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Discovery of a potential MCR-1 inhibitor that reverses polymyxin activity against clinical *mcr-1*-positive *Enterobacteriaceae*

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SUMMARY

The recent emergence of the plasmid-mediated colistin resistance gene *mcr-1* poses a substantial clinical threat to the severe infections caused by CRE (Carbapenem Resistant *Enterobacteriaceae*), as the treatment failure of the *mcr-1*-positive CRE "Superbug" most likely occurs by using the combination of carbapenem and polymixins. Therefore, our study aims to seek a potent MCR-1 inhibitor to fight this infection. A checkerboard MIC (Minimum Inhibitory Concentration) assay, time-killing assay, MPNP (Modified rapid polymyxin Nordmann/Poirel) test, combined disk test and molecular modelling analysis were performed on different *mcr-1*-positive strains to confirm the synergistic effects of the combination of colistin and osthole (OST). And a thigh mouse infection model was also used to evaluate such synergies. We identified that OST regained the bactericidal activity of polymyxins (FIC (Fractional Inhibitory Concentration) index = 0.11±0.04 – 0.29±0.10) against *mcr-1*-positive *Enterobacteriaceae* including *Escherichia coli* and *Klebsiella pneumoniae*. The in-vitro time-killing assays showed that either OST or polymyxins failed to eradicate *mcr-1*-positive *Enterobacteriaceae*, but the combination eliminated *mcr-1*-positive *Enterobacteriaceae* by 3–7-h post-inoculation. The mouse infection model demonstrated that the combination therapy significantly reduced the bacterial load in the thighs following subcutaneous administration. Our results established that OST is a promising natural compound that could be used to extend the life of polymyxins and to tackle the inevitability of serious infections caused by polymyxin-resistant bacteria.

Introduction

The relentless increase in antibiotic resistance in multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria has posed a severe global clinical threat, especially carbapenem-resistant *Enterobacteriaceae* (CRE) in human clinical settings is now recognized as one of the most serious global threats to public health.[1] Novel and effective antibiotics are urgently needed to treat these bacteria infections, but the development pace of new antimicrobial agents has declined in recent decades, thus, the major clinical drugs used for these infections are the older polymyxins, including colistin (polymyxin E) and polymyxin B.[2] In human clinical chemotherapy, neither polymyxin B nor polymyxin E is usually an option for monotherapy, mainly because the dose escalation that is required to achieve sufficiently high concentrations with the currently recommended dosing protocols has risks, including the rapid onset of nephrotoxicity.[3]

Furthermore, the discovery of a transferable and plasmid-mediated colistin resistance gene *mcr-1*, which modifies the lipid A component of lipopolysaccharide (LPS) with phosphoethanolamine, thereby inhibiting the binding of polymyxins on target bacteria, appears to have already spread worldwide.[4–6] Subsequently, isolates with the *mcr-1* gene have been identified in various animals, meats, vegetables and humans in more than 40 countries.[6] The MCR-1- and NDM-1-carrying clinical *E. coli* isolate, ZJ478, has been increasingly identified in China, and MCR-1- and NDM-5-coproducing *E. coli* isolates (MCR1_NJ) have been discovered in the United States.[7,8] These discoveries will bring humanity into a situation where there are no available antibiotics for this category of clinical bacterial infections. Colistin resistance is mostly directed against modifications of the lipid A moiety of LPS, which is the primary target of polymyxin.[5,9] Importantly, as *mcr-1* is plasmid-mediated, resistance to polymyxins is no longer only associated with the chromosome but can also be acquired by horizontal transmission.[10] The loss of these last-line-of-defence

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Table 1
MIC values of the different antibiotics and OST combination therapy for each of the tested bacterial isolates.

