Polish Journal of Microbiology
2019, Vol. 68, No 4, 477–491
https://doi.org/10.33073/pjm-2019-047

ORIGINAL PAPER

Inhibition of Drug Resistance of *Staphylococcus aureus* by Efflux Pump Inhibitor and Autolysis Inducer to Strengthen the Antibacterial Activity of β-lactam Drugs

WENJING LUAN[1#], XIAOLEI LIU[1#], XUEFEI WANG[1], YANAN AN[1], YANG WANG[1], CHAO WANG[1], KESHU SHEN[2], HONGYUE XU[1], SHULIN LI[1], MINGYUAN LIU[1, 3] and LU YU[1*]

[1] Key Laboratory for Zoonosis Research, Ministry of Education, Institute of Zoonosis, Department of Infectious Diseases of First Hospital of Jilin University, College of Veterinary Medicine Jilin University, Changchun, China
[2] Jilin Hepatobiliary Hospital, Changchun, China
[3] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, China

Submitted 20 June 2019, revised 20 September 2019, accepted 21 September 2019

Abstract

This study explored a potential treatment against methicillin-resistant *Staphylococcus aureus* (MRSA) infections that combines thioridazine (TZ), an efflux pump inhibitor, and miconazole (MCZ), an autolysis inducer, with the anti-microbial drug cloxacillin (CXN). *In vitro,* the combination treatment of TZ and MCZ significantly reduced 4096-fold (Σ (FIC) = 0.1 – 1.25) the MIC value of CXN against *S. aureus*. *In vivo*, the combination therapy significantly relieved breast redness and swelling in mice infected with either clinical or standard strains of *S. aureus*. Meanwhile, the number of bacteria isolated from the MRSA135-infected mice decreased significantly ($p = 0.0427 < 0.05$) after the combination therapy when compared to monotherapy. Moreover, the number of bacteria isolated from the mice infected with a reference *S. aureus* strain also decreased significantly ($p = 0.0191 < 0.05$) after the combination therapy when compared to monotherapy. The pathological changes were more significant in the CXN-treated group when compared to mice treated with a combination of three drugs. In addition, we found that combination therapy reduced the release of the bacteria-stimulated cytokines such as IL-6, IFN-γ, and TNF-α. Cytokine assays in serum revealed that CXN alone induced IL-6, IFN-γ, and TNF-α in the mouse groups infected with ATCC 29213 or MRSA135, and the combination of these three drugs significantly reduced IL-6, IFN-γ, and TNF-α concentrations. Also, the levels of TNF-α and IFN-γ in mice treated with a combination of three drugs were significantly lower than in the CXN-treated group. Given the synergistic antibacterial activity of CXN, we concluded that the combination of CXN with TZ, and MCZ could be developed as a novel therapeutic strategy against *S. aureus*.

Key words: *Staphylococcus aureus*, mastitis, thioridazine (TZ), miconazole (MCZ), cloxacillin (CXN), combination therapy

Introduction

Staphylococcus aureus infection and drug resistance problems have caused increasing public health problems. The increase in antimicrobial resistance coupled with intracellular infection makes this bacteria the third-largest threat to human health according to the WHO (Lowy 1998; Demon et al. 2012). MRSA is of particular concern because of its ability to spread extensively and rapidly, along with its multi-drug resistance to β-lactam and aminoglycoside antibiotics (Boucher et al. 2009; Kolendi 2010). MRSA infection is always associated with chronic or recurrent infections, including osteomyelitis, pulmonary infection, and endocardial inflammation (Que et al. 2005). In China, almost 10% of *S. aureus* clinical isolates were considered resistant to penicillin in recent years (Hu et al. 2016; Chen et al. 2017). Several new targets have been discovered and addressed in recent drugs, including ClpP protease and FtsZ of the cell division machinery. Resistance can be modified and inactivated by enzymatic drugs, enzymatic modification of drug-binding sites, drug efflux, and the others. Studies on the resistance of the current antibiotics have been reported also using drug

[#] These authors contributed equally to this work.

* Corresponding author: L. Yu, College of Veterinary Medicine Jilin University, Changchun, China; **e-mail: yu_lu@jlu.edu.cn**

combinations (Foster et al. 2017). Several synergistic combinations of small molecules and antibiotics have also been provided to treat *S. aureus* infection by reversing the resistance mechanisms, attenuating *S. aureus* virulence and/or interfering with quorum sensing (Vermote et al. 2017). Thus, the urgency is required in the development of the new strategies for antimicrobial drug combinations against MRSA.

One novel strategy is to utilize helper compounds in combination with traditional antibiotics. Helper compounds are drugs approved for other therapeutic purposes that also possess antibacterial activity (Dickey et al. 2017). Thioridazine (TZ) is primarily an antipsychotic drug and also functions as an efflux pump inhibitor, which can be used as a helper compound (Klitgaard et al. 2008; Pule et al. 2016). Several *in vitro* studies have shown that TZ significantly increases the susceptibility of MRSA to β-lactam antibiotics (Poulsen et al. 2013). It has been shown that cytoderm synthesis and autolysis are linked since the inhibition of the former activates the latter. Thus, the destruction of the cytoderm is an important step in the bactericidal process of penicillin and other antibiotics (Zore et al. 2011). Miconazole (MCZ) is an antifungal drug that is considered as an autolysis inducer, which causes a release of cellular K^+ at low concentrations, and MCZ at the minimum inhibitory concentration (MIC) showed a certain antibacterial effect on clinically isolated MRSA (Falk et al. 2010). In our study, we aimed to suppress the multiple drug resistance of MRSA by combining cloxacillin (CXN) with an autolysis inducer MCZ and an efflux pump inhibitor TZ.

The previous studies have shown that the innate immune response, including pattern recognition receptors (PRR), was activated upon infection with *S. aureus* (Elazar et al. 2010). The release of cytokines is an important indicator for the evaluation of antibiotics. When inoculated with *S. aureus*, immune cells produce the inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), and interleukin 6 (IL-6) in high concentrations (Chen et al. 2017). *S. aureus* may also stimulate nuclear factor-κB inhibitor (IKB), nuclear factor-κB (NF-KB), and mitogen-activated protein kinase phosphorylation (Gao et al. 2015). The previous studies have shown that TNF-α is the earliest and primary endogenous mediator and plays crucial role in both inflammatory and neuropathic hyperalgesia (Zhang et al. 2007). In these studies, TNF-α and IL-6 after infection were released at high concentrations for 24 h, and there was no time gradient for IFN-γ detection after infection, which was consistent with previous reports (Trigo et al. 2009; Hu et al. 2010). In the studies on the mechanism of action of antibiotics, the level of cytokines was measured at 12–24 h (Wei W et al. 2009; Fu Y et al. 2014). The inflammatory cells including macrophages regulate inflammatory responses by the induction of significant inflammation and release of inflammatory cytokines (IL-1β, IL-6, and TNF-α) and chemokines (Kim et al. 2015) at the high concentrations.

Our study is the first to explore the combination of TZ, MCZ, and CXN in the treatment of mastitis using a mouse model to provide a basis for subsequent combined antibacterial therapy.

Experimental

Materials and Methods

Ethics statement. The BALB/c mice were housed in micro-isolator cages and received food and water freely. The laboratory temperature was 24 ± 1°C, and relative humidity was 40–80%. All animal studies were conducted according to the experimental practices and standards approved by the Animal Welfare and Research Ethics Committee at Jilin University (no: IZ-2009-008). The protocols were reviewed and approved by the committee. All animal studies were performed under isoflurane anesthesia, and every effort was made to minimize suffering.

Strains and growth conditions. *S. aureus* was obtained from the China Type Culture Collection (CTCC) (American Type Culture Collection [ATCC] 29213), and *S. aureus* isolates were derived from subclinical mastitis. The three antimicrobial agents used were CXN, MCZ (Yeyuan, Shanghai China), and TZ (MedChemexpress).

Antimicrobial susceptibility testing. To determine the MIC values for CXN, TZ, and MCZ against *S. aureus* a microdilution assay was performed according to the CLSI (formerly NCCLS) guidelines. The determination of the MIC of CXN for the mastitis isolates of *S. aureus* was performed using the Mueller-Hinton agar dilution assay according to the CLSI guidelines (CLSI 2008). Plates were incubated at 35–38°C for 16–20 h.

Interpretation of synergy tests. The synergy test was performed in a 96-well microtiter plate containing two or three antimicrobial agents that were distributed in a two- or three-fold dilution on the day of the assay in a checkerboard pattern. Each well contained 0.1 ml of an individual antimicrobial composition or broth control. The final inoculate concentration was maintained at $3–5 \times 10^5$ CFU/ml. The plate was incubated for 20–24 h, and the MIC value was determined. *S. aureus* ATCC 29213 was used as the quality control strain.

