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Plasmid-mediated colistin resistance in *Escherichia coli* from the Arabian Peninsula

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ABSTRACT

Objectives: Searching for the presence of the *mcr-1* gene in colistin resistant *Enterobacteriaceae* in countries of the Arabian Peninsula.
Methods: Seventy-five independent, colistin resistant *Enterobacteriaceae* strains isolated from clinical cases in Bahrain, Kuwait, Oman, Saudi Arabia and the United Arab Emirates were tested by PCR for the *mcr-1* gene. *mcr-1* positive strains were genotyped, and their antibiotic susceptibility was established. The *mcr-1* containing plasmids were mobilized into *Escherichia coli* K-12 and their sequence was determined.
Results: Four *E. coli* isolates (two from Bahrain, one from Saudi Arabia and one from the United Arab Emirates) were identified carrying the *mcr-1* gene on conjugative plasmids. They belonged to global multidrug resistant *E. coli* clones, i.e. ST648, ST224, ST68 and ST131, respectively. One strain carried the bla_{NDM-1} carbapenemase gene. Three strains carried *mcr-1* on IncI2 type plasmids, one of them also harboring a $bla_{CTX-M-64}$ gene. In the fourth strain *mcr-1* was located on a 240 kb IncHI2 plasmid co-harboring 13 other resistance genes.
Conclusions: This is the first report on the presence of the plasmid-coded *mcr-1* gene in a variety of multi-resistant clinical isolates from the Arabian Peninsula indicating that several commonly used antibiotics can potentially facilitate the spread of *mcr-1* carrying strains, or directly, *mcr-1* containing plasmids.

1. Introduction

The recent emergence and spread of carbapenem resistant *Enterobacteriaceae* (CRE), *Pseudomonas* and *Acinetobacter* strains has led to the re-introduction of colistin, i.e. a drug once considered to be inconvenient and too toxic for routine parenteral use, into daily clinical application.[1] As for these pathogens colistin often represents the last resort of treatment options, resistance to it commonly leads to more severe complications and increased mortality.[2] Deletions, insertions or mutations in several chromosomal genes affecting the composition of the cell wall, thus preventing the efficient binding of the drug, can lead to different levels of decreased colistin susceptibility.[3] The recent discovery of Liu et al. describing a mobile colistin resistance gene (*mcr-1*) located on a transferable plasmid has implied that colistin resistance can also spread efficiently from strain to strain.[4] Indeed, *mcr-1* emerged in a remarkable variety of strains recovered from healthy carriers, food and environmental sources and clinical isolates as well (reviewed in [5]). Most of the *mcr-1* positive isolates carried different resistance genes, including carbapenemase genes, namely bla_{NDM-9},[6] bla_{NDM-5},[7] bla_{VIM-1},[8] $bla_{OXA-48-type}$,[9] and bla_{KPC-2}.[10] Importantly, in some cases other resistance genes were also co-located on the *mcr-1* carrying plasmids.[8,11–14]

Although *mcr-1* expressing strains have been found in Asia, Europe, North America and Africa,[5] so far they have not been reported from countries of the Arabian Peninsula. From these countries nation-wide, surveillance-based data on antibiotic

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resistance, including the rate of carbapenem or colistin resistance, is not available.[15] In a recent, limited scale study, the rate of colistin resistance among CRE strains collected in four countries of the region was found to be 4.1%.[16] Here we present data showing that *mcr-1* positive strains, represented by multi- drug resistant (MDR), high risk clones, such as ST131, or by ones harbouring bla_{NDM-1} carbapenemase gene, are indeed present in countries of the region, some carrying an *mcr-1* plasmid also containing a large cluster of resistance genes.

2. Materials and methods

2.1. Strains

Seventy-five colistin resistant (MIC >2 mg/L) *Klebsiella pneumoniae* and *Escherichia coli* strains were included in the study. The strains were partly from our collection of CRE continuously submitted to our laboratory for molecular typing, or had been sent by different laboratories as colistin resistant isolates for the sake of the current study, irrespective of their carbapenem susceptibility. All strains were isolated from samples collected by clinical indications and strains were submitted to us coded, without any patient-identifiers. Eight of the 75 colistin resistant isolates were part of a collection published earlier.[16]

2.2. Antibiotic susceptibility assay

Susceptibilities to most antibiotics were tested by microdilution and were interpreted by CLSI standards.[17] Trimethoprim-sulfamethoxazole sensitivity was assayed on VITEK-2 (BioMérieux), while colistin sensitivity was tested by agar dilution and interpreted by the EUCAST criteria.[18] The minimum inhibitory concentration (MIC) of chlorhexidine, benzalkonium and irgasan was also established by agar dilution. Strains were considered carbapenem non-susceptible if any of the three carbapenems tested (*i.e.* imipenem, meropenem or ertapenem) exhibited an MIC above their respective clinical breakpoints.