Species	Source	*mcr-1* confirmation	Antibiotics	MIC (μg/mL)		FIC index
				Alone	Combination	
E. coli ZJ69	Human urine	+	Colistin	8.00±0.00	1.67±0.58	**0.29±0.10**
E. coli ZJ378	Human feces	+	Colistin	8.00±0.00	1.00±0.00	**0.19±0.00**
E. coli DZ2-12R	Chicken cloacae	+	Colistin	8.00±0.00	1.00±0.00	**0.21±0.04**
K. pneumoniae ZJ02	Remote tertiary care hospital	+	Colistin	26.67±9.24	3.33±1.15	**0.19±0.0**
K. pneumoniae E8.31	Chicken cloacae	+	Colistin	21.33±9.24	3.33±0.00	**0.23±0.07**
K. pneumoniae 13b5	Chicken cloacae	+	Colistin	32.00±0.00	3.33±1.15	**0.17±0.04**
E. coli ATCC25922	Laboratory strain	−	Colistin	1.33±0.58	1.00±0.00	0.90±0.29
S. typhimurium SL1344	Derived from the virulent strain SL1344	−	Colistin	1.33±0.58	1.67±0.58	1.40±0.58
A. Baumanii ATCC19606	Laboratory strain	−	Colistin	1.68±0.58	1.68±0.58	1.06±0.00
K. pneumoniae K7	The People's Hospital of Jilin Province	−	Colistin	1.00±0.00	1.00±0.00	1.06±0.00
E. coli ZJ478	Human intra-abdominal fluid (bla_{NDM-1}-carrying)	+	Colistin	10.67±4.62	1.33±0.58	**0.19±0.00**
			Imipenem	16.00±0.00	16.00±0.00	1.06±0.00
			Penicillin	682.67±295.60	682.67±295.60	1.06±0.00
			Streptomycin	85.33±36.95	85.33±36.95	0.90±0.29
			Kanamycin	85.33±36.95	106.67±36.95	1.40±0.58
			Gentamycin	341.33±147.80	341.33±147.80	1.06±0.00
			Chloramphenicol	1.00±0.29	1.00±0.29	1.06±0.00
			Erythromycin	85.33±36.95	85.33±36.95	1.06±0.00
			Acheomycin	170.67±73.90	170.67±73.90	1.0.6 ± 0.00
K. pneumoniae ZJ05	Remote tertiary care hospital	+	Colistin	32.00±0.00	2.67±1.15	**0.11±0.04**
			Imipenem	0.67±0.29	0.67±0.29	1.06±0.00
			Penicillin	1024.00±0.00	1024.00±0.00	1.06±0.00
			Streptomycin	16.00±0.00	16.00±0.00	1.06±0.00
			Kanamycin	21.33±9.24	21.33±9.24	1.06±0.00
			Gentamycin	2.00±0.00	1.67±0.58	0.90±0.29
			Chloramphenicol	16.00±0.00	16.00±0.00	1.06±0.00
			Erythromycin	106.67±36.95	106.67±36.95	1.06±0.00
			Acheomycin	128.00±0.00	128.00±0.00	1.06±0.00
E. coli BL21(DE3) (pET28a-*mcr-1*)	Laboratory strain (carried a *mcr-1* gene that originated from K. *pneumoniae* ZJ05)	+	Colistin	13.33±4.62	2.00±0.00	**0.23±0.07**
			Imipenem	0.25±0.00	0.25±0.00	1.06±0.00
			Penicillin	2.67±1.15	2.67±1.15	1.06±0.00
			Streptomycin	8.00±0.00	8.00±0.00	1.06±0.00
			Kanamycin	512.00±0.00	512.00±0.00	1.06±0.00
			Gentamycin	1.67±0.58	1.67±0.58	1.06±0.00
			Chloramphenicol	0.67±0.29	0.67±0.29	1.06±0.00
			Erythromycin	42.67±18.48	42.67±18.48	1.06±0.00
			Acheomycin	10.67±4.62	10.67±4.62	1.06±0.00
E. coli BL21(DE3) (pET28a)	Laboratory strain	−	Colistin	0.83±0.29	0.67±0.29	0.86±0.29
			Polymyxin B	1.00±0.00	0.67±0.29	0.73±0.29
E. coli ZJ40	Remote tertiary care hospital (*mcr-1* located in chromosome)	+	Colistin	53.33±18.48	5.33±2.31	**0.23±0.04**
			Polymyxin B	53.33±18.48	6.67±2.31	**0.25±0.00**
K. pneumoniae 16ZJJ9-19BC	Chicken cloacae (Polymyxin-resistant *mcr-1*-negative)	−	Colistin	32.00±0.00	13.33±4.62	0.48±0.14
			Polymyxin B	21.33±9.24	13.33±4.62	0.73±0.29

All MICs were determined in triplicate. The concentration of OST was 32 μg/mL in all bacterial isolates. The FIC values of all *mcr-1*-positive isolates were indicated in bold. The data were presented as the mean ± standard deviation.
+: *mcr-1*-positive species, −: *mcr-1*-negative species.

antibiotics has necessitated the development of novel and effective strategies to address the serious challenges posed by MCR-1, which will require the investment of large amounts of manpower and resources. Restoring the efficacy of polymyxin to treat CRE-mediated infection could be a useful strategy.[11] It was reported that a significant synergy was observed both *in vitro* and *in vivo* for inactivating colistin-resistance enzymes or efflux pumps combined with polymyxins.[12,13] Here, we discovered a novel MCR-1 inhibitor, OST (7-methoxy-8-(3-methyl-2-butenyl) coumarin, which was isolated from the dried root and rhizome of *Cnidium monnieri* and other medicinal plants and enhanced the therapeutic effects of polymyxins both *in vitro* and *in vivo*.[14] Previous studies revealed that OST has various pharmacological activities, mainly anti-inflammation, anti-oxidation, anti-allergy, oestrogen-like and anti-hepatitiseffects.[15–17] Antitumour effects, by inhibiting tumour cell growth and inducing apoptosis by OST, have also been reported.[18] However, the effects of OST on the inhibition of resistance enzyme and the possible mechanisms behind it remain unclear.

Materials and methods

Bacterial strains and chemicals

The bacterial strains used in this study are listed in Table 1. The *mcr-1*-positive isolates ZJ478, ZJ69, ZJ378, DZ2-12R, 16ZJJ9-19BC, ZJ40, ZJ02 and ZJ05 were collected as described in our previous studies.[7,12,19] *E. coli* BL21(DE3)(pET28a-*mcr-1*) carried a *mcr-1* gene that also originated from *K. pneumoniae* ZJ05. *K. pneumoniae* E8.31 and *K.pneumoniae* 13b5 were collected from chicken cloacae (in Shandong in 2006 and Shanghai in 2013, China). The polymyxin-resistant *mcr-1*-negative *K. pneumoniae* 16ZJJ9-19BC was obtained from chicken cloacae in Zhejiang, China. The *mcr-1*-negative *K. pneumoniae* K7 was obtained from a patient in the People's

Hospital of Jilin Province, China, and the mcr-1-negative *Salmonella Typhimurium* strain was derived from the virulent strain SL1344.[12] The *E. coli* ATCC25922 was used as a quality control strain. In view of the importance of human clinical colistin-resistant Gram-negative strains, human clinical MCR-1-producing isolates ZJ478, ZJ02 and ZJ05 were used in most of the experiments. MCR-3-producing strains *E. coli* W3110 (pUC19-*mcr-3*) was also used in this study.[20]

OST was purchased from Sigma-Aldrich, St. Louis, MO, USA. Colistin sulfate, polymyxin B sulfate, penicillin, imipenem, gentamycin sulfate and chloramphenicol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Streptomycin sulfate, kanamycin sulfate, erythromycin and acheomycin were purchased from Dalian Meilun Biotechnology Co., LTD. Dalian, China. Dimethylbenzene and phenol red were purchased from Biotopped technology Co., LTD. Beijing, China. Stock solutions of OST were prepared in dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA).