For the first clear well in each row of the microtitre plate containing both antimicrobial agents, the fractional inhibitory concentration (FIC) of each agent was calculated as follows:

FIC of drug A (FIC_A) = MIC of drug A in combination / MIC of drug A alone

FIC of drug B (FIC_B) = MIC of drug B in combination / MIC of drug B alone

The summation of both FICs (Σ FIC) in each well ($FICI = \Sigma\ FIC = FIC_A + FIC_B$) was used to classify the combination of antimicrobial agents at the given concentrations as synergistic (Σ FIC, ≤ 0.5), partially synergistic (Σ FIC, > 0.5 and ≤ 1.0), indifferent (Σ FIC, > 1 and ≤ 4), or antagonistic (Σ FIC, > 4) (Zore et al. 2011).

Using three-dimensional checkerboard microdilution with CXN, TZ, and MCZ the combined concentrations of each antibiotic showed synergy when their sum, FICI (Σ FICs) was lower than 1.0.

Mouse *S. aureus* mastitis model. The *S. aureus* mastitis mouse model has been used to investigate the novel prevention and treatment methods for *S. aureus* mastitis, as reported previously. Briefly, the mice weighed approximately 50 g at the beginning of the experiment. The pups were weaned 1–2 h before bacterial inoculation of the mammary glands. A mixture of oxygen and isoflurane (2–3%) was inhaled to anesthetize the lactating mice. A syringe with a 32-gauge blunt needle was used to inoculate both L4 (on the left) and R4 (on the right) glands of the fourth abdominal mammary gland pair, with approximately 10^7 CFU of *S. aureus*. A total of 18 mice were used for each mice group infected with a single strain of *S. aureus*, either ATCC 29213 or MRSA135. The 18 mice of each group were divided into the following groups, with three mice in each group: blank (I), infection control (II), MCZ monotherapy (III), TZ monotherapy (IV), CXN monotherapy (V), and CXN + TZ + MCZ treatment (VI) groups. The mice were observed for 24 h following infection before treatment was initiated, and then the results were obtained after 72 h of treatment in each drug group. In *in vivo* experiment, the concentration of cloxacillin was 20 mg/kg/d, thioridazine – 16 mg/kg/d, and miconazole – 11 mg/kg/d in the single-agent treatment group. In the combination treatment group the concentration of: cloxacillin was 0.75 mg/kg/d, thioridazine – 12 mg/kg/d, and miconazole – 1.5 mg/kg/d.

Cytokines in the mastitis mouse model. At 24 h after *S. aureus* inoculation the animal blood was centrifuged. Cytokines were detected by the double sandwich enzyme-linked immunosorbent assay technique. Different groups were compared using an independent samples *t*-test, and a paired samples *t*-test was used to analyze any significant differences in the data that originated from the same group at different time points (Moon et al. 2007).

Antimicrobial susceptibility testing. The MIC of CXN against 47 strains of *S. aureus* was determined, and the values ranged from 4 to 512 μg/ml. The MIC value of CXN against *S. aureus* ATCC 29213 was 4 μg/ml and that of MRSA135 was 256 μg/ml. The drug susceptibility results showed that 23 strains of *S. aureus* were resistant to CXN, while 24 strains of *S. aureus* were sensitive to CXN. The MIC of TZ for 47 strains of *S. aureus* was determined, ranging from 16 μg/ml to 64 μg/ml. The MIC value of TZ for *S. aureus* ATCC 29213 was 16 μg/ml and that of MRSA135 μg/ml was 32 μg/ml. The MIC of MCZ for 47 strains of *S. aureus* was determined, and the values ranged from 1 μg/ml to 8 μg/ml. The MIC value of MCZ for *S. aureus* ATCC 29213 was 4 μg/ml and that of MRSA135 was 4 μg/ml (Table I).

Table II
Summary of thioridazine, miconazole and cloxacillin activity in combination (expressed as the MIC value) against *Staphylococcus aureus* strains.

Antimicrobial agents		MIC (μg/ml)		
		Range	50%	90%
Cloxacillin	Thioridazine	0.125–512	16	512
	Miconazole	0.25–512	4	512
	Thioridazine + Miconazole	0.000972–16	0.5	8

MIC – minimum inhibitory concentration

Table I
The MICs values of individual antimicrobial agents against *Staphylococcus aureus* isolates.

Antimicrobial agents	MIC (μg/ml)		
	Range	50%	90%
Cloxacillin	4–512	16	512
Thioridazine	16–64	32	64
Miconazole	1–8	4	8

MIC – minimum inhibitory concentration

Drug synergy results against *S. aureus* isolates. The FICI index, used as a predictor of synergy, was evaluated using the TZ and MCZ agents combined with CXN (Table II). The results showed that both the combination of the two drugs and the combination of the three drugs reduced the MIC value of the drugs to varying degrees. Anti-*S. aureus* activity of two drugs, CXN and TZ, was shown in Table III. Among the 47 strains tested, the combined FICI ranged from 0.14 to 1.13, of which 24 strains had synergistic effects (0.14–0.5), 13 strains had partial synergistic effects (0.56–0.75), and 10 strains had an unrelated effect (1.06–1.25). Anti-*S. aureus* activity of a combination of CXN and MCZ was reported in Table IV. Among the 47 strains tested, the combined FICI ranged from 0.14 to 2.25, of which 15 had synergistic effects (0.14–0.5), 15 had partial synergistic effects (0.51–1), and 17 had irrelevant effects (1.03–2.25). The activity of a combination of CXN, TZ, and MCZ against *S. aureus* MRSA strains was shown in Table V. The FICI ranged from 0.19 to 0.75 among

Table IV
The activity of the combination of cloxacillin and miconazole against *Staphylococcus aureus* strains *in vitro*.

Strains	FICI
MRSA14	0.63
MRSA15	0.75
MRSA16	0.38
MRSA20	0.31
MRSA21	0.31
MRSA22	2.25
MRSA25	0.53
MRSA29	2.13
MRSA30	0.26
MRSA64	0.14
MRSA65	0.26
MRSA75	1.00
MRSA76	1.13
MRSA92	0.26
MRSA94	0.51
MRSA97	0.52
MRSA98	0.26
MRSA125	0.50
MRSA126	1.50
MRSA134	1.50
MRSA135	0.27
MRSA142	1.01
MRSA162	0.63
ATCC 29213	0.50
MSSA10	0.56
MSSA13	0.31
MSSA14	2.00
MSSA31	1.13
MSSA36	2.02
MSSA41	1.50
MSSA42	0.27
MSSA44	1.06
MSSA50	1.03
MSSA51	1.00
MSSA54	1.50
MSSA56	1.00
MSSA62	1.50
MSSA65	1.03
MSSA66	0.53
MSSA67	0.31
MSSA68	0.53
MSSA70	0.52
MSSA72	0.28
MSSA73	1.03
MSSA78	1.06
MSSA79	0.56
MSSA80	0.56

See FICI criteria for details.

Table III
The activity of the combination of cloxacillin and thioridazine against *Staphylococcus aureus* strains *in vitro*.

Strain	FICI
MRSA14	0.38
MRSA15	0.63
MRSA16	0.75
MRSA20	0.25
MRSA21	0.50
MRSA22	0.63
MRSA25	0.31
MRSA29	0.19
MRSA30	1.06
MRSA64	0.25
MRSA65	0.25
MRSA75	1.13
MRSA76	1.13
MRSA92	0.31
MRSA94	0.19
MRSA97	0.25
MRSA98	0.19
MRSA125	0.27
MRSA126	0.50
MRSA134	0.16
MRSA135	0.16
MRSA142	0.63
MRSA162	0.38
ATCC 29213	0.28
MSSA10	0.31
MSSA13	0.56
MSSA14	0.63
MSSA31	0.63
MSSA36	0.38
MSSA41	0.63
MSSA42	0.56
MSSA44	0.38
MSSA50	0.38
MSSA51	0.14
MSSA54	0.38
MSSA56	0.63
MSSA62	0.63
MSSA65	0.63
MSSA66	1.13
MSSA67	1.13
MSSA68	1.13
MSSA70	1.13
MSSA72	1.06
MSSA73	0.63
MSSA78	1.25
MSSA79	1.25
MSSA80	0.25

See FICI criteria for details.

Table V
The activity of the combination of cloxacillin, thioridazine, and miconazole against MRSA strains *in vitro*.

