Journal of Microbiology, Immunology and Infection 58 (2025) 189–197

Contents lists available at ScienceDirect

Journal of Microbiology, Immunology and Infection

journal homepage: www.e-jmii.com

Synergistic effect of repurposed mitomycin C in combination with antibiotics against *Aeromonas* infection: In vitro and in vivo studies

Cheng-Fa Yeh [a,b], Chi-Chung Chen [c], Chih-Cheng Lai [d], Jin-Wei Liu [b], Hung-Jen Tang [b,*], Wen-Pin Su [a,e,f,**]

[a] Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[b] Department of Internal Medicine, Chi Mei Medical Center, Tainan, Taiwan
[c] Medical Research, Chi Mei Medical Center, Tainan, Taiwan
[d] Department of Intensive Care Medicine, Chi Mei Medical Center, Tainan, Taiwan
[e] Department of Oncology, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan
[f] Department of Internal Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University, Tainan, Taiwan

ARTICLE INFO

Keywords:
Aeromonas infections
Antimicrobial combination therapy
Mitomycin C
Synergistic effect

ABSTRACT

Background: *Aeromonas* infections pose a significant threat associated with high mortality rates. This study investigates the potential of mitomycin C (MMC), an anticancer drug, as a novel antimicrobial agent against *Aeromonas* infections.

Methods: We evaluated the minimum inhibitory concentrations (MICs) of MMC and antibiotics against clinical *Aeromonas* isolates using broth microdilution. Synergistic effects of MMC with antibiotics were determined via time-kill studies. MMC's intracellular killing effects were analyzed using a representative *Aeromonas* isolate. Efficacy of combined therapies was assessed in a neutropenic mouse model. MMC-induced SOS response was evaluated using cell elongation method, and RNA extraction and quantitative real-time PCR.

Results: Combining 1/8× MIC of mitomycin C (MMC) with either 1× or 1/2× MIC of LVX demonstrated significant synergistic effects over 24 h in vitro. In a neutropenic mouse model, the combination of MMC (2 mg/kg or 1 mg/kg) with LVX achieved survival rates of 100 % and 80 %, respectively, compared to 0 % survival with monotherapy. MMC induced marked cell elongation and division inhibition in response to escalating doses. However, the combination therapy's enhancement did not surpass the effects of individual drug treatments. Notably, combination therapy reduced *recA* activator levels below those observed with either drug alone, suggesting rapid bacterial cell death curtailed further expression of *recA* and *lexA*. Alternatively, extensive DNA damage may have overwhelmed bacterial DNA repair mechanisms, rendering them ineffective.

Conclusions: These findings suggest that MMC may serve as a potential antimicrobial agent, particularly when used in combination with antibiotics. The integration of MMC with antibiotic therapy offers a promising approach for the treatment of *Aeromonas* infections and holds potential for future clinical applications.

1. Introduction

Aeromonas, a common pathogen to both farm-raised and wild fish, poses a significant health threat, causing severe illnesses, including septic arthritis, diarrheal enteritis, skin and soft tissue infections, meningitis, and bacteremia.[1,2] In Southern Taiwan, the annual incidence of *Aeromonas* bacteremia averaged 76 cases per million inhabitants from 2008 to 2010, exceeding rates in Western countries.[3] *A. hydrophila*, *A. caviae*, and *A. veronii biovar sobria* are common etiological agents, with virulence factors including cytotoxins, proteases, hemolysins, lipases, adhesins, agglutinins, enterotoxins, and VacB.[4,5] Early and aggressive surgical debridement and broad-spectrum antibiotics, including the combination of doxycycline (DOX) with either ciprofloxacin (CIP) or ceftriaxone, is crucial.[6]

Given the slow pace of antimicrobial agent development, there is an urgent need for novel strategies to enhance antibiotic efficacy against *Aeromonas* infections. Mitomycin C (MMC), derived from *Streptomyces caespitosus*, disrupts DNA by inducing cross-linking and inhibiting

* Corresponding author. Department of Internal Medicine, Chi Mei Medical Center, No.901, Zhonghua Road, Tainan, 710, Taiwan.
** Corresponding author. Institute of Clinical Medicine, College of Medicine, National Cheng Kung University, No.138, Sheng Li Road, Tainan, 704, Taiwan.
E-mail addresses: 8409d1@gmail.com (H.-J. Tang), wpsu@mail.ncku.edu.tw (W.-P. Su).

https://doi.org/10.1016/j.jmii.2024.12.005
Received 13 June 2024; Received in revised form 3 December 2024; Accepted 19 December 2024
Available online 27 December 2024

synthesis, showing the potential anti-bacterial effect.[7] A previous study reported that MMC combined with a tobramycin-ciprofloxacin hybrid exhibits synergistic effects against multidrug-resistant (MDR) Gram-negative bacteria.[8] Combining antibiotics with non-antibiotic compounds that enhance their activity is crucial for combating severe infectious diseases.

This study explored the repurposing of MMC in combination with antimicrobial agents to target *Aeromonas* species, aiming for minimal effective dosages to reduce side effects.

2. Materials and methods

2.1. Bacterial isolates

The thirty-one *Aeromonas* isolates, including 11 isolates of *Aeromonas hydrophila*, 10 isolates of *A. caviae*, and 10 isolates of *A. sobria*, were identified by MALDI-TOF MS (Bruker Daltonik, Bremen, Germany) and *rpoB* sequencing.[9,10] These isolates were isolated from clinical specimens, including blood, ascites, pus, and wound exudates from patients. The species were identified by DNA sequence matching of *rpoD* and *gyrB* (or *rpoB* if necessary). The sequences of amplified DNA products were compared with reference sequences available in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Isolates with a dissimilarity value of ≤1 % were considered the same species. These isolates were stored at −80 °C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, UK) before investigation.

All primers utilized in PCR/qPCR assays must be accompanied by either their reference citations or complete sequence information (Supplementary appendix 1). PCR amplicons were sequenced on an ABI PRISM 3730 sequence analyzer (Applied Biosystems, Foster City, CA, USA).

2.2. Antimicrobial susceptibility testing

Standard amikacin (AMK), aztreonam (ATM), cefazolin (CZ), cefmetazole (CMZ), cefotaxime (CTX), cefpirome (CPO), chloramphenicol (CHL), CIP, DOX, fosfomycin (FOS), gentamicin (GM), imipenem (IPM), levofloxacin (LVX), meropenem (MEM), minocycline (MIN), tigecycline (TGC), and MMC were used for antimicrobial susceptibility testing.