Antibacterial activity assays in vitro

Construction of MCR-1 and its mutants

The sequence of *mcr-1* was amplified from K. *pneumoniae* ZJ05 with the primers MCR-1-WT, cloned into the expression vector pET-28a followed by a digestion with the endonucleases *BamHI* and *XhoI*. Site-directed mutagenesis of *mcr-1* was performed using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) to produce S330A, D331A and F344A with pET-28a-*mcr-1* as the template. The pET-28a-*mcr-1* and mutant vectors were transformed into E. *coli* BL21 (DE3) for protein expression.

MIC determination and growth curves

The checkerboard microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI, USA)[21] was applied to evaluate synergies between OST and antibiotics (listed in Table 1) against polymyxin-resistant strains (positive for MCR-1), polymyxin-resistant strains (negative for MCR-1) and polymyxin-sensitive strains (negative for MCR-1).[22] These combinations were evaluated by calculating the fractional inhibitory concentration (FIC) index values; the FIC index was calculated as follows: FIC index=(FIC of polymyxin)+(FIC of OST).[23] A growth curve assay was performed to evaluate the effect of OST on the growth of the tested strains. As previously described,[24] the tested strains were cultured in Luria-Bertani medium at 37 °C with shaking at 200 rpm to obtain a starting $OD_{600} = 0.3$. OST (or the dimethyl sulfoxide control) was added to the five cultures at 0 μg/mL, 16 μg/mL, 32 μg/mL, 64 μg/mL, and 128 μg/mL. The bacteria were cultured at 37 °C with shaking, and the growth of the bacteria was estimated by measuring the OD_{600} every 30 min for 6–7 h.

Time-killing assays

The potential bactericidal effect of OST in combination with colistin was evaluated via time-killing assays.[25,26] Mid-logarithmic-phase bacterial cells were diluted to 1×10^5 CFU/ml in Luria-Bertani broth and incubated in colistin (2 μg/mL for *E. coli* BL21(DE3)(pET28a-*mcr-1*) or ZJ478 and 4 μg/mL for *K. pneumoniae* ZJ02 or ZJ05), OST (32 μg/mL), colistin in combination with OST or DMSO (as a normal control). Serial 10-fold dilutions of the samples were spread onto drug-free Luria-Bertani agar plates. The number of colonies was determined after an incubation of 24 h at 37 °C.

Combined disk test

The combined disk test (CDT) was determined as described previously.[27,28] Initially, according to the growth curve assay and Checkerboard MIC studies, 10 μL of various concentrations of OST (0 μg/mL, 8 μg/mL and 32 μg/mL) without antibacterial activity against all screened strains were added to colistin 10 μg disks (Oxoid Ltd. Basingstoke, United Kingdom). For each bacterium, these disks were then placed on Luria Broth agar plates following inoculation with an $OD_{600} = 0.1$ bacterial suspension. Inhibition zone diameters around the colistin disks (with or without OST) were measured and compared after 18–24 h of incubation at 37 °C.

Modified polymyxin NP (MPNP) test Western blot *assay*

The MPNP test was based on the NP test proposed for the identification of polymyxin resistant/sensitive *Enterobacteriaceae*.[29] Briefly, 150 μL of colistin-free NP solution (PH of NP solution was 7.8 ± 0.2), colistin-containing (due to the different MIC of the test isolates, the concentration of colistin was 4 μg/mL for *K. pneumoniae* and 2 μg/mL for *E. coli*) NP solution, colistin-free NP solution plus OST (32 μg/mL) and colistin-containing (the concentration of colistin was 4 μg/mL for *K. pneumoniae* and 2 μg/mL for *E. coli*) NP solution plus OST (32 μg/mL) were poured into sterile 96-well polystyrene plates respectively to confirm the effect of MCR-1 inhibitor. For each isolate, 50 μL of a OD_{600}=1.0 bacterial suspension (approximately 1×10^9 CFUs/mL) was dispensed and mixed with 150 μL of NP solution in each of the four wells, and the first column was filled with 50 μL of 5 M NaCl solution. Subsequently, the plates were incubated at 37 °C with shaking for 4–6 h, and visual changes in the colour of the wells were monitored and detected by measuring the optimal OD of the mixture at 492 nm.

Western blot assay was performed as previously described.[30] In short, the test strains were cultured at 37 °C in LB supplemented with different concentrations of OST (0, 8 and 32 μg/mL) and grown for 4 h. The bacterial pellet was boiled with BUFFER, separated with SDS-PAGE, and transferred onto polyvinylidene fluoride (PVDF) membranes (Roche, Basel, Switzerland) using a semi-dry transfer method (Bio-Rad, Munich, Germany). The blots were developed with antibodies using Amersham ECL Western blotting detection reagents (GE Healthcare, UK).

Molecular modelling

In this work, the initial structure of MCR-1 was obtained from the 3D structure of X-ray (PDB code: 5GOV). To obtain the starting structure of the ligand/MCR-1 complex for the molecular dynamic (MD) simulation, a standard docking procedure for a rigid protein and a flexible ligand was performed with AutoDock 4.[31,32] Subsequently, the molecular dynamic simulation of the complexing systems was performed, and the detailed processes of the computational biology method were described in previous reports.[33,34]

Cytotoxicity assays

Human lung epithelial cells (A549, ATCC) and Africa green monkey SV40 transformation cell (COS-1, ATCC) were seeded into 96-well plates at 2×10^4 cells per well and incubated with various concentrations of OST (0, 2, 4, 8, 16, 32, 64, 128 μg/mL) for 5 h and 24 h at 37 °C. The released LDH was measured using a cytotoxicity detection kit (LDH, Roche) on a microplate reader (Tecan, Austria) at an absorbance of 490 nm. The positive control used was 0.02% Triton X-100, and the untreated sample served as a negative control.