2.3. PCR for antibiotic resistance genes and virulence factors

The presence of the mcr-1 gene,[4] and that of other resistance genes (bla_{TEM}, bla_{CTX-M}, bla_{SHV}, bla_{PER}, bla_{AmpC}, bla_{NDM}, $bla_{OXA-48-like}$, bla_{KPC}, bla_{VIM}, bla_{IMP}, *armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtF, qnrS, qepA, aac6-1b-cr, emrE, qacΔE, qacF, qacE, mdfA, ydgE, ydgF, sugE*(c), *sugE*(p), *cepA*) was established as described.[19,20] Specific alleles of beta-lactamase genes were determined by direct sequencing of the respective amplicons on a 3130X genetic analyzer (Applied Biosystems). The presence of the virulence factor genes (*cdt, hlyA, papC, papA, papEF, fimH*, PAI, *fyuA, bmaE, sfa/foc DE, iutA, traT, focG, cvaC, gafD, sfaS, cnf1, afa/dra, nfaE, rfc, ibe A, iha, upaH, upaB*, Kps MTII, K5, K1, Kps MTIII, *sat, ireA, iroN, usp, csgA, crl, bcsA*) was tested as described.[21,22]

2.4. Strain typing

Strains carrying *mcr-1* were subjected to macrorestriction analysis with *XbaI* (NE Biolabs, USA) digestion combined with pulsed field gel electrophoresis.[16] Strains were also subjected to multilocus sequence typing.[23]

2.5. Plasmid work

Plasmids were detected and sized by the alkaline lysis method using episomes in *E. coli* 39R861 and *E. coli* V517 as molecular mass controls.[24] The *mcr-1*-containing plasmids were conjugally transferred into *E. coli* J53RAZ or, if unsuccessful as a single plasmid, by

Table 1
Characteristics of the strains producing mcr-1

Strain	Country	Sample	Date of isolation	MLST	Phyl.[a]	Virulence factor genes	Resistance genes	Size (bp) and Inc type of the *mcr-1* plasmid	Resistance genes located on the mcr-1 plasmid[b]	GenBank accession number of the *mcr-1* carrying plasmid
BA76	Bahrain	Tissue swab from sacral bed sore	August 2015	ST648	D	*papC, fimH, PAI, csgA, crl, bcsA*	*mcr-1*, $bla_{CTX-M-64}$, *rmtB, aac6-1b-cr, qacΔE, mdfA, ydgE, ydgF, sugE(C)*	64942 IncI2	*mcr-1*, $bla_{CTX-M-64}$	KX013540
BA77	Bahrain	Urine	September 2015	ST224	B1	*fimH, csgA, crl*	bla_{TEM-1}, $bla_{CTX-M-15}$, *mdfA, ydgE, ydgF, sugE(C), qacF*	62661 IncI2	*mcr-1*	KX013539
SA26	KSA[c]	Blood	June 2012	ST68	D	*fimH, PAI, KpsMTII, K5, csgA, crl, bcsA*	bla_{NDM-1}, bla_{TEM-1}, *rmtC, aac6-1b-cr, mdfA, ydgE, ydgF, sugE(C, qacF, qacE*	240367 IncHI2	*mcr-1*, bla_{TEM-1}, **dfrA14, mphA, mefB, aadA2, aadA1, sat1, aph(3')-Ia, strA, strB, floR, sul3, tet(A)**	KU743384
ABC149	UAE[d]	Blood	May 2013	ST131	B2	*fimH, PAI, fyuA, iutA, afq/dra, iha, upaH, upaB, KpsMTII, K5, sat, usp, csgA, crl, bcsA*	$bla_{CTX-M-15}$, *aac6-1b-cr, emrE, qacΔE, mdfA, ydgE, ydgF, sugE,*	61228 IncI2	*mcr-1*	KX013538