Antibiotic MICs were determined by the broth microdilution method, and the interpretation criteria were based on the recommendations of the Clinical and Laboratory Standards Institutes (CLSI, 2023)[11] or the US Food and Drug Administration (FDA).[12] The normal inoculum 5×10^5 CFU/mL and high inoculum 5×10^7 CFU/mL were detected by this method. Quality control testing was performed using *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, and *Pseudomonas aeruginosa* ATCC 27853.[13]

2.3. Combination killing method

Eight *A. hydrophila* isolates were randomly selected for in vitro measurement of the inhibitory effect of combination regimens, modified as reported by Tang et al.[14] In brief, bacterial suspensions were diluted to 5×10^7 CFU/mL in fresh Mueller–Hinton broth. The drug concentrations of CIP, LVX, or TGC were adjusted to 1× MIC, 1/2× MIC, 1/4× MIC, and 1/8× MIC combined with 1× MIC, 1/2× MIC, 1/4× MIC, and 1/8× MIC of MMC. Bacterial counts were measured at 24 h by enumerating the colonies in 10-fold serially diluted specimens of 100-μL aliquots plated on the nutrient agar ((Difco™ Laboratories, Sparks, MD, USA) at 37 °C. All experiments were performed in duplicate. The statistical significances were compared with that of each drug alone and no drug control:*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

2.4. Cell cytotoxicity test

The PrestoBlue assay was used to evaluate cytotoxicity.[15] Briefly, RAW 264.7 cells were seeded in 24-well plates at a density of 5×10^5 cells/well for 24 h. After the culturing medium was removed, the cells were washed with PBS twice prior to treatment with MMC of different concentrations (1×, 1/2× and 1/4× MIC) for 0, 2, 4, 24 h. After removing the medium, the cells were treated with 10 % PrestoBlue reagent (Invitrogen Corporation, San Diego, USA) for 2 h. The absorbance wavelengths at 570 nm and 600 nm were measured. The cell survival rate of the control treatment was set at 100 %. The experiments were conducted in three independent repeats, and all data were presented as the mean ± S.D.

2.5. Assessment of the intracellular antibacterial activity of antibiotics

RAW 264.7 cells diluted to 5×10^5 CFU/mL cells in 24-well culture plates. The cells were incubated with 5×10^6 CFU/mL well of *Aeromonas* strain Aero01-243. Thus, the ratio of viable bacteria to cells was 10:1. One hour later, the culture plates were incubated with 8 μg/mL gentamicin for 2 h at 37 °C to kill the extracellular bacteria and then washed twice with phosphate-buffered saline (PBS). The drug concentration of MMC and LVX were adjusted to those of 1/2×, and 1/4× MIC. Each drug alone and the combination of LVX were test. At selected time intervals (0, 2, 4, 24 h), bacterial loads in the wells were counted. The cells were washed with ice-cold PBS. Resuspension the bacteria in 0.5 mL of 1 % Triton X-100 in PBS, and the lysates with released bacteria were serially diluted (1:10 in PBS), plated on MH agar plates, and cultured overnight prior to bacterial counting. The limit of detection via plate counting of bacterial loads was about 10 CFU/mL.[16–18]

2.6. Murine infection model

Female BALB/c mice (18–20 g, 6–8 weeks old, National Science Council, Taipei, Taiwan) were used. This study was approved by the Chi-Mei Medical Center Institutional Animal Care and Use Committee and conducted per National Research Council recommendations and were provided food and water ad libitum. Mice were rendered neutropenic by intraperitoneal injections with 100 and 250 mg/kg of body weight of cyclophosphamide (Sigma-Aldrich, St. Louis, MO, USA) given one and four days prior to inoculation, respectively.[19] The *Aeromonas* strain was cultured centrifuged, and adjusted to the desired turbidity for intramuscular injection (0.05 mL) into ten mice. Antimicrobials were initiated 2 h after inoculation for 48 h.

Mice received MMC (2 mg/kg every 24 h) and LVX (20 mg/kg every 12 h). The selection of the 2 mg/kg MMC dosage in vivo was based on converting the clinically administered human dose (10 mg/m^2) to the mouse-equivalent dose.[20] Low MMC doses (1 and 0.5 mg/kg) were also tested. Survival was recorded at 8-h intervals for 7 days.

2.7. Cell elongation method

Aeromonas strains were grown in Mueller-Hinton broth (MHB) medium at 37 °C for 90min, then diluted to a 0.5 McFarland standard. Bacteria were incubated at 37 °C with MMC (at 1/2×, 1/4×, or 1/8× MICs) for 180 min in a final volume of 3 mL. Fluid samples were Gram stained to evaluate cell length. Slides were treated with crystal violet for 60 s, Gram iodine for 60 s, decolorized with 95 % ethyl alcohol for 5–10 s, and counterstained with safranin for 45 s. This protocol produced clear images, facilitating accurate assessment of cell morphology.[21]

2.8. RNA extraction and quantitative real-time PCR (Q-PCR)

Bacteria were grown overnight in MHB at 37 °C then the mild-log phase cultures were adjusted to match the turbidity standard of 0.5 McFarland. This experiment was performed different MMC

concentrations (1/2×, 1/4×, 1/8× MICs) at 2 h. Treated and untreated cultures were extracted total RNA using Bioanalysis, DNA, RNA, protein purification Kit (Macherey-Nagel, Düren, Germany). 250 ng of total RNA from each sample were reverse transcribed to cDNA using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scien-tific, Vilnius, Lithuania). qPCR was carried out in triplicate on each sample using Applied Biosystems™ Fast SYBR™ Green Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) in a StepOnePlus™ Real-Time PCR Systems (Thermo Fisher Scientific). Forward and reverse primers for *recA*, *lexA*, *uvrB*, *uvrA*, *umuD* and *umuC* were designed with an amplicon length of about 200 bp from NCBI Reference Sequence (accession number: NZ_BAFL01000032.1). The expression levels of target genes were normalized using the 16 S rRNA housekeeping genes as endogenous controls (Supplementary appendix 2).[22]

3. Results

3.1. MIC results for various antibiotics and mitomycin C

Overall susceptibility rates exceeding 90 % under normal inoculum conditions (5 × 10^5 CFU/mL) were observed for AMK, GM, ATM, CTX, CPO, CIP, LVX, MIN, TGC, FOS, and MEM. However, susceptibility rates fell below 90 % for CZ, CMZ, CHL, DOX, and IPM (Table 1). Notably, only three antibiotics—CIP, LVX, and TGC—maintained susceptibility rates above 90 % under high inoculum conditions (5 × 10^7 CFU/mL). Additionally, differential susceptibility rates for *Aeromonas hydrophila*, *A. caviae*, and *A. sobria* were observed with AMK, CPO, and MEM (Supplementary Appendix 3).

3.2. Time-kill studies

The synergistic effects of MMC combined with antibiotics were investigated against eight *Aeromonas hydrophila* isolates with a high inoculum 5 × 10^7 CFU/mL. Broth microdilution methods were conducted for 24 h using MMC at 1×, 1/2×, 1/4×, 1/8×, and 0× MICs combined with CIP, LVX, and TGC at similar MICs (Table 2). Significant synergistic effects were observed with MMC at 1×, 1/2×, and 1/4× MICs combined with CIP at 1× MIC ($p < 0.001$; Table 2A), and with MMC at 1×, 1/2×, 1/4×, and 1/8× MICs combined with LVX at 1× MIC ($p < 0.001$; Table 2B). However, TGC exhibited a less effect with MMC ($p < 0.001$; Table 2C). Consequently, LVX was selected for further in vivo studies due to its superior synergistic performance with MMC.

Table 1
The minimum inhibitory concentrations (MICs) values were determined using broth microdilution for sixteen antimicrobial agents against 31 isolates of the *Aeromonas* species.