An in vivo *thigh infection mouse model for* E. coli *ZJ478 and* K. pneumoniae *ZJ05*

Then, 6–8-week-old female BALB/c mice weighing 20 ± 2 g were purchased from the Experimental Animal Centre of Jilin University (Changchun, Jilin, China). Animal experiments were approved by and conducted in accordance with the guidelines of the Animal Care and Use Committee of Jilin University. Thigh infection was induced in the mice as described previously.[35] The mice were

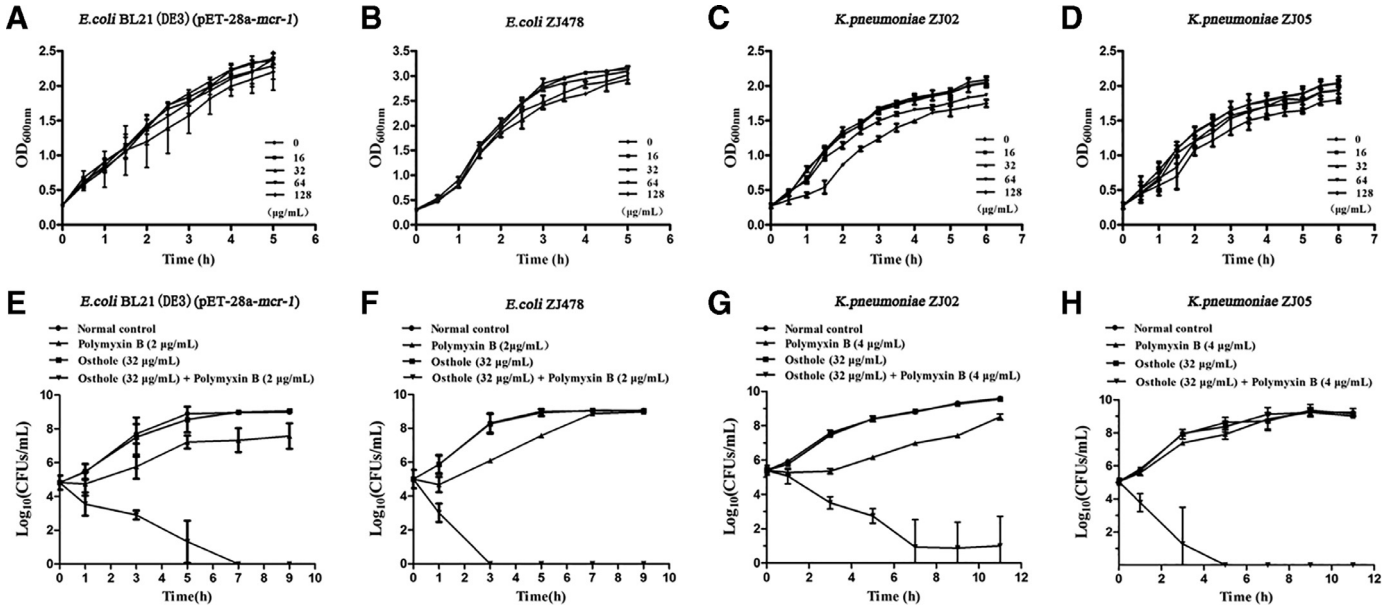

Fig. 1. Growth curves for four *mcr-1*-positive strains (A)–(D) cultured in the presence of various concentrations of OST (0–128 μg/mL). Time-killing curves for colistin, OST, combination and control treatment (medium only) against on four *mcr-1*-positive strains (E)–(H). Values represent the averages of three independent experiments.

Table 2
Combined disk tests for colistin in combination with OST for each of the tested bacterial isolates.

Species	Inhibition zone diameter (mm)			
	Assay	Colistin (10 μg)	Colistin (10 μg)+OST (8 μg/mL)	Colistin (10 μg)+OST (32 μg/mL)
K. pneumoniae ZJ02	Mean	8.50±0.50	11.33±0.29*	14.17±0.29*
E. coli ZJ478	Mean	9.33±0.29	11.17±0.29*	14.33±0.29*
E. coli BL21(DE3) (pET28a-*mcr-1*)	Mean	9.33±0.29	11.50±0.50*	14.00±0.50*
E. coli ATCC 25922	Mean	13.00±0.50	13.33±0.76	13.33±0.29

The combined disk test method was performed in triplicate. Three 10-μg colistin disks with OST (0, 8, and 32 μg/mL) were used. The mean inhibition zone diameters were indicated in italics.

* $P < 0.01$ compared with the colistin 10-μg disk alone based on two-tailed Student's *t*-tests. The data were presented as the mean ± standard deviation.

infected thigh intramuscularly with a dose of 2×10^7 CFU of E. *coli* ZJ478 and *K. pneumoniae* ZJ05. The infected mice were subcutaneously administered colistin (5 mg/kg), OST (50 mg/kg, intraperitoneally), a combination of OST and colistin, or solvent on the same schedule 2 h after inoculation. The thigh were harvested, homogenized, diluted and plated to determine the thigh bacterial load at 72 h post-infection.

Statistical analysis

The IBM Statistical Program for Social Sciences (SPSS) version 19.0 (IBM Corp. Armonk, NY, USA) was used to analyse the experimental data, and the data are presented as the mean ± standard deviation. An independent Student's *t*-test was used to determine significant differences, and differences were considered statistically significant when *P* values were less than 0.05.

Results

OST restores mcr-1-*positive* Enterobacteriaceae *sensitivity to polymyxins* in vitro

To determine the synergistic effect of OST (Fig. 3(A)) in combination with polymyxin, *mcr-1*-positive and *mcr-1*- negative bacteria isolates were examined in this study. The results of the checkerboard microdilution method indicated that a synergistic effect between OST and polymyxins solely occurred in the *mcr-1*-positive strains (FIC index from 0.19±0.00 to 0.25±0.00), in the presence of 32 μg/mL OST, whereas no synergies were observed in any of the other tested antibiotics (Table 1). Meanwhile, there were significant differences between the synergistic effects of *mcr-1*-negative polymyxin-resistant isolate *K. pneumoniae* 16ZJJ9-19BC and *mcr-1*-positive isolates (Table 1). Additionally, a synergistic effect between OST and colistin was also observed in *mcr-3*-carrying strains *E. coli* W3110 (pUC19-*mcr-3*) (Table 4).