Strain	CXN	TZ	MCZ	CXN	TZ	MCZ	FICI
	MICs (Single)			MICs (Synergy)			
MRSA14	512	32	2	4	4	0.25	0.26
MRSA15	512	32	2	2	4	0.25	0.25
MRSA16	512	16	4	1	4	0.25	0.31
MRSA20	256	32	2	0.25	4	0.25	0.25
MRSA21	128	16	4	0.5	4	0.25	0.32
MRSA22	64	32	4	0.25	4	0.50	0.25
MRSA25	512	16	2	0.5	4	0.50	0.50
MRSA29	256	64	4	4	4	0.50	0.20
MRSA30	512	64	4	0.5	4	0.50	0.19
MRSA64	512	32	8	8	4	0.50	0.20
MRSA65	512	32	4	0.5	4	0.50	0.25
MRSA75	512	32	2	0.5	4	0.50	0.38
MRSA76	512	32	4	8	4	0.50	0.27
MRSA92	512	16	4	0.0078	4	0.50	0.38
MRSA94	512	32	2	0.0078	4	0.50	0.38
MRSA97	256	32	2	0.0156	4	0.50	0.38
MRSA98	512	32	4	0.0078	4	0.50	0.25
MRSA125	256	16	2	0.5	4	0.50	0.50
MRSA126	256	16	1	1	4	0.50	0.75
MRSA134	512	32	1	0.0078	4	0.50	0.63
MRSA135	256	32	4	0.25	4	0.50	0.25
MRSA142	512	32	1	2	4	0.50	0.63
MRSA162	512	32	4	16	4	0.50	0.28
ATCC 29213	4	16	4	0.0156	4	0.50	0.38

See FICI criteria for details.
MCZ – miconazole; TZ – thioridazine; CXN – cloxacillin;
FIC of drug A (FIC_A) = MIC of drug A in combination / MIC of drug A alone;
FIC of drug B (FIC_B) = MIC of drug B in combination / MIC of drug B alone;
Combination FIC (AB) = Σ FIC = $FIC_A + FIC_B$;
Synergistic (Σ FIC ≤ 0.5);
Partially synergistic (Σ FIC > 0.5 and ≤ 1.0);
Indifferent (Σ FIC > 1 and ≤ 4);
Antagonistic (Σ FIC > 4).

the 23 strains tested, of which 20 strains had synergistic effects (0.19–0.5), and three isolates have partial synergy (0.63–0.75). The activity of a combination of CXN, TZ, and MCZ against *S. aureus* MSSA strain (Table VI) showed that the FICI ranged from 0.1 to 1.25 for 23 strains tested, 19 of which had synergistic effects (0.1–0.5), one has a partial synergistic effect (0.69), and three strains have an unrelated effect (1.09–1.25). As the results of the synergistic tests, the concentration of each drug can be lowered. The *in vivo* dose of the compounds administered were referred to the ratio of the MIC value at synergistic combination (cloxacillin : thioridazine : miconazole = 0.25 μg/ml : 4 μg/ml : 0.50 μg/ml) as it was obtained by checkerboard assay *in vitro*, where the synergistic ratio of cloxacillin : thioridazine : miconazole was 1 : 16 : 2. We also, referred to the dose of these compounds when they were single-administered to mouse or calculated from other animal studies already reported *in vivo* (cloxacillin ≤ 50 mg/kg/d, thioridazine ≤ 16 mg/kg/d, miconazole ≤ 20 mg/kg/d) (Hendricks et al. 2003; Choi et al. 2012). Finally, we calculated the corresponding dose of these compounds to be single-administered or in combination with animal experiments *in vivo*. The *in vivo* concentrations of three drugs in a combination treatment was as follows: cloxacillin (0.75 mg/kg/d) : thioridazine (12 mg/kg/d) : miconazole (1.5 mg/kg/d).

Statistical analysis. Comparisons of mean values from three experiments were statistically evaluated by analysis of variance, followed by the One-Way ANOVA

Table VI
The activity of the combination of cloxacillin, thioridazine, and miconazole against MSSA strains *in vitro*.

Strain	CXN	TZ	MCZ	CXN	TZ	MCZ	FICI
	MICs (Single)			MICs (Synergy)			
MSSA10	16	64	4	4	4	0.25	0.38
MSSA13	8	64	8	8	4	0.25	1.09
MSSA14	8	32	2	8	4	0.25	1.25
MSSA31	16	32	2	4	4	0.25	0.50
MSSA36	16	32	1	0.015625	4	0.25	0.38
MSSA41	8	32	1	4	4	0.25	0.88
MSSA42	16	64	8	0.125	4	0.25	0.10
MSSA44	16	32	1	0.5	4	0.25	0.41
MSSA50	16	32	1	0.125	4	0.25	0.38
MSSA51	16	32	2	0.001975	4	0.25	0.25
MSSA54	16	16	2	0.0625	4	0.25	0.38
MSSA56	16	32	2	0.03125	4	0.25	0.25
MSSA62	16	32	2	0.0009715	4	0.25	0.25
MSSA65	16	32	2	0.125	4	0.25	0.26
MSSA66	16	32	4	4	4	0.25	0.44
MSSA67	16	32	4	8	4	0.25	0.69
MSSA68	16	32	4	16	4	0.25	1.19
MSSA70	16	32	4	0.015625	4	0.25	0.19
MSSA72	16	64	8	0.5	4	0.25	0.13
MSSA73	16	32	2	0.0625	4	0.25	0.25
MSSA78	8	16	2	0.03125	4	0.25	0.38
MSSA79	8	16	4	0.03125	4	0.25	0.32
MSSA80	16	32	4	0.0078125	4	0.25	0.19
ATCC 29213	4	16	4	0.0156	4	0.50	0.38

See FICI criteria for details.
MCZ – miconazole; TZ – thioridazine; CXN – cloxacillin;
FIC of drug A (FIC_A) = MIC of drug A in combination / MIC of drug A alone;
FIC of drug B (FIC_B) = MIC of drug B in combination / MIC of drug B alone;
Combination FIC(AB) = Σ FIC = $FIC_A + FIC_B$;
Synergistic (Σ FIC ≤ 0.5);
Partially synergistic (Σ FIC > 0.5 and ≤ 1.0);
Indifferent (Σ FIC > 1 and ≤ 4);
Antagonistic (Σ FIC > 4).

analysis. Differences with 2-sided $p < 0.05$ were considered statistically significant. All statistical analyses were performed using the GraphPad Prism 5 software (version 11.5; SPSS).

Results

Mouse mastitis treatment results. Two *S. aureus* mastitis mouse models were constructed, each injected with a single strain of *S. aureus*, either ATCC 29213 or the MRSA135 strain (Fig. 1A). Clinical observations showed that none of the mice in either the ATCC 29213 or MRSA135 groups died during the experiment, and their mental states were normal. The areolas of both bacterial infection groups were swollen and red. The areolas in the MCZ or TZ monotherapy group were pale red and swollen, but there were no significant changes in the other groups. In the MRSA-infected groups, the mammary glands varied in color; those of the blank control group were milky white, those of the infection control group were purple, those of the MCZ or TZ treated group were red, those of a portion of the CXN treated group were red but most were milky white, and most were also milky white in the group treated with the combination of the three drugs (Fig. 1B). In the ATCC 29213-infected groups, the mammary glands varied in color; the blank control group had a normal mammary gland color, the infection control group had purple mammary glands, most of the glands in the