	Normal inoculum			High inoculum		
	MIC $_{50}$, mg/L	MIC $_{90}$, mg/L	susceptible	MIC $_{50}$, mg/L	MIC $_{90}$, mg/L	susceptible
AMK	4	8	100	16	32	67.86
GM	1	2	96.42	4	8	71.43
ATM	≦0.03	0.25	100	1	16	85.71
CZ	>128	>128	0	>128	>128	0
CMZ	16	>64	53.57	>64	>64	35.71
CTX	0.25	1	92.86	2	16	64.29
CPO	0.06	0.5	92.86	16	>32	39.29
CHL	1	4	89.29	2	8	89.29
CIP	0.12	0.5	96.42	0.25	1	92.86
LVX	0.12	0.25	100	0.25	0.5	100
DOX	4	8	71.43	8	16	25
MIN	2	4	100	4	8	75
TGC	1	2	100	1	2	96.42
FOS	4	16	100	16	64	89.29
MEM	0.12	1	96.42	32	>32	35.71
IPM	2	8	79.31	>64	>64	21.43
MMC	0.12	0.5	–	0.25	0.5	–

AMK, amikacin; GM, gentamicin; ATM, aztreonam; CZ, cefazolin; CMZ, cefmetazole; CTX, cefotaxime.
CPO, cefpirome; CHL, chloramphenicol; CIP, ciprofloxacin; LVX, levofloxacin; DOX, doxycycline; MIN.
Minocycline; TGC, tigecycline; FOS, fosfomycin; MEM, meropenem; IPM, imipenem; MMC, mitomycin C.

3.3. Intracellular antibacterial activity of mitomycin C

Following a 24-h exposure to MMC at concentrations of 1×, 1/2×, 1/4×, and 0×, the survival rates of RAW 264.7 cells were 87.6 %, 93.8 %, 93.3 %, and 100 %, respectively, indicating a relatively low cytotoxicity associated with MMC.

The intracellular killing effects of MMC, LVX alone, and the combination of MMC and LVX against *Aeromonas* Aero01-243 are presented in Fig. 1. At 24 h, the combination of 1/2× and 1/4× MICs of MMC with 1/2× MIC of LVX resulted in no detectable *Aeromonas* Aero01-243 ($P \leq 0.001$), whereas 1/2×, 1/4× MMC or 1/2× LVX alone, as well as the control group, showed no inhibition, with bacterial counts exceeding log 5. The combination of 1/2× and 1/4× MICs of MMC with 1/2× MIC of LVX significantly reduced intracellular colony counts.

3.4. Animal study

Neutropenic mice were intramuscularly administered *Aeromonas* Aero01-243 at concentrations of 1.8 × 10^2, 1.8 × 10^3, 1.8 × 10^4, and 1.8 × 10^5 CFU/mL (Fig. 2). The group with inoculum of 1.8 × 10^2 CFU/mL exhibited a 100 % survival rate (10/10) on day 1, 70 % (7/10) on day 2, 60 % (6/10) on day 3, and 0 % (0/10) after day 4. The group inoculated with 1.8 × 10^3 CFU/mL exhibited a 100 % survival rate (10/10) on day 1, dropping to 0 % (0/10) after day 2. The 1.8 × 10^4 CFU/mL group exhibited a 0 % survival rate (0/10) on day 1. Remarkably, mice inoculated with 1.8 × 10^4 CFU/mL and treated with 2 mg/kg MMC maintained a 100 % survival rate (10/10) until day 7, whereas those with 1.8 × 10^5 CFU/mL treated with 2 mg/kg MMC exhibited a 30 % survival rate (3/10) on day 1 and 0 % (0/10) after day 2. The 1.8 × 10^4 CFU/mL group exhibited significantly higher survival rate compared to the other groups.

3.5. Synergy of mitomycin C with levofloxacin

In neutropenic mice, *Aeromonas* Aero01-243 was administered intramuscularly at 0.99 × 10^5 CFU/mL (Fig. 3 A) or 0.99 × 10^6 CFU/mL (Fig. 3 B), and treated with MMC, LVX, or a combination of both. At 0.99 × 10^5 CFU/mL, the control group exhibited a 0 % survival rate (0/10) by day 1, the MMC group declined to 0 % (0/10) by day 2, the LVX group exhibited 60 % (6/10) after day 4, and the combination group maintained 100 % survival rate (10/10) until day 7. At 0.99 × 10^6 CFU/mL, both the control and MMC groups had 0 % survival, while the LVX group had 0 % survival (0/10) after day 4, and the combination group maintained 100 % survival (10/10) until day 7. The combination of MMC and LVX demonstrated significant synergistic effects, achieving a 100 % survival rate at both bacterial concentrations, compared to 0 % survival with MMC alone and 60 % (at 0.99 × 10^5 CFU/mL) or 0 % (at 0.99 × 10^6 CFU/mL) with LVX alone.

3.6. Efficacy of lower dosages of mitomycin C in combination with levofloxacin

Survival rates were assessed using a reduced dosage of MMC in combination with LVX against a higher inoculum (1.89 × 10^6 CFU/mL) of *Aeromonas* Aero01-243 (Fig. 4). On day 1, the control group showed 0 % survival (0/10). The LVX group had 100 % survival (10/10) on day 1, 20 % (2/10) on day 2, and 0 % (0/10) post day 3. The MMC group, combined with LVX, demonstrated survival rates of 20 % (2/10), 80 % (8/10), and 100 % (10/10) on day 7 at MMC doses of 0.5, 1, and 2 mg/

Table 2
The 24- killing effect of the antibiotic combination on the 8 isolates of *Aeromonas* species with a high inoculum (5×10^7 CFU/mL) using broth methods with 1×, 1/2×, 1/4×, 1/8× and 0× mitomycin C (MMC) in 1×, 1/2×, 1/4×, 1/8× and 0× minimum inhibitory concentrations (MICs) of ciprofloxacin (CIP) (A), levofloxacin (LVX) (B), and tigecycline (TGC) (C). Colony counts are shown as the log means ± standard deviations. P value was compared with that of each drug alone and no drug control. *: $P < 0.05$. **: $P < 0.01$. ***: $P < 0.001$.

A

24hr	1× CIP	1/2× CIP	1/4× CIP	1/8× CIP	0× CIP
1× MMC	0.83 ± 1.49 ***	3.14 ± 2.61 ***	6.46 ± 1.59 *	7.16 ± 0.81	7.51 ± 0.11
1/2× MMC	2.24 ± 2.37 ***	5.00 ± 2.41 ***	7.14 ± 1.39 *	8.00 ± 0.40	8.11 ± 0.45
1/4× MMC	3.70 ± 2.35 ***	5.68 ± 2.49 ***	7.78 ± 0.35 ***	8.14 ± 0.41	8.31 ± 0.35
1/8× MMC	5.06 ± 2.45 **	7.42 ± 0.57 ***	7.86 ± 0.37 ***	8.12 ± 0.41	8.36 ± 0.31
0× MMC	7.18 ± 0.81	8.09 ± 0.39	8.40 ± 0.28	7.46 ± 2.91	8.59 ± 0.08