The results of the growth curve assay showed that various concentration of OST (0–128 μg/mL) did not affect the growth of the *mcr-1*-positive *E. coli* and *K. pneumoniae* (Fig. 1(A)–(D)). And the bacteriostatic activity of OST combined with colistin was measured in mm with a ruler in the CDT. The *mcr-1*-positive *E. coli* and *K. pneumoniae* isolates were analysed with an increase from 8.50 ± 0.50 mm to 14.33 ± 0.29 mm (Table 2) in the size of inhibition zones with approximately 10 μg colistin-plus-OST (32 μg/mL) in comparison to the inhibition zones of 10 μg colistin without OST (Fig. S1). The synergistic effect of OST combined with colistin was then further evaluated using time-killing assays. When colistin was used alone, it had little effect on growth. However, the tested isolates were killed completely by the combination after 1–3 h (Fig. 1(E)–(H)).

The MPNP test was determined by the colour change and by measuring the OD of the mixture at 492 nm. In contrast, only the colistin-resistant E. *coli* isolates BL21(DE3)(pET28a-*mcr-1*) and K. *pneumoniae* ZJ02 showed a positive MPNP test (positive MPNP test indicated by changed colour of phenol red in combination well) (Fig. 2(A)), and the value of OD_{492} in combination wells significantly decreased when compared to other wells (colistin treatment, OST treatment and the control) (Fig. 2(B)). Due to the *mcr-1*-negative *K. pneumoniae* K7 was sensitive to colistin, thus, the

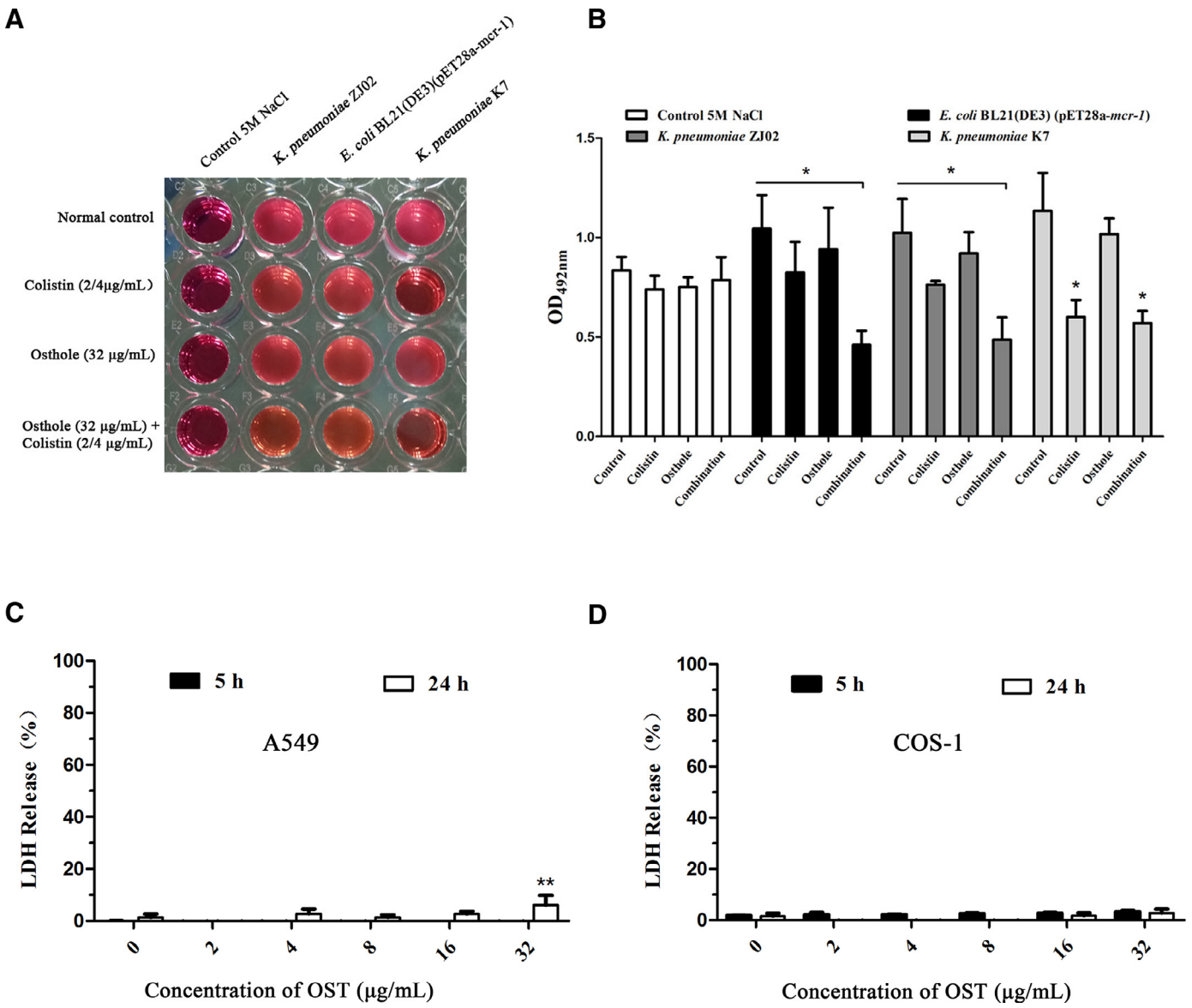

Fig. 2. The first to fourth row of wells were filled with 150 μL of only NP solution, colistin-containing (4 μg/mL for *K. pneumoniae* and 2 μg/mL for *E. coli*) NP solution, OST-containing (32 μg/mL) NP solution and combination NP solution plus OST(32 μg/mL).The different columns were filled with 50 μL of bacterial suspension of the tested isolates (A). Visual changes were detected by measuring the optimal OD of the mixture at 492 nm (B). LDH release by A549 (C) and COS-1 (D) cells co-cultured with various concentrations of OST (0–128 μg/mL). Each value is the average of three independent experiments. ** indicates $P < 0.01$; * indicates $P < 0.05$.

colour of the wells containing colistin was different from the others.