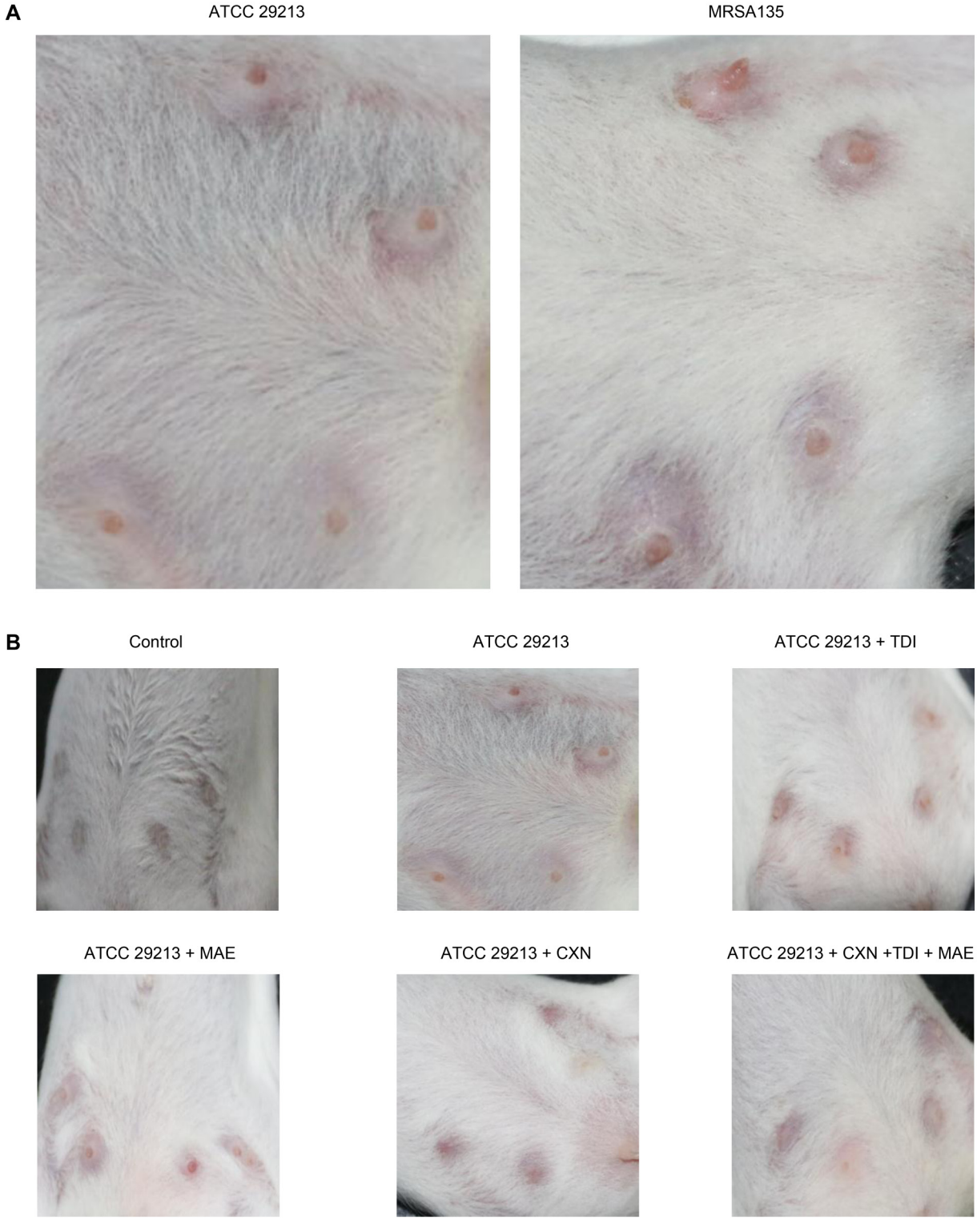

Fig. 1A i 1B. Clinical observations of mammary tissue of mouse infected with *Staphylococcus aureus* ATCC 29213 or MRSA135 before and after treatment with the drugs.

MCZ- and TZ-treated groups were red, and those of the CXN-treated and combination-treated groups were mostly milky white (Fig. 1C).

Histopathology of mammary tissue by the hematoxylin and eosin stain (H&E) was observed (Fig. 2A, and 2B). No obvious pathological changes were found in the blank control group, which displayed normal acinar mammary glands and neat rows of acinar epithelial cells with prolactin. A large number of bacterial lumps and detached cells were seen in the infection control group, and both had red blood cell infiltrate observed in the interstitial space. The agglomerated cell mass was seen

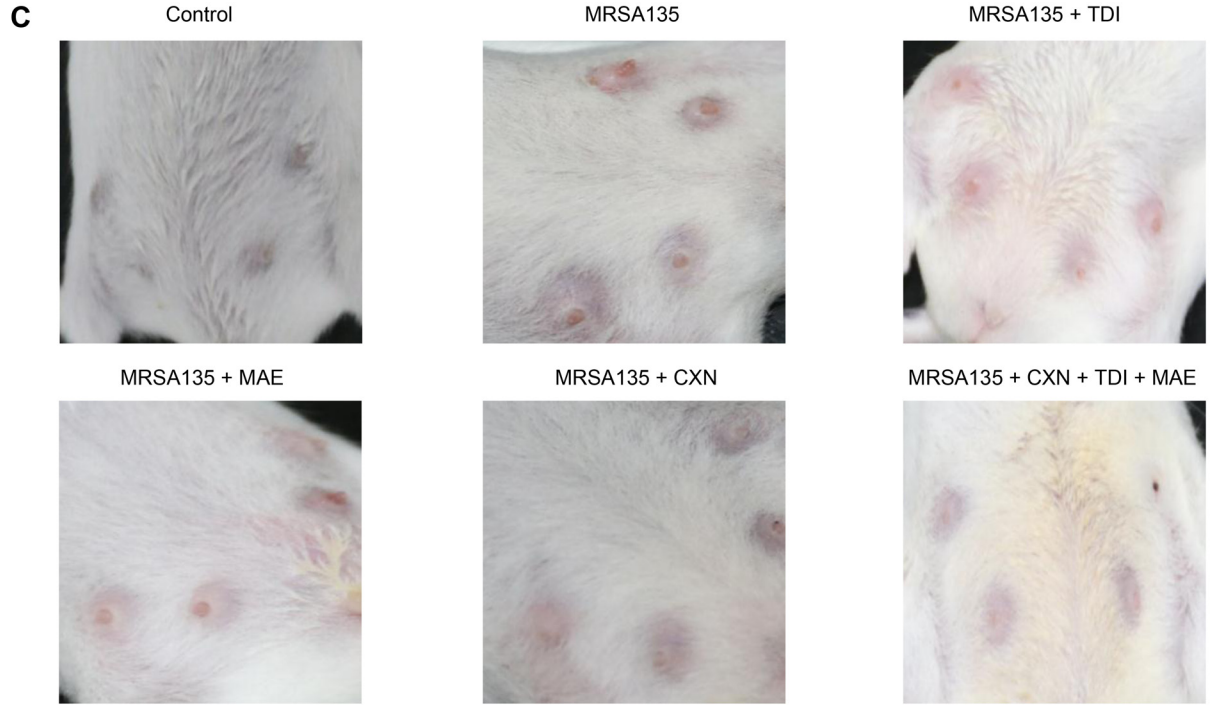

Fig. 1C. Clinical observations of mammary tissue of mouse infected with *Staphylococcus aureus* ATCC 29213 or MRSA135 before and after treatment with the drugs.

in the acinus, and large numbers of lymphocyte infiltrate were observed in the interstitium. In the MCZ or TZ monotherapy groups, epithelial cells were swollen, obvious bacterial masses were visible, and lymphocytes had infiltrated the stroma. Few lymphocytes and certain bacterial masses were observed in the CXN group. In the combination group, few lymphocytes and very few bacteria were visible, in addition to a lack of cell shedding. The results showed that, in addition to the known effect of CXN monotherapy, the combination of CXN, TZ, and MCZ has obvious therapeutic effects against infection by both strains of *S. aureus* tested. In the group treated with the combination of the three drugs, most of the pathological changes were milder than the CXN treatment group.

***S. aureus* count results.** Colony counts from mice of the clinical strain MRSA135-infected group showed that the bacterial concentration was 6.96×10^7 CFU/ml without therapy. Following treatment, the bacterial concentration in mice of the MCZ-treatment group was 2.23×10^7 CFU/ml; in the TZ-treatment group it was 1.73×10^7 CFU/ml; after CXN treatment it was 3.20×10^6 CFU/ml ($p = 0.0447 < 0.05$), and after the combination therapy with CXN, TZ, and MCZ it was 1.10×10^6 CFU/ml ($p = 0.0427 < 0.05$) (Fig. 3B). There was no significant difference between the CXN monotherapy- and the combination therapy groups (Fig. 3C). The colony counts after the mice were infected with the reference strain ATCC 29213 showed the following: the bacterial concentration without therapy was 2.34×10^5 CFU/ml; in the MCZ-treatment group it was 1.56×10^5 CFU/ml; in the TZ-treatment group it was 7.91×10^4 CFU/ml; after CXN treatment it was 1.17×10^4 CFU/ml ($p = 0.0212 < 0.05$); and after the combination therapy with CXN, TZ, and MCZ was 4.43×10^3 CFU/ml ($p = 0.0191 < 0.05$) (Fig. 3A). The difference between the CXN monotherapy and the three-drug treatment groups was significant ($p = 0.0040 < 0.01$) (Fig. 3D).