[a] Phylogenetic group
[b] Genes printed bold were identified by analysis of the sequence of the plasmid, the others were also confirmed by PCR.
[c] Kingdom of Saudi Arabia, [d] United Arab Emirates

transformation into DH5α, as previously described[25] using selective plates containing 2 mg/L colistin. For transformation and for sequencing purposes plasmids were purified using the Plasmid Maxi Prep kit (Qiagen, Germany). Plasmid incompatibility types were determined by PCR-based replicon typing (PBRT),[26] and by analysing the complete sequences of the plasmids. *mcr-1* containing plasmids were submitted for commercial complete sequencing performed by the CCIB DNA Core Facility at Massachusetts General Hospital (Cambridge, MA). If the assembly could not unambiguously determine the entire plasmid sequence, PCR mapping and direct sequencing of the products was used for gap closure. Antibiotic resistance genes on the assembled plasmid sequences were identified by ResFinder 2.1.[27] Sequences were annotated using Sequin (http://www.ncbi.nlm.nih.gov/Sequin) and submitted to GenBank.

3. Results

Seventy-five independent, colistin resistant *Klebsiella pneumoniae* and *Escherichia coli* strains collected in the Kingdom of Bahrain, Kingdom of Saudi Arabia (KSA), Kuwait, Oman and in the United Arab Emirates (UAE) were studied. The species, carbapenem susceptibility, carbapenemases produced and the countries of origin of the strains are shown in Suppl. Table 1. Four *E. coli* strains carrying the *mcr-1* gene, two from Bahrain, one from Saudi Arabia and one from the UAE were detected in this collection, respectively. Two *E. coli* were isolated from blood in 2012 and in 2013, a urine and a wound isolate were recovered in 2015 (Table 1.). Besides colistin, all four strains were uniformly resistant to 3rd generation cephalosporins, tetracycline, trimetoprime/sulfamethoxasole and gentamicin, and variably resistant to amikacin, carbapenems, chloramphenicol and ciprofloxacin (Table 2.).

Genotyping results (Table 1.) showed that the isolates were clonally unrelated and belonged to different globally distributed sequence types (STs), including ST131 (strain ABC149), associated with the MDR phenotype. All isolates carried various types of ESBL or carbapenemase genes. (Table 1). Isolate SA26 carried the bla_{NDM-1} gene on a 95 kb non-typable plasmid, and expressed high level of carbapenem resistance (Table 2.). Strain BA76 was intermediately susceptible to some of the carbapenems (Table 2.) while not carrying any of the carbapenemase genes tested. In this case no further attempts were made to investigate the basis of the decreased carbapenem susceptibility. One of the Bahraini and the Saudi strain also carried 16S methylase genes, *rmtB* (BA76) or *rmtC* (SA26), respectively.

Although all strains carried genes associated with disinfectant resistance[20] (Table 1.), no increase in MIC to the disinfectants tested was detected compared to *E. coli* K12 laboratory strains (Table 2).

All plasmids carrying the *mcr-1* gene were transferred into *E. coli* J53RAZ by conjugation. In case of ABC149, however, these attempts have always resulted in transconjugants containing multiple plasmids, suggesting that pABC149-mcr-1 is not self-conjugative, but can be mobilized in trans by other conjugative plasmid present in the same donor cell. Therefore, pABC149-mcr-1 was transferred by transformation into *E. coli* DH5α.

In three of the four strains, BA76, BA77 and ABC149, respectively, the *mcr-1* gene was located on approximately 60 kb size IncI2 plasmids, which in case of pBA76-mcr-1 also harboured an *ISEcp1*-driven $bla_{CTX-M-64}$ inserted independently of *mcr-1* at position 8357 of the plasmid scaffold (GenBank Acc. No. **KX013540**). The backbones of all three plasmids, i.e. pBA76-mcr-1, pBA77-mcr-1 (GenBank Acc. No. **KX013539**) and pABC149-mcr-1 (GenBank Acc. No. **KX013538**), respectively were highly similar to that of the mcr-1 plasmid first described (pHNSHP45),[4] with some variations in the shufflon region of the conjugative apparatus. The genetic surroundings of the *mcr-1* gene in the four plasmids, together with examples available in GenBank, are shown in Figure 1A. Upstream structures identical to those seen in pABC149-mcr-1, pBA76-mcr-1 and pBA77-mcr-1 have been observed earlier.[28]