B

24hr	1× LVX	1/2× LVX	1/4× LVX	1/8× LVX	0× LVX
1× MMC	0.00 ± 0.00 ***	3.13 ± 2.25 ***	5.68 ± 2.40 *	5.87 ± 2.46 *	7.25 ± 0.43
1/2× MMC	1.62 ± 1.69 ***	5.25 ± 2.25 ***	6.31 ± 2.50 *	7.45 ± 0.35 **	7.86 ± 0.37
1/4× MMC	3.09 ± 2.09 ***	6.46 ± 1.28 ***	7.51 ± 0.50 ***	7.90 ± 0.42 ***	8.45 ± 0.18
1/8× MMC	3.83 ± 2.48 ***	7.43 ± 0.37 ***	8.15 ± 0.47 *	8.25 ± 0.47	8.57 ± 0.11
0× MMC	6.31 ± 1.07	8.01 ± 0.45	8.34 ± 0.34	8.49 ± 0.27	8.68 ± 0.17

C

24hr	1× TGC	1/2× TGC	1/4× TGC	1/8× TGC	0× TGC
1× MMC	4.10 ± 2.67 ***	6.84 ± 0.81**	7.42 ± 0.56	7.69 ± 0.71	7.57 ± 0.51
1/2× MMC	5.34 ± 2.36 **	7.44 ± 0.70 **	7.82 ± 0.41	7.90 ± 0.72	8.10 ± 0.46
1/4× MMC	5.88 ± 2.49 *	7.89 ± 0.65	8.19 ± 0.46	8.09 ± 0.73	8.33 ± 0.40
1/8× MMC	7.24 ± 0.71	8.17 ± 0.49	8.34 ± 0.46	8.25 ± 0.52 *	8.54 ± 0.16
0× MMC	7.44 ± 0.59	8.27 ± 0.48	8.44 ± 0.34	8.63 ± 0.22	8.75 ± 0.16

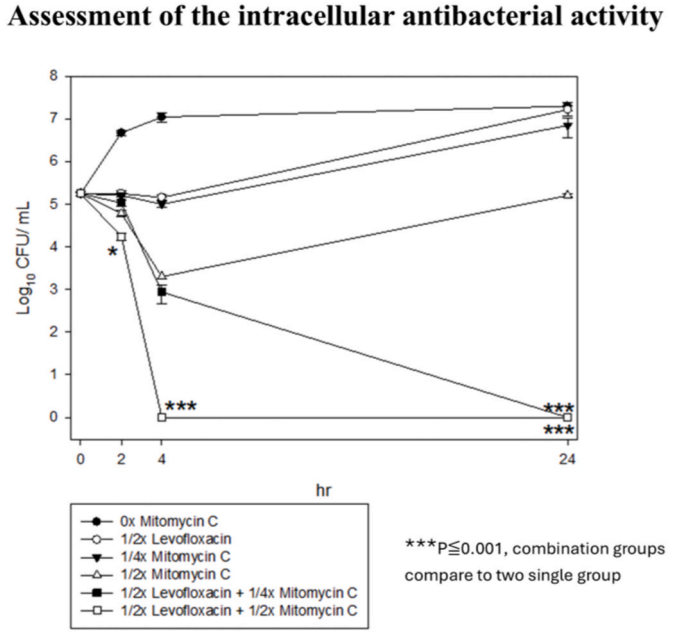

Fig. 1. The intracellular killing effects of MMC, LVX against Aeromonas Aero01-243.

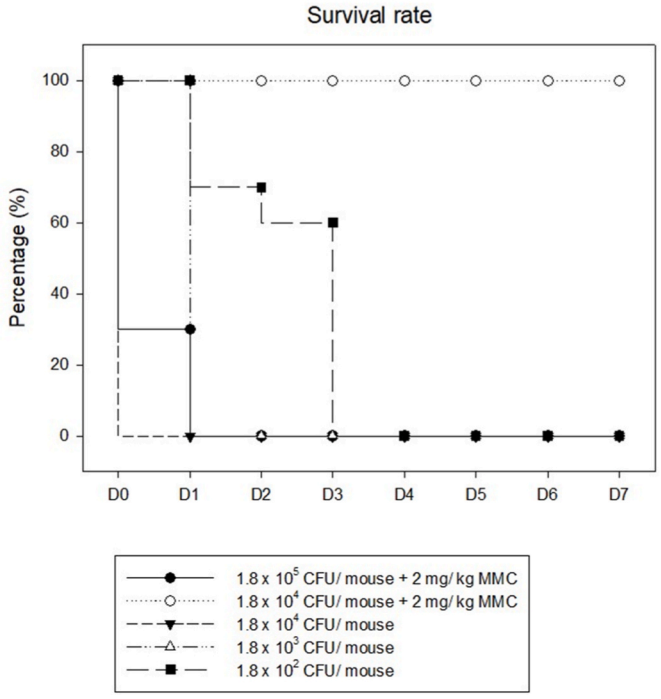

Fig. 2. Survival rates of neutropenic mice infected Aeromonas Aero01-243 at various inoculum size treated with MMC compared to the control group.

kg, respectively.

3.7. Cell elongation induced by mitomycin C

Investigating the role of MMC, we examined MMC-induced SOS-related cell elongation. MMC treatment induced significant SOS-related cell elongation, with cell lengths measuring 7.23 ± 2.25 μm at 1/2× MIC, 5.37 ± 2.30 μm at 1/4× MIC, 3.99 ± 1.44 μm at 1/8× MIC, compared to 1.06 ± 0.22 μm in the control group (mean ± SD). Cell counts were consistently lower in the MMC-treated groups than in controls (Fig. 5A). Significant increases in cell length were observed at 1/2× MIC versus 1/8× MIC and control ($p < 0.001$), at 1/2× MIC versus 1/4× MIC ($p < 0.01$), and at 1/4× MIC versus 1/8× MIC and control ($p < 0.001$), with 1/8× MIC also showing significant elongation compared to control ($p < 0.001$) (Fig. 5B).