Toxicity often limits the clinical application of novel lead molecules. Therefore, the cytotoxicity of OST was preliminarily assessed using the LDH assay. Although cytotoxicity for A549 following OST treatment (32 μg/mL) for 24 h was observed, OST hardly exhibited cytotoxicity to different sources of cells (A549 cells and COS-1 cells) at concentrations no more than 32 μg/mL for 5 h (Fig. 2(C) and (D)). Thus, our results established that OST treatment restores polymyxin antimicrobial activity against *mcr-1*-positive *Enterobacteriaceae* at the concentration of 32 μg/mL without cytotoxicity no more than 5 h.

A direct engagement of OST inhibits MCR-1 activity

Through a computational biology method, the potential binding mode of OST with MCR-1 in the active site was explored in the study. The OST was bound to MCR-1 and the binding mode is given in Fig. 3(C). It is clear that OST can bind to MCR-1 via hydrogen bonding and hydrophobic interactions. During the time course of the simulation, OST localized to the binding pocket of MCR-1 (residue 330–350). In detail, the binding model of OST with the MCR-1 revealed that the 2-methyplent-2-ene moiety of OST can form a strong interaction with Ser330 and Lys348. Moreover, the 2*H*-chromen-2-one group plane of OST was parallel to the benzene ring plane of Phe344, indicating that the $\pi-\pi$ interaction exists between OST and Phe344. In addition, the side chains of Asp331, Ser332, Met336, Asp337, and Lys341 also formed interactions with OST (Fig. 3(C)).

To explore the energy contributions from the residues of the binding sites in the MCR-1-OST complex, the energy decomposition was calculated for the MCR-1 and OST complex system. As shown in Fig. 3(B), Met336, Lys341, and Phe344 had the strong total binding energy contribution, with a ΔE_{total} of ≤ -0.5 kcal/mol. Of these, residue Phe344 had the most appreciable, strong binding energy contribution, with a ΔE_{total} of ≤ -1.7 kcal/mol, which is

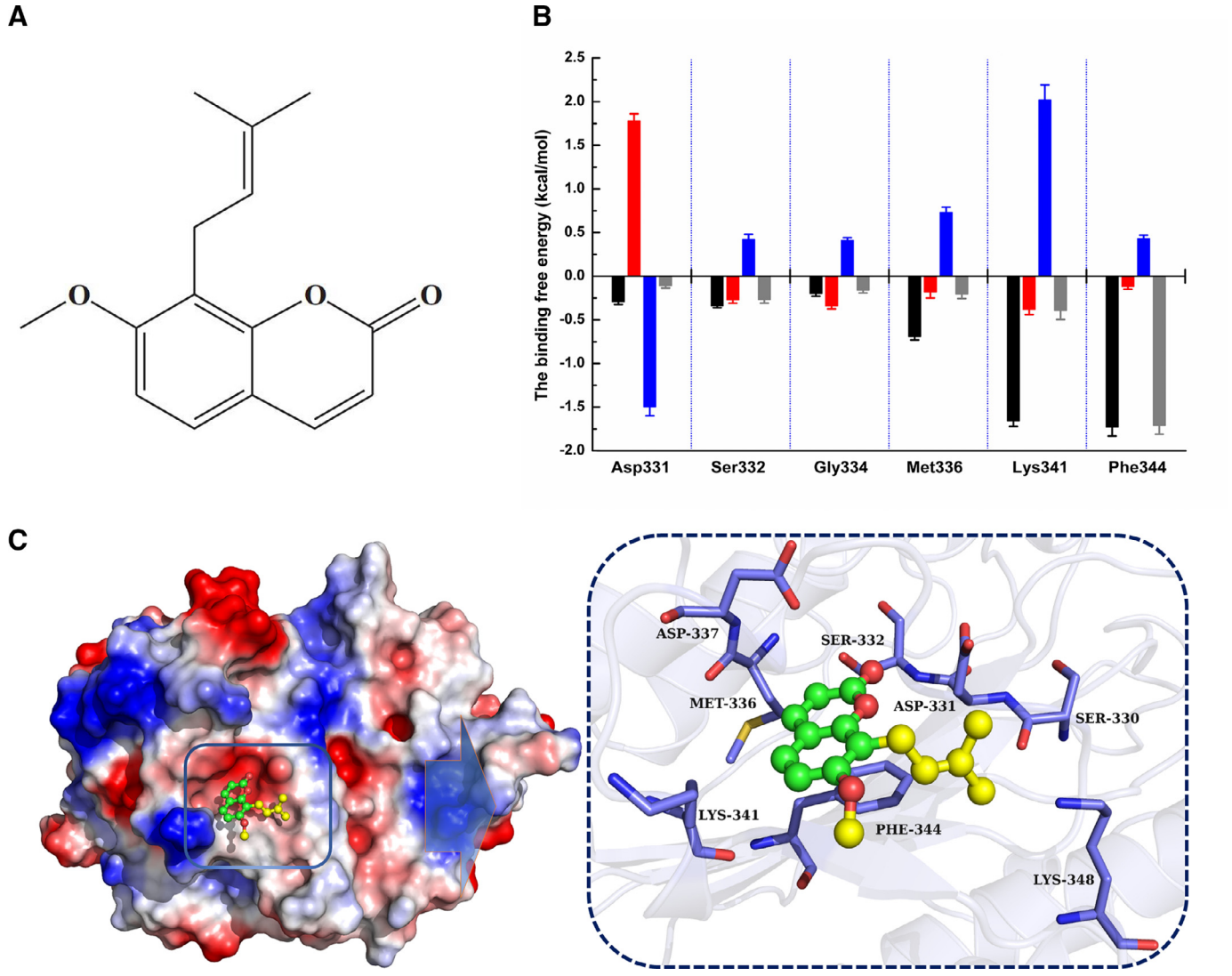

Fig. 3. The structure of OST (A). Decomposition of the binding energy on a per-residue basis in the binding sites of the MCR-1-OST complex (B). The summations of the per residue interaction free energies were separated into van der Waals (black column), electrostatic (red column), solvation (blue column), and the total contribution (grey column). The 3D structure determination of MCR-1 with the OST complex by the molecular modelling method (C).