The downstream region in all three IncI2 MCR-plasmids was identical to that in the originally described pHNSHP45.[4]

Table 2
Antibiotic susceptibility of the *mcr-1* donor and recipient strains

Antibiotics[a]		Strains									
		BA76	J53RAZ (pBA76-MCR-1)	BA77	J53RAZ (pBA77-MCR-1)	SA26	J53RAZ (pSA26-MCR-1)	ABC149	DH5α (pABC149-MCR-1)	J53RAZ	DH5α
		W[b]	TC[c]	W	TC	W	TC	W	TF[d]	R[e]	R
MIC (mg/L)	COL	16	4	16	4	16	4	16	8	0.25	0.25
	AMP	>256	>256	>256	4	>256	>256	>256	4	4	2
	CAZ	128	8	16	<0.25	>128	<0.25	128	<0.25	<0.25	<0.25
	CTX	>128	128	>128	<0.25	128	<0.25	>128	<0.25	<0.25	<0.25
	AZT	>128	16	64	<0.25	<0.25	<0.25	128	<0.25	<0.25	<0.25
	ETP	1	<0.125	<0.125	<0.125	>64	<0.125	0.25	<0.125	<0.125	<0.125
	MEM	2	0.5	1	0.5	>128	0.5	1	0.5	0.5	<0.25
	IMP	<0.25	<0.25	<0.25	<0.25	16	<0.25	<0.25	<0.25	<0.25	<0.25
	CIP	>64	<0.125	16	<0.125	0.25	0.25	>64	<0.125	<0.125	<0.125
	AMK	>256	<0.5	4	<0.5	>256	2	8	2	1	2
	GEN	>256	<0.5	64	<0.5	>256	1	128	1	<0.5	<0.5
	KAN	>256	1	>256	1	>256	>256	128	2	0.5	2
	STR	64	1	256	1	>256	32	128	4	2	4
	TMP-SMX	>320	<20	>320	<20	>320	>320	>320	<20	<20	<20
	CHL	256	4	32	4	256	64	8	2	8	2
	TET	128	<0.5	64	<0.5	256	32	256	<0.5	<0.5	<0.5
	ERY	>256	64	64	64	256	64	>256	4	32	4
	BAC	128	64	128	64	128	64	128	8	128	8
	CHX	2	2	2	2	2	2	2	0.5	2	0.5
	IRG	0.25	<0.125	0.25	<0.125	0.25	<0.125	0.25	<0.125	0.25	<0.125

[a] Antibiotics: COL – colistin, AMP – ampicillin, CAZ – ceftazidime, CTX – cefotaxime, AZT – aztreonam, ETP – ertapenem, MEM – meropenem, IMP – imipenem, CIP – ciprofloxacin, AMK – amikacin, GEN – gentamicin, KAN – kanamycin, STR – streptomycin, TMP-SMX – trimethoprim-sulphamethoxazole, CHL – chloramphenicol, TET – tetracycline, ERY – erythromycin, BAC – benzalkonium, CHX – chlorhexidine, IRG - irgasan
[b] W – Wild type, [c] TC – Transconjugant, [d] TF – Transformant, [e] R – Recipient