3.8. Modulation of SOS gene expression in response to mitomycin C therapy

The fold changes in gene expression at various concentrations are

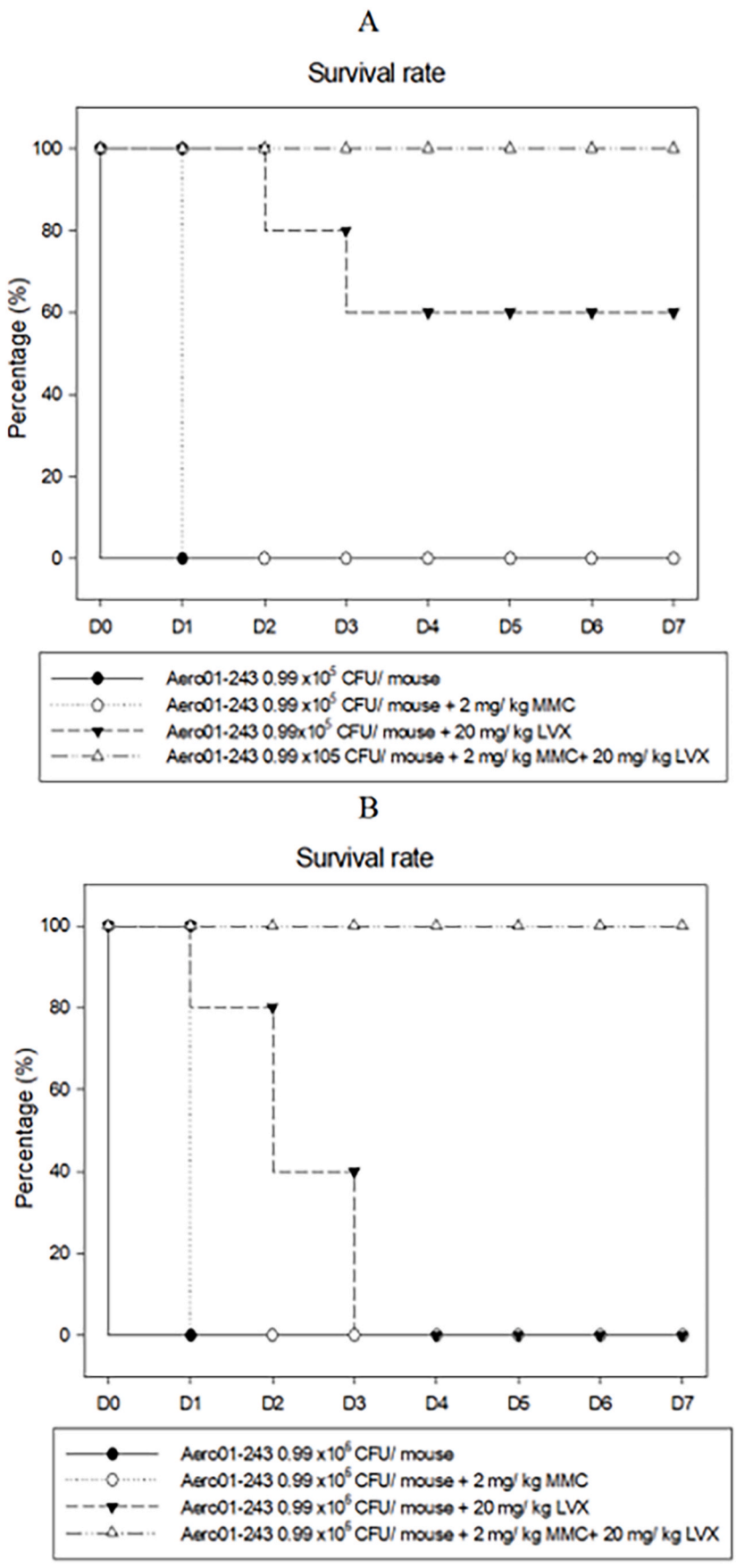

Fig. 3. Survival rates in neutropenic mice infected with a clinical isolate of Aeromonas Acro 01-243 at a normal inoculum (0.99×10^5 CFU/mL) (Fig. 3 A) or high inoculum (0.99×10^6 CFU/mL) (Fig. 3 B) treated with MMC, levofloxacin or a combination of MMC and levofloxacin.

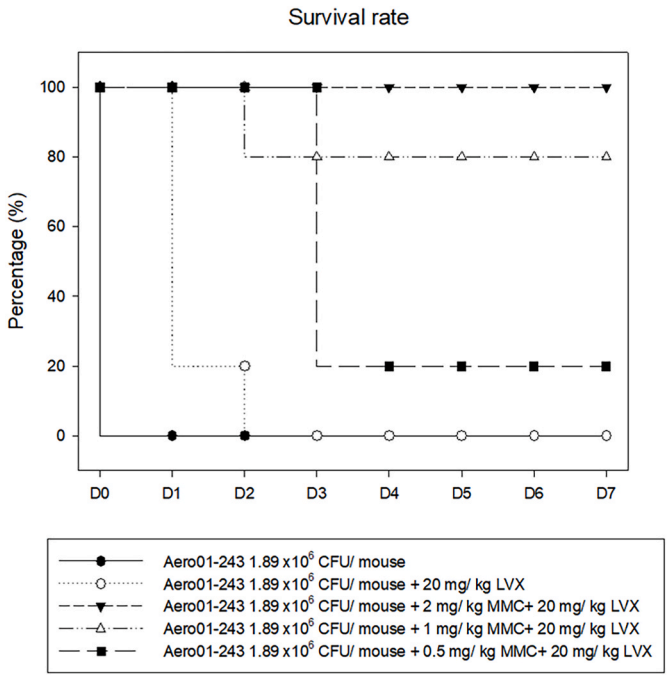

Fig. 4. Survival rates in neutropenic mice infected with Aeromonas Aero01-243 at a higher inoculum (1.89×10^6 CFU/mL) with various doses of MMC in combination with levofloxacin.

shown in Fig. 6. For *recA, lexA, uvrA, and umuD*, fold changes at 1×, 1/2×, 1/4×, and 1/8× MIC of MMC demonstrated significant increases compared to the control. For *umuC*, fold changes at 1×, 1/2×, and 1/4× MIC of MMC also showed significant upregulation compared to the control. These findings suggest that the MMC-induced SOS response regulates the expression of *recA, lexA, uvrA, umuD, and umuC*. The fold changes in gene expression at 1/2× MIC of MMC in combination with 1/2× MIC of LVX is presented in Fig. 7. For *recA, lexA, uvrA, uumuD, and umuC*, fold changes at 1/2× MIC of MMC, 1/2× MIC of LVX alone, or 1/2× MIC of MMC combined with 1/2× MIC of LVX exhibited significant increases in gene expression compared to the control. These results suggest that the MMC and LVX combination induces an SOS response that regulates the expression of *recA, lexA, uvrA, umuD, and umuC*.

4. Discussion

We found a notably low MIC of MMC against *Aeromonas* species: MIC_{50}/MIC_{90} values were 0.12/0.5 mg/L at a normal inoculum of 5×10^5 CFU/mL and 0.25/0.5 mg/L at a high inoculum of 5×10^7 CFU/mL. The inoculum effect for MMC was less pronounced than for other antibiotics. However, only CIP, LVX, TGC, and MMC maintained susceptibility rates above 90 % at the high inoculum. Therefore, we investigated synergistic effect of combining MMC with three antibiotics under high inoculum in a time-kill assay. The results showed significant synergy when using 1/8× MIC of MMC with 1× MIC of LVX compared to each drug alone and a no-drug control ($p < 0.001$).

Intracellular survival of *Aeromonas* within host macrophages is crucial in infection outcomes due to complex biochemical mechanisms.[23] Intracellular bacteria, shielded from high extracellular antibiotic levels, elevate drug resistance risk. Additionally, eukaryotic cell efflux pumps reduce intracellular antibiotic concentrations, diminishing efficacy against intracellular bacteria.[24,25] LVX demonstrated a remarkable 300-fold reduction in intracellular bacterial load at clinically relevant concentrations.[26] Our study demonstrated that the combination of 1/2× and 1/4× MICs of MMC with 1/2× MIC of LVX resulted in no detectable *Aeromonas* Aero01-243 ($P \leq 0.001$) in vitro. Consequently, LVX was selected for further investigation in combination with MMC in an animal study.