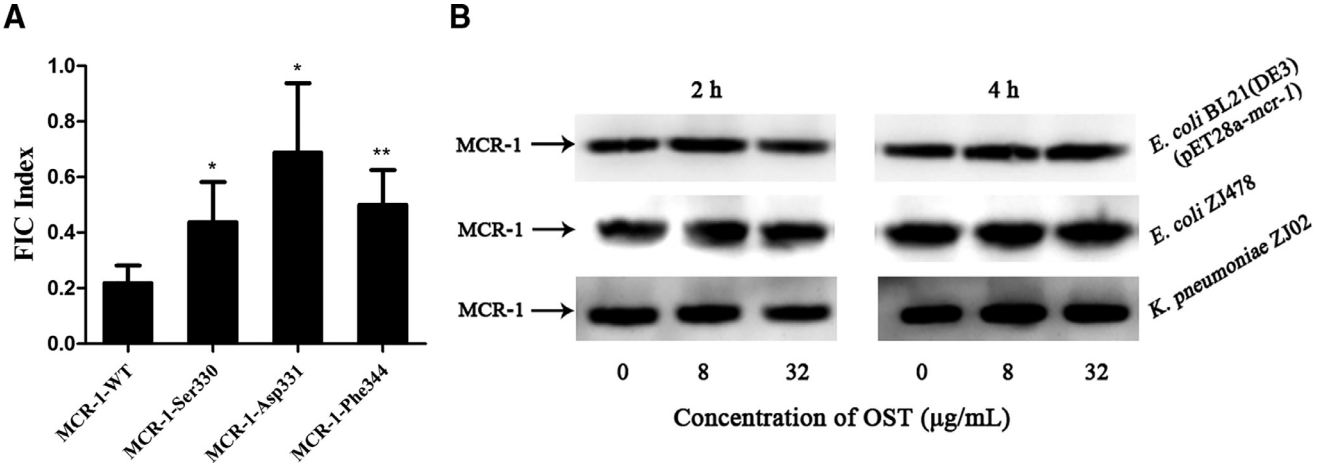

Fig. 4. FIC Index values of colistin and OST combination therapy for each of the variants (A). *Western blot* assays of MCR-1 production (B). *E. coli* BL21(DE3)(pET28a-*mcr-1*), *E. coli* ZJ478 and *K. pneumoniae* ZJ02 were used as tested. ** indicates $P < 0.01$; * indicates $P < 0.05$.

Table 3
MIC values of colistin and OST combination therapy for each of the point mutations.

Primers name	Oligonucleotide (5′–3′)[a]	Antibiotics	MIC (μg/mL)		FIC index
			Alone	Combination	
MCR-1-WT	F:CGCGGATCCATGATGCAGCATACTTCT R:CCGCTCGAGTCAGCGGATGAATGCGGT	Colistin	14.00±4.00	2.00±0.00	**0.22±0.06**
MCR-1-Ser330	F:GGCGTGATAATAAT**GCG**GACTCAAAAGGCG R:CGCCTTTTGAGTCCGCATTATTATCACGCC	Colistin	16.00±0.00	6.00±2.31	**0.44±0.14**
MCR-1-Asp331	F:GCGTGATAATAATTCG**GCG**TCAAAAGGCGTGATGG R:CCATCACGCCTTTTGACGCCGAATTATTATCACGC	Colistin	14.00±4.00	8.00±0.00	**0.69±0.25**
MCR-1-Phe344	F: GCTGCCAAAAGCGCAAGCCGGCCGATTATAAATCCG R: CGGATTTATAATCGGCCGCTTGCGCTTTTGGCAGC	Colistin	16.00±0.00	7.00±2.00	**0.50±0.13**

[a] Restriction endonuclease recognition sites or mutated codons are underlined. The data were presented as the mean ± standard deviation.

Table 4
MIC values of colistin and OST combination therapy for *Escherichia coli* W3110 (pUC19-*mcr-3*).

Species	Antibiotics	MIC (μg/mL)		FIC index
		Alone	Combination	
E. coli W3110 (pUC19-*mcr-3*)	Colistin	8.00±0.00	1.00±0.00	**0.19±0.00**

The MICs were determined in triplicate, and the fold change is indicated in parentheses in bold. The data were presented as the mean ± standard deviation.

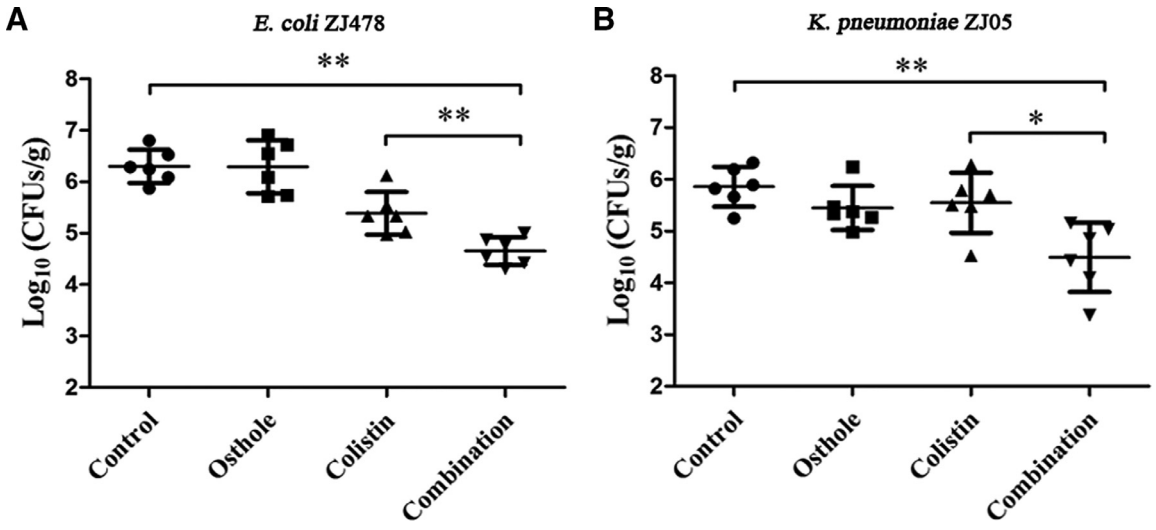

Fig. 5. Effects of OST and colistin combination therapy *in vivo*. The mice were infected intramuscularly in the thigh with *E. coli* ZJ478 and *K. pneumoniae* ZJ05, and the bacterial burden of the thighs was calculated (A), (B). $^{**}P < 0.01$; $^{*}P < 0.05$.