Cytokine detection in a mouse model of mastitis. Serum supernatants were assayed for TNF-α, IL-6 and IFN-γ levels using an ELISA kit (Fig. 4). The cytokines measured in the sera of mice infected with ATCC 29213 (the ATCC 29213 infected group) were as follows: the infected group had significantly increased levels of TNF-α, IL-6, and IFN-γ when compared to those in the control group ($p = 0.0260$, $p = 0.0348$, $p < 0.0001$, respectively) (Fig. 4A, 4C, and 4E). There was no significant difference in TNF-α, IL-6, and IFN-γ levels between mice treated with MCZ when compared with those of the control group. The levels of IL-6 and IFN-γ in the TZ-treated group were significantly lower than those in the ATCC 29213-infected group ($p = 0.0176$, $p = 0.0046$), but there was no significant difference in TNF-α levels. The levels of TNF-α, IL-6, and IFN-γ in the CXN-treated group were significantly lower than those in the infected group ($p = 0.0016$, $p = 0.0245$, $p < 0.0001$, respectively). The levels of TNF-α, IL-6, and IFN-γ in the group of mice treated with three drugs together were significantly lower than those in

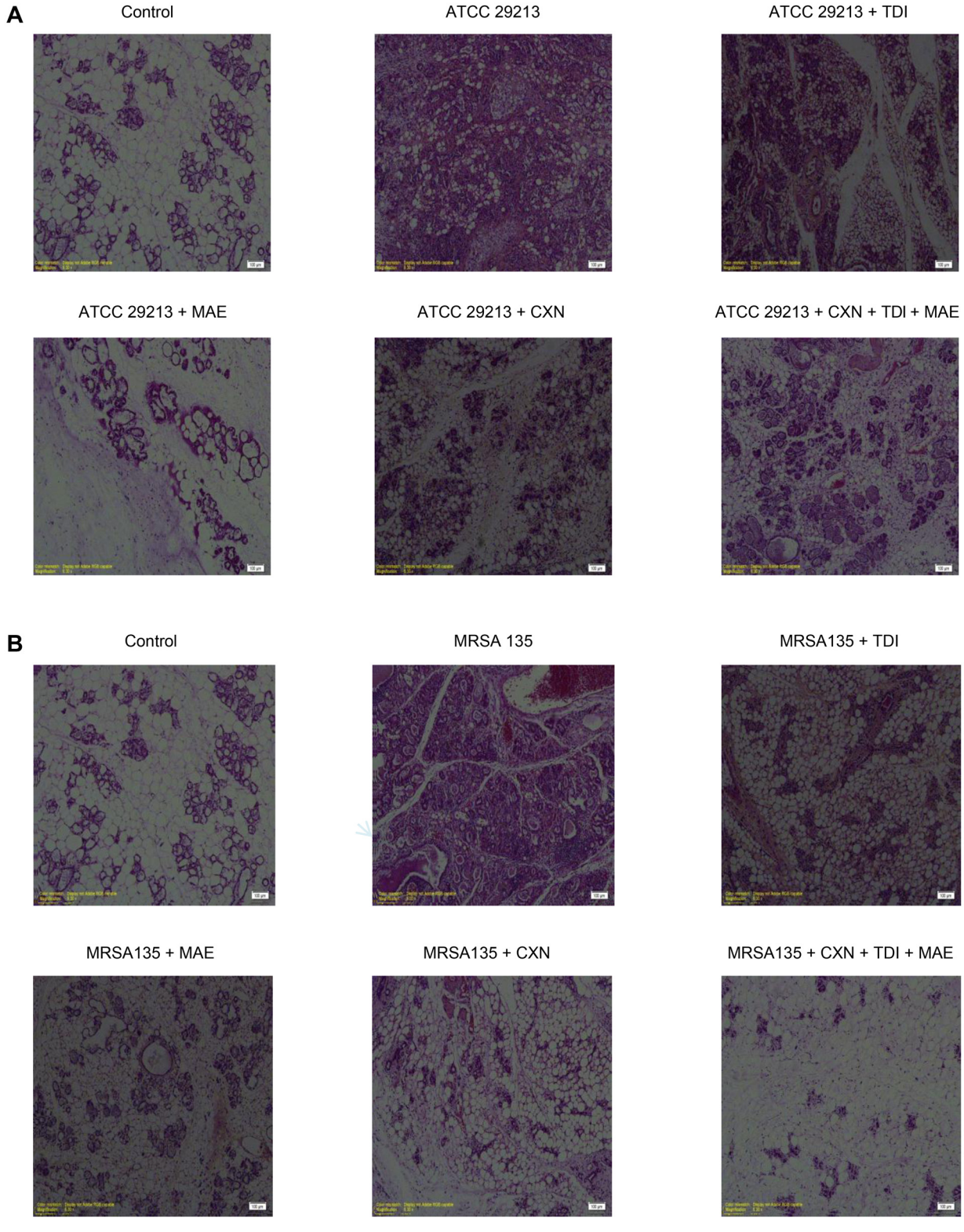

Fig. 2. Histopathological observations for each group of mice (control, infected, and treated with the drugs).

the ATCC 29213-infected group ($p = 0.0004$, $p = 0.0136$, $p < 0.0001$). The levels of TNF-α and IFN-γ in the group of mice treated with three drugs together were significantly lower than those in the ATCC 29213-infected group ($p = 0.0084$, $p = 0.0280$). The cytokine results in the MRSA135-infected mice (the MRSA135-infected group) were as follows: the MRSA135-infected group had significantly higher levels of IL-6 and IFN-γ than those in the control group ($p = 0.0185$, $p = 0.0148$, respectively) (Fig. 4B, 4D, and 4F). The levels of TNF-α and IL-6 in the MCZ-treated group were not significantly different from those the MRSA135-infected