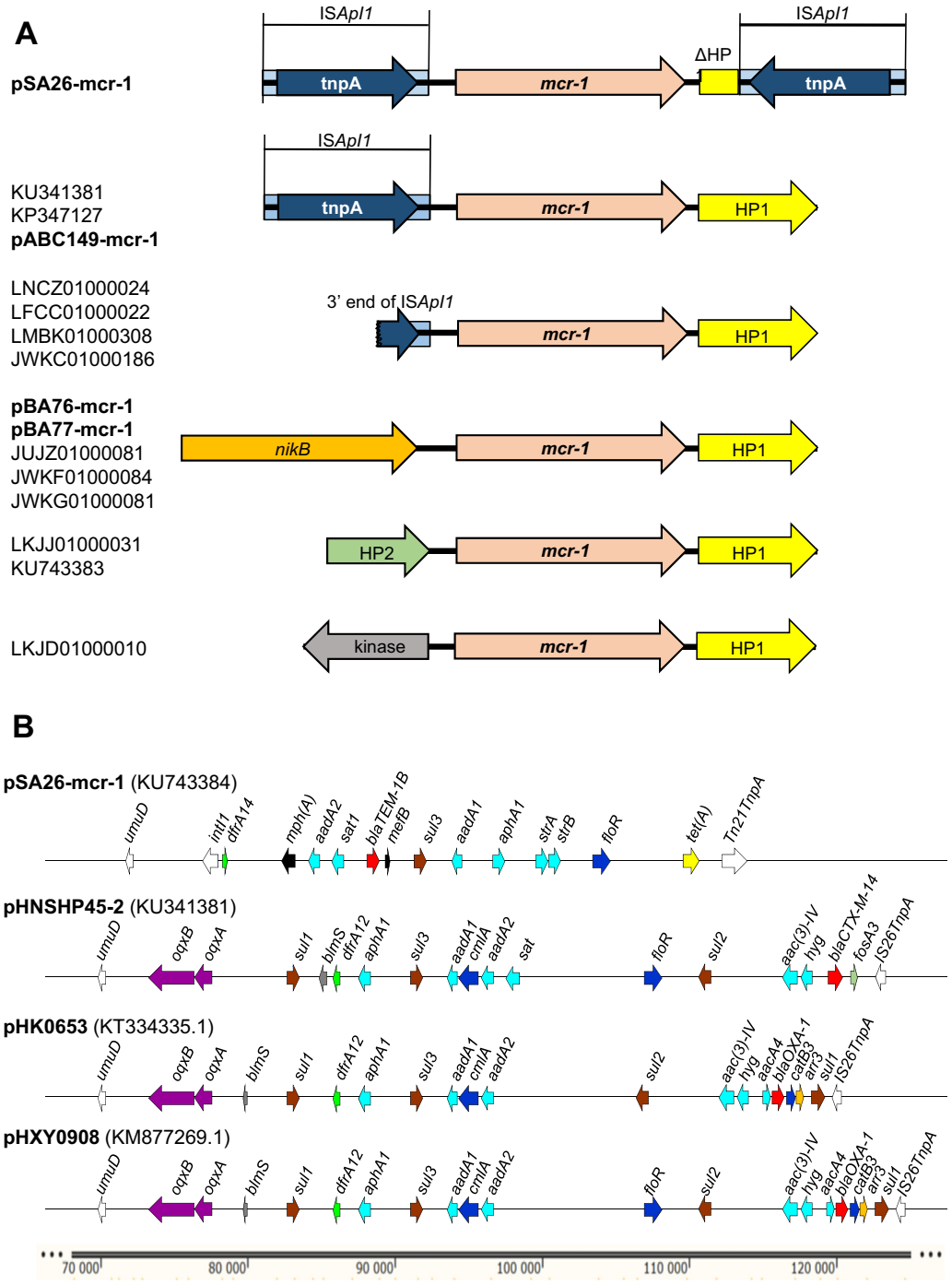

Figure 1. Comparison of the flanking regions of the *mcr-1* gene in different plasmids (A) and the antibiotic resistance gene cluster in pSA26-mcr-1 (B) to that in other plasmids.
A. Plasmids of the current study are printed bold, for other plasmids the respective GenBank numbers are provided. HP – hypothetical protein
B. Genes coding resistance to the different antibiotic classes are color-coded.

A completely different plasmid was identified in SA26. pSA26-mcr-1 was a 240367 bp, IncHI2 type plasmid (GenBank Acc. No **KU743384**). It exhibited extensive similarity to the backbone of the *mcr-1* negative, multidrug resistant plasmids pHK0653 (GenBank Acc. No. **KT334335**) and pHXY0908 (GenBank Acc. No. **KM877269**) carried by *Salmonella* Typhimurium strains isolated from human and poultry samples in China, and to the more recently identified second *mcr-1* plasmid from the SHP45 pig isolate, pHNSHP45-2 (GenBank Acc. No. **KU341381**).[29] However, in pSA26-mcr-1 a resistance island located between positions 76369 and 113971 was substantially different from the ones seen in these plasmids, respectively (Figure 1B). pSA26-mcr-1 contained 13 different resistance genes conferring aminoglycoside, beta-lactam, phenicol, tetracycline, sulfonamide, trimethoprim and macrolide resistance. The *mcr-1* gene was not part of this resistance island, but was identified in a different region, located between positions 160238 and 161863, flanked both upstream and downstream by a complete IS*Apal1* (Figure 1A).