consistent with the above result (Fig. 3(C)). These results suggest that these three residues are key residues for OST and indicate that the information generated by the MD simulation on the MCR-1-OST complex is reliable. Due to the binding of inhibitor, OST, with the binding region (residues of Asp331, Ser332, Gly334, Met336, Lys341, and Phe344), the biological activity of MCR-1 was inhibited.

Western blot analysis was used to further determine the production of MCR-1 by *mcr-1*-positive isolates. As shown in Fig. 4(B), OST at the indicated concentrations had no influence on the production of MCR-1. To further verify the potential mechanism by which OST affects the activity of MCR-1, the checkerboard microdilution method was applied to evaluate synergies between OST and colistin against variants with amino acid mutations at Ser330, Asp331 and Phe344. As expected, the synergistic effect on variants was significantly reduced compared with *E. coli* BL21(DE3)(pET-28a-*mcr-1*) (Fig. 4(A), Table 3). Together, our results suggest that OST is a potential MCR-1 inhibitor by directly occupying the amino acids Ser330, Asp331 and Phe344.

Combination therapy of OST and colistin synergistically attenuates the mcr-1*-positive bacteria burden* in vivo

To determine whether the synergistic effects could be replicated *in vivo* in the mouse model of thigh infection by *K. pneumoniae* ZJ05 or *E. coli* ZJ478, the bacterial burden was assessed at 72 h post-infection. The combination of colistin and OST resulted in a significant reduction of the bacterial load in the thigh compared with the monotherapy treatments ($P < 0.01$) (Fig. 5(A) and (B)), although the colistin-treated group also showed a significant decrease in CFU compared with the control group ($P < 0.05$). Thus, our results indicate that OST combined with colistin significantly reduced the bacteria burden in the mouse model of thigh infection.

Discussion

The emergence and spread of antibiotic-resistant bacteria that causes severe clinical infections is of great concern for researchers.

We were pleased to find that OST, targeting *mcr-1*-positive colistin-resistant *Enterobacteria* demonstrated efficacy both *in vitro* and *in vivo*. Although the pharmacological effect of OST needs further study *in vivo*, OST as an agent can be considered a promising leading compound for clinical infection by *mcr-1*-resistant bacteria in the future.[36] OST is only effective against MCR-1, plasmid-mediated polymyxin resistance or chromosomal-carrying polymyxin resistance, without synergy with any other antibiotics, and it is relatively inferior against *mcr-1* negative polymyxin-resistant clinical isolates. This specific synergy further demonstrated that OST may directly focus on MCR-1 and influence its activity. Additionally, the water solubility of OST limits its maximum pharmacological activity *in vitro/in vivo*.[37] Therefore, the molecular structure of OST requires further modification.

Clinical drugs such as Chinese medicinal formulae have limited treatment efficacy owing to their poor intestinal absorption.[14,38] In this study, we attempted to subcutaneously administer OST and colistin to the infected mice and achieved favourable therapeutic effects. OST is the major pharmaceutical ingredient in *C. fructus*, which has been widely used in traditional Chinese medicinal herb formulae to treat infectious diseases such as suppurative dermatitis and vaginitis.[39] It was reported that no deaths or obvious adverse reactions were observed in the BALB/c mice after oral administration with a concentration of OST (100 mg/kg) for 6 weeks, and it indicated the absence of obvious toxic effects of osthole on Chang liver cells and HepG2 cells using the MTT assay.[37] In addition, OST was intraperitoneally injected administered at a dosage of 30 mg/kg twice daily, which did not show obvious toxic effects.[40]

The molecular dynamic simulation for the MCR-1-OST complex system was carried out to explore the interaction mechanism between OST and MCR-1. By means of the molecular dynamic simulation, we found that OST could localize to the binding pocket of MCR-1 (residue 330–350). Due to the binding of OST with MCR-1, the binding of substrate with MCR-1 was blocked, leading to the loss of biological activity of MCR-1. For polymyxin-resisitance, a variety of polymyxin resistance mechanisms are present in *Enterobacteriaceae* species, with some strains containing two or more resistance enzymes. Naturally, only *E. coli* BL21(DE3)(pET28a-*mcr-1*) and K. *pneumoniae* ZJ02 were inhibited by OST and showed a positive MPNP test.[29]

In summary, our study shows that a combination of polymyxins and OST may be an alternative treatment option against MCR-1-positive polymyxin-resistant *Enterobacteriaceae*. Furthermore, the effect of OST combined with colistin against these bacteria was validated in this study. Additional studies are necessary to further confirm the universality of the synergistic effect of the MCR-1 inhibitor, OST, on MCR-producing isolates by testing other *Enterobacteriaceae*, which carries *mcr-1* or its variants isolated worldwide.

Transparency declarations

None to declare.

Conflicts of interest

The authors declare no competing financial interest.

Author contributions

Study design: Xuming Deng, Yang Wang, Yonglin Zhou; Experimental studies: Yonglin Zhou, Jianfeng Wang, Yan Guo; Data analysis/interpretation: Shunli Liu, xinqi Liu, Xiaodi Niu; Statistical analysis: Yonglin Zhou, Jianfeng Wang; Manuscript preparation: Xuming Deng, Yang Wang, Yonglin Zhou.

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Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jinf.2019.03.004.

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