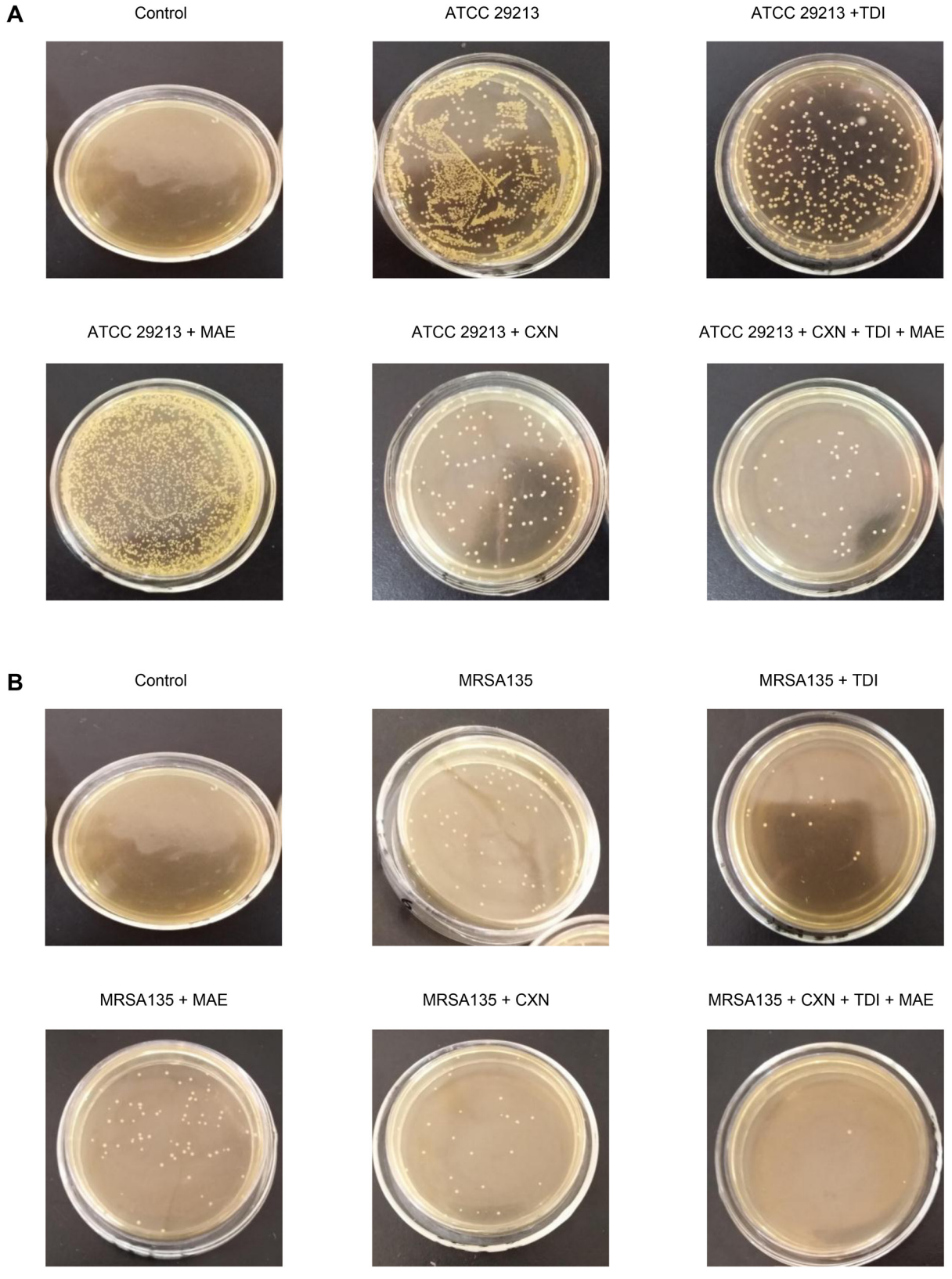

Fig. 3A i 3B

group, but the IFN-γ levels were significantly decreased ($p = 0.0434$). There were no significant differences in TNF-α, IL-6, and IFN-γ levels in the TZ-treated group compared with those of the MRSA135-infected group. The levels of IL-6 and IFN-γ in the CXN-treated group were significantly lower than those the MRSA135-infected group ($p = 0.0191$, $p = 0.0262$, respectively), but there was no significant difference in TNF-α levels.

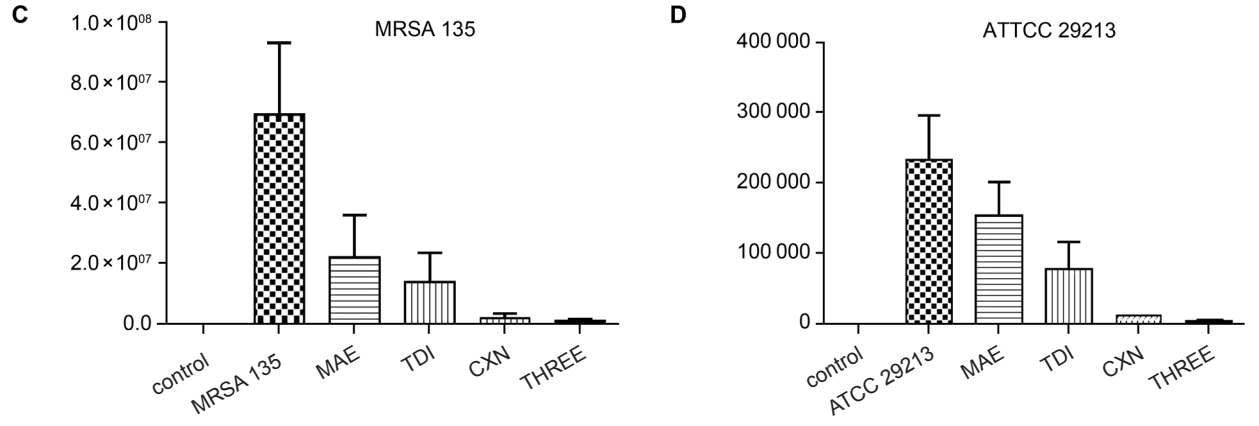

Fig. 3. The culture of *Staphylococcus aureus* isolated from each group of mice (control, infected, treated with the drugs).

MCZ – miconazole; TZ – thioridazine; CXN – cloxacillin; MCZ + TZ + CXN – the combination of miconazole, thioridazine, cloxacillin. There was no significant difference between the cloxacillin-treated and three-drugs-treated mice ($P = 0.5649$). The * on the horizontal line indicates a significant difference analysis between the CXN group and the three-drug group.* $P < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

The levels of TNF-α, IL-6, and IFN-γ in mice treated with the three drugs together were significantly lower than those of the MRSA135-infected group ($p = 0.0230$, $p = 0.0051$, $p = 0.0060$, respectively). The TNF-α levels in mice treated with the three drugs together were significantly lower than those of the MRSA135-infected group ($p = 0.0010$). In summary, treatment with CXN alone or with the combination of three drugs was capable of inhibiting the expression of TNF-α and IL-6.

Discussion

A recent report indicated that developing of new natural compounds or combination therapies should be a focus on the fight against *S. aureus* (Celenza et al. 2012; Dickey et al. 2017) Combinations of antibiotics have been previously used to treat bacterial infections, including pathogens that cannot be suppressed or killed by a single antibiotic or infection with a multiplicity of microbial species (Navon-Venezia et al. 2005). A single antibiotic is hardly capable of killing bacteria that possess multiple drug resistance mechanisms against broad-spectrum β-lactams and aminoglycosides antibiotics (Wax 2008). However, bacteria can be inactivated through the joint use of a synergistically active antibacterial agent along with the antibiotic (Mascaretti 2003; Tegos and Mylonakis 2012). Combination therapy can improve the antibacterial effect and reduce the risk of drug resistance during treatment, thereby reducing drug toxicity (Tegos and Mylonakis 2012; Breser et al. 2018). In addition, it has been found that when synthetic peptides of host defense bind to conventional antibiotics, synergistic effects can reduce the concentration of antibiotics required to eradicate certain bacterial strains of interest (Rudilla et al. 2016). Alternatively, the two antimicrobial agents combination can also neutralize the biofilm development (Hwang et al. 2013). However, the antibiotic enhancement remains a challenge, and clinical treatment of bovine mastitis also lacks preclinical animal and clinical data to validate its utility (Tse et al. 2017). This study demonstrates that the drug combination provides good effects in the *in vitro* assays and in the *in vivo* treatment of mouse mastitis, and can provide a basis for clinical development. Following the CLSI recommendations, supplementary tests should be performed, even when the penicillin MIC (≤ 0.12 mg/l) is within the drug-sensitive range *in vitro* (CLSI 2015). The combination of TZ and β-lactam antibiotics may enhance efficacy against *S. aureus* as a synergistic effect. In this study, the results of *in vivo* experiments indicated that the concentration of cloxacillin alone (20 mg/kg/d) was higher than in the combination with the two other drugs (0.75 mg/kg/d), and the treatment effect was better for the combination of drugs. Thus, this study indicates the concentration of drugs that reduce drug resistance when used synergistically, providing some new ideas for drug resistance research.

Previous studies reported that TZ functions as an external pump inhibitor, and MCZ acts as an autolytic inducer against *S. aureus* (Pule et al. 2016). Therefore, we investigated these two drugs in combination with CXN to inhibit the resistance of *S. aureus* through the induction of bacterial autolysis, thus enhancing the antibacterial effect of CXN. In this study, the drug susceptibility results showed that *S. aureus* had particular resistance to CXN. Again, the two drugs showed a synergistic effect in inhibiting *S. aureus*. The synergistic effect of CXN and MCZ was better that of CXN and TZ. The synergistic effect of the three drugs studied not only reduced the concentration of CXN required but also enhanced the antibacterial effect. Altogether, our results suggested that

