniabactin, especially within colistin resistant CPE [58].

#### 5. Conclusion

This study explored the presence of colistin resistance among ESBL- CP- enterobacteria in humans in Cambodia. The presence of multiple *mcr* variants, emergence of *mcr-8.2*, chromosomal mutations in TCS and co-occurrence of both *mcr* genes and TCS modifications on resistant isolates underscore the need for continuous surveillance, antimicrobial stewardship, and infection control measures to mitigate the spread of colistin resistance.

#### Ethics statement

Permission for the study was obtained from the National Ethics Committee for Health Research (NECHR) in Cambodia (Approval letter number 074NECHR).



## Declaration of competing interest

None.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2024.06.017.

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