In immunocompromised individuals, including those with conditions like diabetes, cancer, liver cirrhosis, or HIV, *Aeromonas* infections pose a serious threat, especially bloodstream infections, which can have mortality rates as high as 68 %.[27,28] Treatment with MMC at a lower initial bacterial load (1.8×10^4 CFU/mL) resulted in 100 % survival, contrasting starkly with the 0 % survival seen at a higher initial load (1.8×10^5 CFU/mL). While monotherapy with MMC is effective for mild infections at low bacterial levels, its efficacy diminishes as the bacterial load increases. Although MMC has a short plasma half-life of approximately 20 min[29] mainly due to its rapid hepatic clearance, adverse effects such as bone marrow suppression, renal and hepatic toxicity have rarely occurred after chemotherapy with large doses of this drug for bladder and peritoneal carcinomas.[30] Considering that most adverse effects of MMC, such as myelosuppression, nausea, vomiting, diarrhea, stomatitis, cognitive impairment, and alopecia, are dose-dependent. The selection of the 2 mg/kg MMC dosage in vivo was based on converting the clinically administered human dose (10 mg/m^2) to the mouse-equivalent dose. In a neutropenic mouse model, we evaluated the synergy of combining MMC with LVX against high bacterial inoculums (1.89×10^6 CFU/mL) of *Aeromonas* Aero01-243. Mice receiving 2 mg/kg MMC with 20 mg/kg LVX exhibited a 100 % survival rate, compared to 0 % for MMC or LVX alone, indicating significant clinical potential for MMC-antibiotic combinations. Further, adjusting MMC doses to 1 mg/kg and 2 mg/kg with LVX yielded survival rates of 80 % and 100 %, respectively.

MMC induces DNA cross-links, activating the bacterial SOS response, which regulates DNA damage. A key feature of this response is cellular elongation, dependent on protein synthesis. This intricate pathway is tightly controlled by the *lexA* repressor and the *recA* activator.[31,32] *RecA* triggers *umuD* autolytic cleavage, yielding *umuD′*, which then complexes with *umuC* to form *umuD′2C* (Polymerase V). Concurrently, *uvrA* gene repression is swiftly lifted post-DNA damage, crucial for excision repair. In contrast, *umuDC* expression occurs later during SOS induction.[33] MMC-induced SOS response produce the *lexA*-regulated gene exhibit a role of inhibition of cell division.[34] In our study, escalating doses of MMC significantly increased cell elongation and inhibited cell division, indicating an amplified SOS response, as evidenced by the upregulation of *recA, lexA, uvrB, uvrA, umuD, and umuC*. MMC monotherapy demonstrated a 0 % survival rate at high concentrations of 0.99×10^5 CFU/mL and 0.99×10^6 CFU/mL, likely due to the induction of the SOS response by MMC.

A prior study demonstrated that a range of moxifloxacin concentrations induced *recA* expression, with a peak observed at 2× MIC (0.25 μg/mL), rather than at higher concentrations of 8× or 32× MIC.[35] Another investigation reported that susceptibility to fluoroquinolones is influenced by the cellular concentration of active topoisomerase molecules and the extent of SOS response induction.[36] Notably, the enhancement observed with combination therapy did not exceed the effects of either drug used individually. As shown in Fig. 7, the data demonstrates that the level of *recA* activator during combination therapy was lower than that observed with either drug administered alone. This suggests that the bacteria may undergo rapid cell death under combination therapy, curtailing the further expression of *recA* and *lexA*. Alternatively, excessive DNA damage might overwhelm the bacterial DNA repair mechanisms, rendering them ineffective.

5. Conclusion

Our study reveals MMC's inhibitory effect on intracellular colony counts. Notably, in neutropenic mice, administering MMC and LVX together resulted in 100 % survival, even with reduced dosages, maintaining an impressive 80 % survival rate. These findings strongly support the synergistic efficacy of MMC and LVX against *Aeromonas* species, observed both in vitro and in vivo. Combining MMC with antibiotics for the treatment of *Aeromonas* infections represents a promising

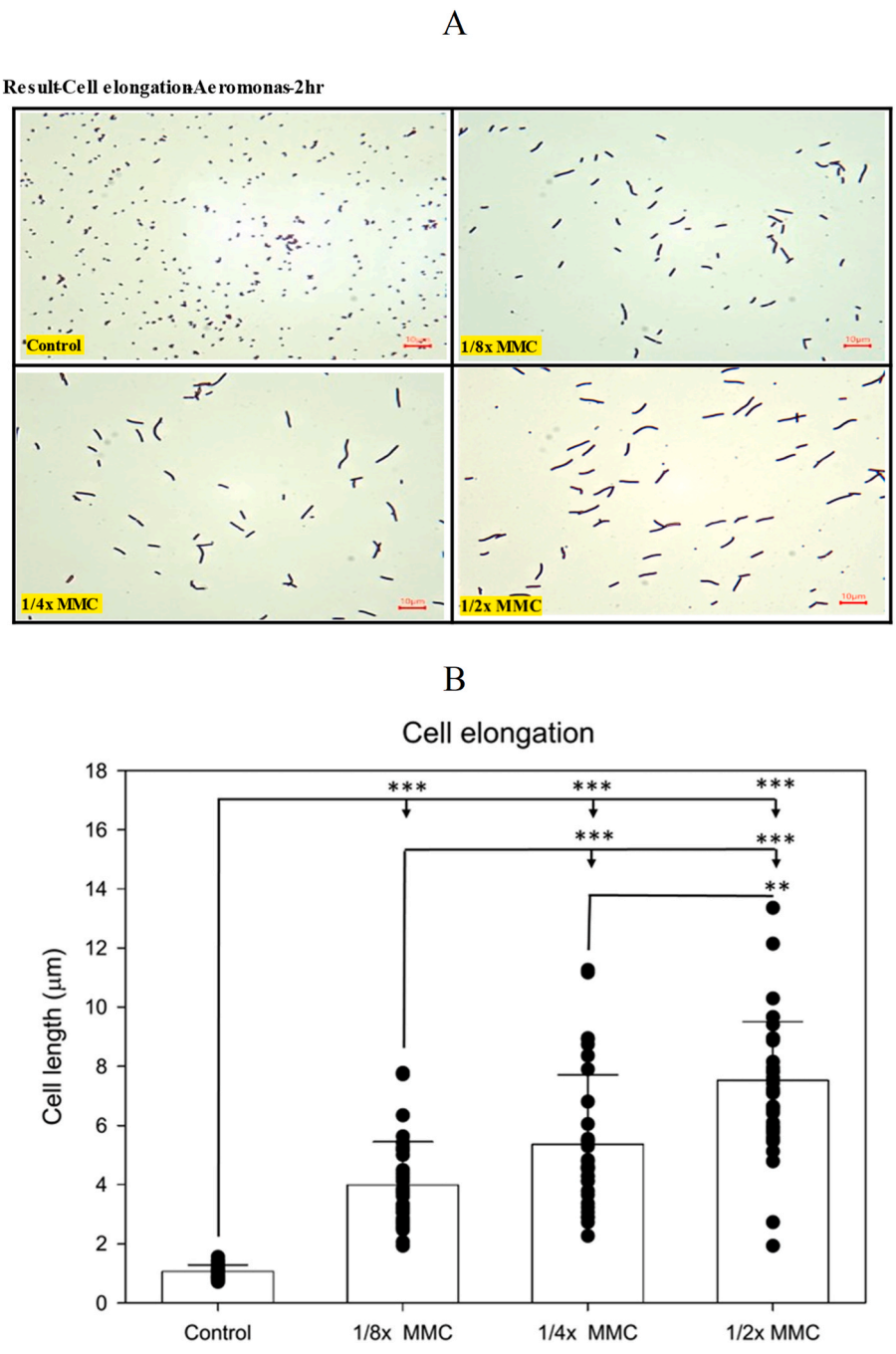

Fig. 5. (Fig. 5A). The mitomycin C (MMC)-induced cell elongation response related to SOS in Aeromonas strain with varying doses of MMC. (upper left) Control without MMC. (upper right) Treated with MMC, 1/8*x* minimum inhibitory concentration (MIC). (lower left) Treated with MMC, 1/4*x* MIC. (lower right) Treated with MMC, 1/2x MIC (Fig. 5B). Dose response of MMC-induced cell clongation in Aeromonas strains. MMC dose is represented in different MICs (horizontal axis), bacterial cell length is measured in μm (vertical axis). P values were compared to different MICs of MMC and a no-drug control.: $P < 0.05$. **: $P < 0.01$ ***: $P < 0.001$.

therapeutic strategy and may hold potential for future clinical applications.