4. Discussion

mcr-1-mediated colistin resistance, commonly found in carbapenem susceptible strains, have been described in CRE, as well.[6–10] While the former group can be instrumental in spreading colistin

resistance, in case of a latter one it also seriously restricts therapeutic options. Our current study confirms the presence of this resistance mechanism both in carbapenem susceptible and non-susceptible *Enterobacteriaceae* in countries of the Arabian Peninsula.

Three of the four *mcr-1* plasmids identified exhibited considerable similarities to the approximately 60 kb IncI2 plasmids described earlier.[4] Although isolates producing *mcr-1* and ESBL enzymes are widespread, and even plasmids co-harboring ESBL genes and *mcr-1* have been described earlier,[5] to the best of our knowledge pBA76-mcr-1 is the first IncI2 type plasmid found to carry $bla_{CTX\text{-}M\text{-}64}$ and *mcr-1* simultaneously, which indicates that this type of plasmids could also be co-selected by commonly used beta-lactam antibiotics.

To the best of our knowledge, pSA26-mcr-1 is the first completely sequenced IncHI2 plasmid carrying *mcr-1* and also resistance genes to several other classes of antibiotics, from a human *E. coli* isolate. Actually, MDR plasmids with this backbone structure have been reported from different parts of the world from animals and they increasingly seem to be important carriers of *mcr-1*. Such IncHI2 group *mcr-1* plasmid was first identified[30] by probing the contigs in GenBank in a *S.* Typhimurium strain isolated in 2011 in Portugal from food sample (**NZ_LFCC01000022**). Subsequently, from Belgium, an *E. coli* strain containing plasmid pKH-475-3-BE isolated from animal stool was reported. While considerably smaller (approximately 80 kb) than the original *S.* Typhimurium plasmids pHK0653 and pHXY0908, pKH-475-3-BE also showed extensive homology to their backbones and carried a cluster of at least 7 resistance genes beyond *mcr-1*.[13] It should be noted that further large plasmids, conferring resistance to multiple antibiotics isolated from animal sources in Vietnam[12] and in France, the latter having the same IncHI2 type, have also been described.[11] Further *mcr-1*-containing IncHI2 plasmids, without characterizing other antibiotic resistance genes located on it, have been reported from various sources in Germany.[10] Although no details of its sequence was available, the *mcr-1* gene in the IncHI2 plasmid from a human isolate in that study was flanked by IS*Apl1* transposase genes,[10] just as in plasmid pSA26-mcr-1 in this study (Figure 1A).

Although, pSA26-mcr-1 has a nearly identical backbone and size to the pig *E. coli* SHP45 IncHI2 plasmid, pHNSHP45-2, it contains a considerably different array of 13 resistance genes (Figure 1B). The fact that pSA26-mcr-1, a multidrug resistant *mcr-1*-containing plasmid, was detected in a human isolate indicates that these plasmids, earlier reported in animal isolates, only[10–12,29,30] did spread into human pathogens, as well. As beyond colistin, pSA26-mcr-1 also confers resistance to several antibiotics, some of them broadly administered even to non-hospitalized patients, treatment with these drugs could easily promote the inter-strain, even inter-species spread of colistin resistance in the community, as well.

While our data clearly showed the presence of *mcr-1* mediated colistin resistance in three countries of the Arabian Peninsula, due to the limitations of compiling the pool of strains for the current study one cannot draw any conclusion regarding the frequency of *mcr-1* in the different countries.

Nevertheless, the fact that 5.3% of the colistin resistant strains tested carried the *mcr-1* gene indicates that the presence of such isolates in the region should be anticipated. The detection of *mcr-1* in globally spreading MDR clones of *E. coli* in this region is of grave concern.

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Competing interest: None to declare.

Ethical approval: not required. All strains were isolated on the basis of clinical indications, i.e. no samples were collected for the sake of the study and were routinely preserved, as multi-resistant isolates, at the isolating laboratories. Strains were submitted for molecular studies without any patients' identifiers.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2016.07.007.

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