CRediT authorship contribution statement

Cheng-Fa Yeh: Writing – review & editing, Writing – original draft, Project administration, Investigation, Funding acquisition, Conceptualization. **Chi-Chung Chen:** Methodology, Formal analysis, Data curation. **Chih-Cheng Lai:** Writing – review & editing, Formal analysis. **Jin-Wei Liu:** Software, Data curation. **Hung-Jen Tang:** Supervision, Funding acquisition, Conceptualization. **Wen-Pin Su:** Writing – review & editing, Conceptualization.

Data availability

All data generated or analyzed during this study are included in this published article. Raw data is available from the corresponding author upon request.

Ethics approval and consent to participate

The study and waiver from the informed consent process were approved by the Institutional Review Board (IRB) of the Chi Mei Medical Center, Tainan City, Taiwan (IRB Serial number LAC. SOP102-104).

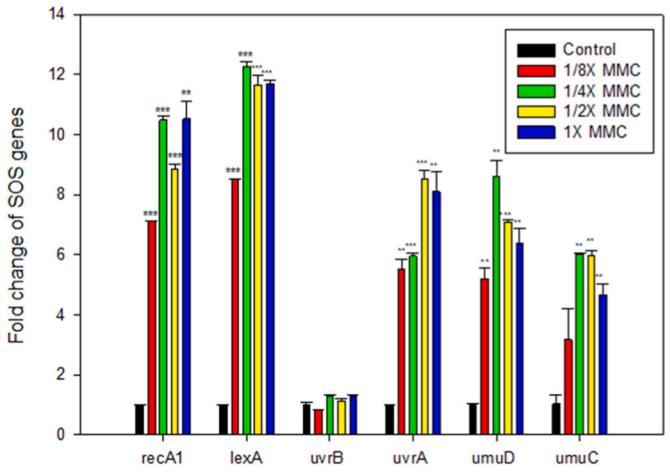

Fig. 6. Fold change in SOS gene expression in Aeromonas strains in response to varying dosages of mitomycin C (horizontal axis), fold change of SOS genes (vertical axis). The statistical significances were compared with that of no drug control: *P < 0.05 **P < 0.01 and ***P < 0.001.

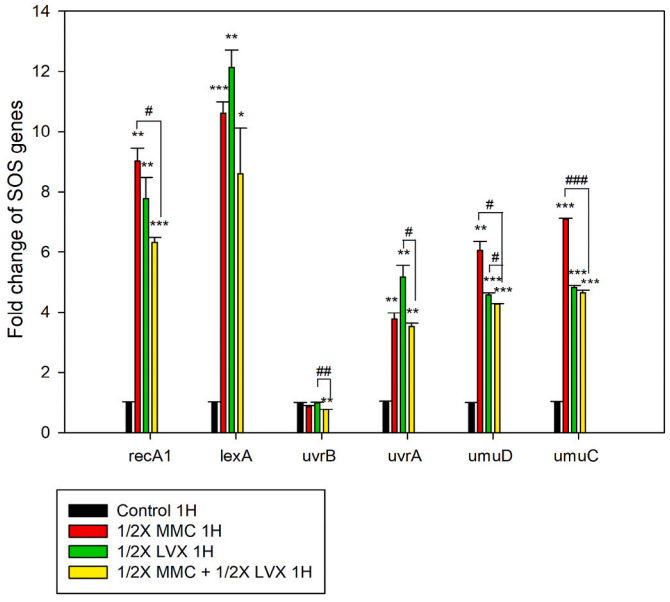

Fig. 7. Fold change in SOS gene expression in Aeromonas strains in response to 1/2? MMC in combination therapy with 1/2? LVX (horizontal axis), fold change of SOS genes (vertical axis). The statistical significance was assessed against each drug alone and no-drug control: *P < 0.05 **p < 0.01, ***p < 0.001. The statistical significance was assessed by comparing the effects of each drug alone and in combination therapy: P < 0.05 ##P < 0.01, ###P < 0.001.

Consent for publication

This manuscript is approved by all authors for publication.

Funding

This work was supported by the Chi-Mei Medical Center Research Foundation-(CMFHR11069, to **Cheng-Fa Yeh),** (CMFHT11202, to **Hung-Jen Tang**), (CMFHR11311, to **Cheng-Fa Yeh**).

Declaration of competing interest

The authors declare no competing interests.

Acknowledgements

The authors would like to thank Laboratory of Medical Research, Chi Mei Medical Center for the technical assistance in operating time - kill studies, intracellular antibacterial activity, and animal studies.

Abbreviations

MMC	Mitomycin C
MICs	Minimum inhibitory concentrations
CIP	Ciprofloxacin
LVX	Levofloxacin
TGC	Tigecycline
AMK	Amikacin
ATM	Aztreonam
CZ	Cefazolin
CMZ	Cefmetazole
CTX	Cefotaxime
CPO	Cefpirome
CHL	Chloramphenicol
DOX	Doxycycline
FOS	Fosfomycin
GM	Gentamicin
IPM	Imipenem
MEM	Meropenem
MIN	Minocycline
CLSI	Clinical and Laboratory Standards Institutes
FDA	Food and Drug Administration
PBS	Phosphate-buffered saline
IRB	Institutional Review Board

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmii.2024.12.005.

References

1. Jones DC, LaMartina EL, Lewis JR, et al. One health and global health view of antimicrobial susceptibility through the "Eye" of Aeromonas: systematic review and meta-analysis. *Int J Antimicrob Agents*. 2023;62, e106848.
2. Tsai YH, Huang KC, Shen SH, Hsu WH, Peng KT, Huang TJ. Microbiology and surgical indicators of necrotizing fasciitis in a tertiary hospital of southwest Taiwan. *Int J Infect Dis*. 2012;16:159–165.
3. Tang HJ, Lai CC, Lin HL, Chao CM. Clinical manifestations of bacteremia caused by *Aeromonas* species in southern Taiwan. *PLoS One*. 2014;9, e91642.
4. Janda JM, Abbott SL. The genus Aeromonas: taxonomy, pathogenicity, and infection. *Clin Microbiol Rev*. 2010;23:35–73.
5. Erova TE, Kosykh VG, Fadl AA, Sha J, Horneman AJ, Chopra AK. Cold shock exoribonuclease R (VacB) is involved in Aeromonas hydrophilia pathogenesis. *J Bacteriol*. 2008;190:3467–3474.
6. Stevens DL, Bisno AL, Chambers HF, et al. Practice guidelines for the diagnosis and management of skin and soft tissue infections: 2014 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2014;59:147–159.
7. Verweij J, Pinedo HM. Mitomycin C: mechanism of action, usefulness and limitations. *Anti Cancer Drugs*. 1990;1:5–13.
8. Domalaon R, Ammeter D, Brizuela M, Gorityala BK, Zhanel GG, Schweizer F. Repurposed antimicrobial combination therapy: tobramycin-ciprofloxacin hybrid augments activity of the anticancer drug mitomycin C against multidrug-resistant gram-negative bacteria. *Front Microbiol*. 2019;10, e1556.
9. Kitagawa D, Suzuki Y, Abe N, et al. Comparison of MALDI-TOF mass spectrometry and rpoB gene sequencing for the identification of clinical isolates of *Aeromonas* spp. *Heliyon*. 2022;8, e11585.
10. Chen PL, Wu CJ, Chen CS, Tsai PJ, Tang HJ, Ko WC. A comparative study of clinical *Aeromonas dhakensis* and *Aeromonas hydrophila* isolates in southern Taiwan: *A. dhakensis* is more predominant and virulent. *Clin Microbiol Infect*. 2014;20:428–434.
11. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Approved Standard. 33th Informational Supplement. CLSI Document M100-S33*. Wayne, PA: CLSI; 2023.
12. FDA reference: https://www.fda.gov/drugs/development-resources/tigecycline-injection-products.
13. Clinical and Laboratory Standards Institute. *Approved Standard M100-S33 Twenty-Second Informational Supplement*. Wayne, PA: Clinical and Laboratory Standards Institute; 2023.

14. Tang HJ, Lai CC, Chen CC, et al. Cephalosporin-glycopeptide combinations for use against clinical methicillin-resistant Staphylococcus aureus isolates: enhanced in vitro antibacterial activity. *Front Microbiol*. 2017;8, e884.
15. Tu EC, Hsu WL, Tzen JTC. Strictinin, a major ingredient in yunnan kucha tea possessing inhibitory activity on the infection of mouse hepatitis virus to mouse L cells. *Molecules*. 2023;28(3):1080.
16. Tang HJ, Ko WC, Chen CC, et al. In vitro and in vivo intracellular killing effects of tigecycline against clinical nontyphoid Salmonella isolates using ceftriaxone as a comparator. *Antimicrob Agents Chemother*. 2011;55:2755–2759.
17. Ponnusamy D, Kozlova EV, Sha J, et al. Cross-talk among flesh-eating *Aeromonas hydrophilia* strains in mixed infection leading to necrotizing fasciitis. *Proc Natl Acad Sci U S A*. 2016;113:722–727.
18. Lai CC, Chen CC, Huang HL, Chuang YC, ang HJ. The role of doxycycline in the therapy of multidrug-resistant E. coli - an in vitro study. *Sci Rep*. 2016;6, 31964.
19. Crandon JL, Kuti JL, Nicolau DP. Comparative efficacies of human simulated exposures of telavancin and vancomycin against methicillin-resistant *Staphylococcus aureus* with a range of vancomycin MICs in a murine pneumonia model. *Antimicrob Agents Chemother*. 2010;54:5115–5119.
20. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm*. 2016;7:27–31.
21. Otero F, Gosálvez J, Bou G, Fernández JL. Simple and Fast detection of resistance to antibiotic b inhibitors of protein synthesis in gram-negative pathogens through evaluation of mitomycin C-induced cell elongation. *Microb Drug Resist*. 2017;23: 973–981.
22. Soto-Dávila M, Chakraborty S, Santander J. Relative expression and validation of *Aeromonas* salmonicida subsp. salmonicida reference genes during ex vivo and in vivo fish infection. *Infect Genet Evol*. 2022;103, e105320.
23. Aderem A, Underhill DM. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol*. 1999;17:593–623.
24. Thwaites GE, Gant V. Are bloodstream leukocytes Trojan Horses for the metastasis of *Staphylococcus aureus*? *Nat Rev Microbiol*. 2011;9:215–222.
25. Seral C, Carryn S, Tulkens PM, Van Bambeke F. Influence of P-glycoprotein and MRP efflux pump inhibitors on the intracellular activity of azithromycin and ciprofloxacin in macrophages infected by Listeria monocytogenes or Staphylococcus aureus. *J Antimicrob Chemother*. 2003;51:1167–1173.
26. Peyrusson F, Whelan AO, Hartley MG, Norville IH, Harding SV, van Bambeke F. Intracellular activity of antibiotics against *Coxiella burnetii* in a model of activated human THP-1 cells. *Antimicrob Agents Chemother*. 2021;65, e01061, 21.
27. Nolla-Salas J, Codina-Calero J, Valles-Angulo S, et al. Clinical significance and outcome of *Aeromonas* spp. infections among 204 adult patients. *Eur J Clin Microbiol Infect Dis*. 2017;36:1393–1403.
28. Lau SM, Peng MY, Chang FY. Outcomes of Aeromonas bacteremia in patients with different types of underlying disease. *J Microb Immunol Infect*. 2000;33:241–247.
29. Kemp EG, Harnett AN, Chatterjee S. Preoperative topical and intraoperative local mitomycin C adjuvant therapy in the management of ocular surface neoplasias. *Br J Ophthalmol*. 2002;86:31–34.
30. Kuzuya T, Yamauchi M, Ito A, et al. Pharmacokinetic characteristics of 5-fluoro-uracil and mito mycin C in intraperitoneal chemotherapy. *J Pharm Pharmacol*. 1994; 46:685–689.
31. Goodman MF. Coping with replication 'train wrecks' in *Escherichia coli* using Pol V, Pol II and RecA proteins. *Trends Biochem Sci*. 2000;25:189–195.
32. Otero F, Gosálvez J, Bou G, Fernández JL. Simple and Fast detection of resistance to antibiotic inhibitors of protein synthesis in gram-negative pathogens through evaluation of mitomycin C-induced cell elongation. *Microb Drug Resist*. 2017;23: 973–981.
33. Pham P, Seitz EM, Saveliev S, et al. Two distinct modes of *RecA* action are required for DNA polymerase V-catalyzed translesion synthesis. *Proc Natl Acad Sci U S A*. 2002;99:11061–11066.
34. Van der Veen S, van Schalkwijk S, Molenaar D, de Vos WM, Abee T, Wells-Bennik MHJ. The SOS response of *Listeria monocytogenes* is involved in stress resistance and mutagenesis. *Microbiology (Reading)*. 2010;156:374–384.
35. Iacobino A, Piccaro G, Pardini M, Fattorini L, Giannoni F. Moxifloxacin activates the SOS response in *Mycobacterium tuberculosis* in a dose- and time-dependent manner. *Microorganisms*. 2021;9:255–263.
36. Theodore A, Lewis K, Vulic M. Tolerance of *Escherichia coli* to fluoroquinolone antibiotics depends on specific components of the SOS response pathway. *Genetics*. 2013;195:1265–1276.