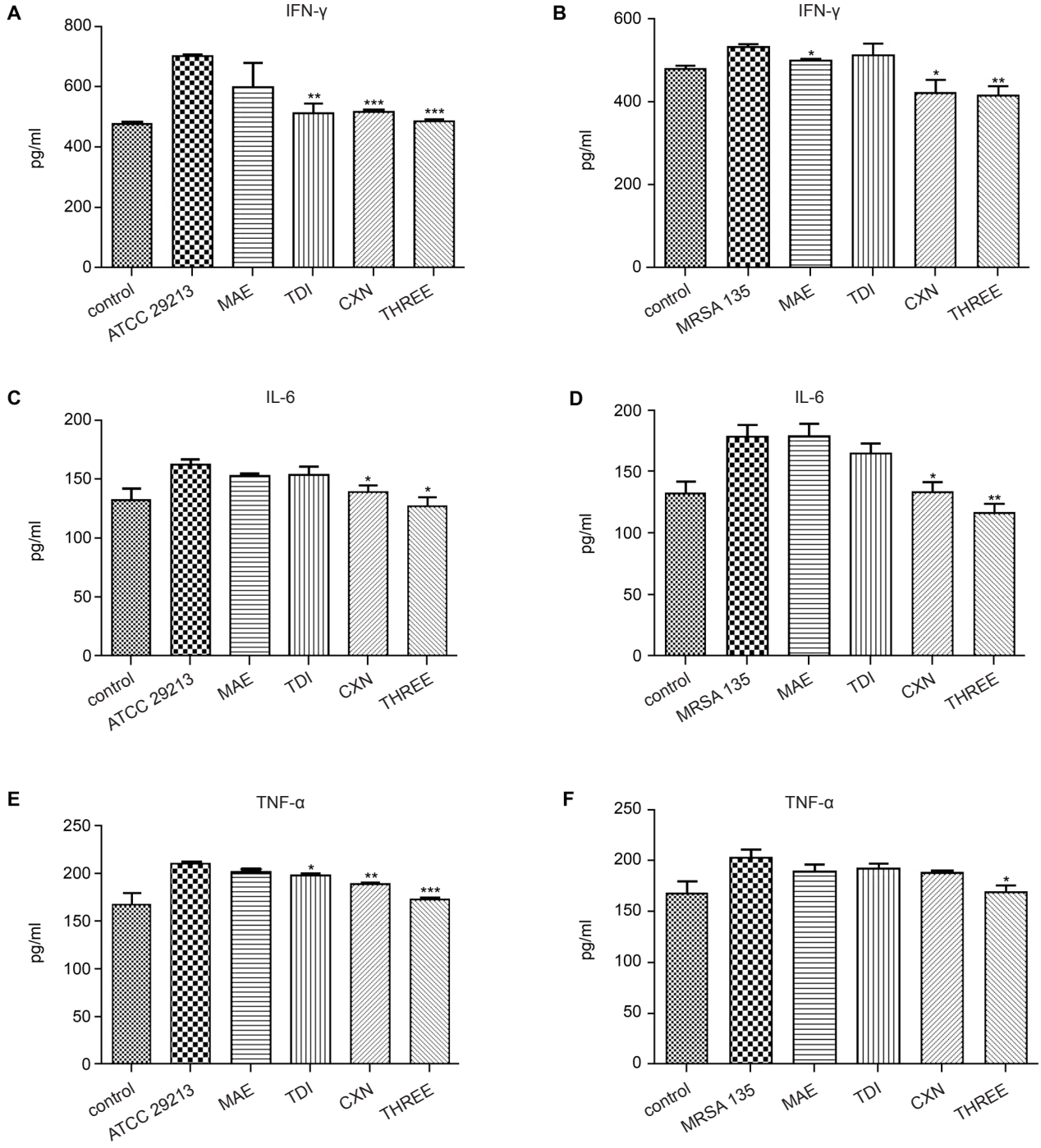

Fig. 4. Drug effects on the cytokine level in mouse mastitis models.

(A) Serum IFN-γ level in mice infected with ATCC 29213; (B) Serum IFN-γ level in mice infected with MRSA135; (C) Serum IL-6 level in mice infected with ATCC 29213; (D) Serum IL-6 level in mice infected with MRSA135; (E) Serum TNF-α level in mice infected with ATCC 29213; (F) Serum TNF-α level in mice infected with MRSA135; MCZ – miconazole; TZ – thioridazine; CXN – cloxacillin; MCZ + TZ + CXN – combination of miconazole, thioridazine, cloxacillin. The * on the horizontal line indicates a significant difference analysis between the CXN group and the three-drug group. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

treatment with a combination of TZ and CXN showed a stronger inhibitory effect against *S. aureus in vitro* when compared to the effect of CXN monotherapy. When TZ and CXN were used in combination, the MIC value was significantly reduced. In the checkerboard test, the FICI model is commonly used to determine the synergy between anti-staphylococcal drugs. Moreover, the *in vivo* dosage of the administered combination compound was determined by the ratio of *in vitro* synergistic combination of the MIC values, the single *in vivo* doses administered in mouse studies or calculated from the other animal studies already reported.

In *S. aureus*-induced mouse mastitis, cytokines released by immune cells can aggravate the inflammatory response of mastitis (De and Mukherjee 2009). It has been suggested that TNF-α, IL-1β, and IL-6 may play an important role in the mechanism of milk rupture (Persson et al. 2003). *In vivo* treatment of *S. aureus*-induced mouse mastitis showed that CXN monotherapy inhibited inflammation and resolved the redness of mouse mammaries. The inflammatory response was lower in both the MCZ-treated and TZ-treated groups compared with the CXN-treated group. Redness of mammary glands was alleviated in the three-drug treatment group, and there were fewer inflammatory cells in the tissue section than in the other groups. In the pathological changes, it was shown that in both mice groups infected with the bacteria, most of the pathological changes were milder in the group treated with the combination of the three drugs than the CXN-treated group. Our results showed that the combination of three drugs could significantly inhibit the expression of TNF-α, IFN-γ, and IL-6, both *in vitro* and *in vivo*. Cytokine assays in mice sera revealed that CXN alone induced IL-6, IFN-γ, and TNF-α in the ATCC 29213-infected mouse groups, and the combination of the three drugs significantly reduced IL-6, IFN-γ, and TNF-α concentrations. The levels of TNF-α and IFN-γ in the sera of mice treated with three drugs simultaneously were significantly lower than those in the CXN-treated group. In the MRSA135-infected mouse group, CXN alone significantly reduced IL-6 and IFN-γ levels but did not significantly reduce the level of TNF-α. The combination of the three drugs significantly reduced IL-6, IFN-γ, and TNF-α concentrations. The level of TNF-α in the sera of mice treated with three drugs simultaneously was significantly lower than those in the CXN-treated group. Therefore, the results demonstrated that the combined use of the three drugs has a significant therapeutic effect on mastitis in mice infected with *S. aureus*, which may be due to the inhibition of the production of inflammatory cytokines by these three drugs applied together.

Conclusions

In our study, we investigated the effects of TZ, MCZ, and CXN on *S. aureus*, as well as the antibacterial effect of the combination of the three drugs, both *in vitro* and *in vivo*. We found that a bacterial efflux pump inhibitor and an autolysis inducer could be used in combination to inactivate the drug resistance of *S. aureus*, thus enhancing the efficacy of the antibiotic CXN. To enhance our resources against the bacterial attack, the research on gene expression effects after the combination therapy should be explored in further studies.

List of abbreviations

MRSA (methicillin-resistant *S. aureus*);
TZ (thioridazine);
MCZ (miconazole);
CXN (cloxacillin);
MIC (the minimum inhibitory concentration);
TNF-α (tumor necrosis factor-α);
IL-1β (interleukin 1β);
IL-6 (interleukin 6); IκB (K-B inhibitor);
NF-KB (nuclear factor K-B);
CTCC (the China Type Culture Collection);
ATCC (American Type Culture Collection);
FIC (the fractional inhibitory concentration).

Authors' contributions

Participated in research design: L. Yu.
Conducted experiments: W. Luan, X. Wang.
Mice model construction: H. Xu, C. Wang.
Performed data analysis: Y. An, S. Li.
Figures making: Y. Wang, K. Shen.
Wrote or contributed to the writing of the manuscript: L. Yu, X. Liu.
Guide all the aspects of the study: M. Liu, L. Yu.

Ethics approval and consent to participate

Mice were housed in miniature isolation cages and were free to receive food and water. The laboratory temperature is 24 ± 1°C and the relative humidity is 40–80%. All animal studies were conducted in accordance with experimental practices and standards approved by the Animal Welfare and Research Ethics Committee of Jilin University (No. IZ-2009-008). *In vivo* studies in mice were performed under isoflurane anesthesia and every effort was made to meet animal welfare requirements.

Funding

This work was supported by the National Key R&D Program of China (2016YFD0501302, 2017YFD0502200); the National Nature Science Foundation of China (No. 81801972, 31172364); the Fund for Science & Technology Development of Jilin Province (20150101108JC); the Important National Science and Technology Specific Projects (2012ZX10003002); the Program for New Century Excellent Talents in University (NCET-09-0434); the Project of the Education Department of Jilin Province (No. 2016444); the Science, Technology and Innovation Commission of Shenzhen Municipality (No. JCYJ2016031100720906, JSGG20160301100442775).

Thanks to all teachers and students of the Institute of Zoonosis for their help in this experiment. Thanks to Xinrui Wang and Yi Xin for the support of this experiment.

Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

Literature

Anand KB, Agrawal P, Kumar S, Kapila K. Comparison of cefoxitin disc diffusion test, oxacillin screen agar, and PCR for mecA gene for detection of MRSA. Indian J Med Microbiol. 2009 Jan-Mar; 27(1):27–29.

Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, Scheld M, Spellberg B, Bartlett J. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009 Jan;48(1):1–12. https://doi.org/10.1086/595011

Breser ML, Felipe V, Bohl LP, Orellano MS, Isaac P, Conesa A, Rivero VE, Correa SG, Bianco ID, Porporatto C. Chitosan and cloxacillin combination improve antibiotic efficacy against different lifestyle of coagulase-negative *Staphylococcus* isolates from chronic bovine mastitis. Sci Rep. 2018 Dec;8(1):5081. https://doi.org/10.1038/s41598-018-23521-0

Celenza G, Segatore B, Setacci D, Bellio P, Brisdelli F, Piovano M, Garbarino JA, Nicoletti M, Perilli M, Amicosante G. *In vitro* antimicrobial activity of pannarin alone and in combination with antibiotics against methicillin-resistant *Staphylococcus aureus* clinical isolates. Phytomedicine. 2012 May;19(7):596–602. https://doi.org/10.1016/j.phymed.2012.02.010

Chen K, Huang Y, Song Q, Wu C, Chen X, Zeng L. Drug-resistance dynamics of *Staphylococcus aureus* between 2008 and 2014 at a tertiary teaching hospital, Jiangxi Province, China. BMC Infect Dis. 2017 Dec;17(1):97. https://doi.org/10.1186/s12879-016-2172-0

Choi JY, Kim CH, Jeon TJ, Kim BS, Yi CH, Woo KS, Seo YB, Han SJ, Kim KM, Yi DI, et al. Effective MicroPET imaging of brain 5-HT_{1A} receptors in rats with [^{18}F]MeFWAY by suppression of radioligand defluorination. Synapse. 2012 Dec;66(12):1015–1023. https://doi.org/10.1002/syn.21607

De UK, Mukherjee R. Expression of cytokines and respiratory burst activity of milk cells in response to *Azadirachta indica* during bovine mastitis. Trop Anim Health Prod. 2009 Feb;41(2):189–197. https://doi.org/10.1007/s11250-008-9174-x

Demon D, Ludwig C, Breyne K, Guédé D, Dörner JC, Froyman R, Meyer E. The intramammary efficacy of first generation cephalosporins against *Staphylococcus aureus* mastitis in mice. Vet Microbiol. 2012 Nov;160(1-2):141–150. https://doi.org/10.1016/j.vetmic.2012.05.017

Dickey SW, Cheung GYC, Otto M. Different drugs for bad bugs: antivirulence strategies in the age of antibiotic resistance. Nat Rev Drug Discov. 2017 Jul;16(7):457–471. https://doi.org/10.1038/nrd.2017.23

Elazar S, Gonen E, Livneh-Kol A, Rosenshine I, Shpigel NY. Neutrophil recruitment in endotoxin-induced murine mastitis is strictly dependent on mammary alveolar macrophages. Vet Res. 2010 Jan;41(1):10. https://doi.org/10.1051/vetres/2009058

Falk SP, Noah JW, Weisblum B. Screen for inducers of autolysis in *Bacillus subtilis*. Antimicrob Agents Chemother. 2010 Sep 01; 54(9):3723–3729. https://doi.org/10.1128/AAC.01597-09

Foster TJ. Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. FEMS Microbiol Rev. 2017 May 01; 41(3):430–449. https://doi.org/10.1093/femsre/fux007

Fu Y, Zhou E, Wei Z, Liang D, Wang W, Wang T, Guo M, Zhang N, Yang Z. Glycyrrhizin inhibits the inflammatory response in mouse mammary epithelial cells and a mouse mastitis model. FEBS J. 2014 Jun;281(11):2543–2557. https://doi.org/10.1111/febs.12801

Gao X, Wang T, Zhang Z, Cao Y, Zhang N, Guo M. Brazilin plays an anti-inflammatory role with regulating Toll-like receptor 2 and TLR 2 downstream pathways in *Staphylococcus aureus*-induced mastitis in mice. Int Immunopharmacol. 2015 Jul;27(1):130–137. https://doi.org/10.1016/j.intimp.2015.04.043

Goering RV, Swartzendruber EA, Obradovich AE, Tickler IA, Tenover FC. Stealth MRSA: emergence of resistance in oxacillin-susceptible MRSA due to mecA sequence instability. Antimicrob Agents Chemother. 2019;68(3):e00558-19.

Hendricks O, Butterworth TS, Kristiansen JE. The *in vitro* antimicrobial effect of non-antibiotics and putative inhibitors of efflux pumps on *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Int J Antimicrob Agents. 2003 Sep;22(3):262–264. https://doi.org/10.1016/S0924-8579(03)00205-X

Hu C, Gong R, Guo A, Chen H. Protective effect of ligand-binding domain of fibronectin-binding protein on mastitis induced by *Staphylococcus aureus* in mice. Vaccine. 2010 May;28(24):4038–4044. https://doi.org/10.1016/j.vaccine.2010.04.017

Hu FP, Guo Y, Zhu DM, Wang F, Jiang XF, Xu YC, Zhang XJ, Zhang CX, Ji P, Xie Y, et al. Resistance trends among clinical isolates in China reported from CHINET surveillance of bacterial resistance, 2005–2014. Clin Microbiol Infect. 2016 Mar;22 Suppl 1:S9–S14. https://doi.org/10.1016/j.cmi.2016.01.001

Hwang I, Hwang JS, Hwang JH, Choi H, Lee E, Kim Y, Lee DG. Synergistic effect and antibiofilm activity between the antimicrobial peptide coprisin and conventional antibiotics against opportunistic bacteria. Curr Microbiol. 2013 Jan;66(1):56–60. https://doi.org/10.1007/s00284-012-0239-8

Intrakamhaeng M, Komutarin T, Pimpukdee K, Aengwanich W. Incidence of enterotoxin-producing MRSA in bovine mastitis cases, bulk milk tanks and processing plants in Thailand. J Anim Vet Adv. 2012;11(5):87–93.

Iwase T, Uehara Y, Shinji H, Tajima A, Seo H, Takada K, Agata T, Mizunoe Y. *Staphylococcus epidermidis* Esp inhibits *Staphylococcus aureus* biofilm formation and nasal colonization. Nature. 2010 May;465(7296):346–349. https://doi.org/10.1038/nature09074

Kim YN, Kim DW, Jo HS, Shin MJ, Ahn EH, Ryu EJ, Yong JI, Cha HJ, Kim SJ, Yeo HJ, et al. Tat-CBR1 inhibits inflammatory responses through the suppressions of NF-κB and MAPK activation in macrophages and TPA-induced ear edema in mice. Toxicol Appl Pharmacol. 2015 Jul;286(2):124–134. https://doi.org/10.1016/j.taap.2015.03.020

Klitgaard JK, Skov MN, Kallipolitis BH, Kolmos HJ. Reversal of methicillin resistance in Staphylococcus aureus by thioridazine. J Antimicrob Chemother. 2008 Sep 10;62(6):1215–1221. https://doi.org/10.1093/jac/dkn417

Kolendi CL. Methicillin-resistant *Staphylococcus aureus* (MRSA): etiology, at-risk populations and treatment. New York (USA): Nova Science Publishers Inc.; 2010.

Koszczol C, Bernardo K, Krönke M, Krut O. Subinhibitory quinupristin/dalfopristin attenuates virulence of *Staphylococcus aureus*. J Antimicrob Chemother. 2006 Jul 01;58(3):564–574. https://doi.org/10.1093/jac/dkl291

Lowy FD. *Staphylococcus aureus* Infections. N Engl J Med. 1998 Aug 20; 339(8):520–532. https://doi.org/10.1056/NEJM199808203390806

Mascaretti OA. Bacteria versus antibacterial agents: an integrated approach. Washington, D.C. (USA): ASM Press; 2003.

Moon JS, Kim HK, Koo HC, Joo YS, Nam H, Park YH, Kang MI. The antibacterial and immunostimulative effect of chitosan-oligosaccharides against infection by *Staphylococcus aureus* isolated from bovine mastitis. Appl Microbiol Biotechnol. 2007 Jun 13;75(5): 989–998. https://doi.org/10.1007/s00253-007-0898-8

Navon-Venezia S, Ben-Ami R, Carmeli Y. Update on *Pseudomonas aeruginosa* and *Acinetobacter baumannii* infections in the healthcare setting. Curr Opin Infect Dis. 2005 Aug;18(4):306–313. https://doi.org/10.1097/01.qco.0000171920.44809.f0

Persson Waller K, Colditz IG, Lun S, Östensson K. Cytokines in mammary lymph and milk during endotoxin-induced bovine mastitis. Res Vet Sci. 2003 Feb;74(1):31–36. https://doi.org/10.1016/S0034-5288(02)00147-9

Poulsen MØ, Jacobsen K, Thorsing M, Kristensen NRD, Clasen J, Lillebæk EMS, Skov MN, Kallipolitis BH, Kolmos HJ, Klitgaard JK. Thioridazine potentiates the effect of a beta-lactam antibiotic against *Staphylococcus aureus* independently of mecA expression. Res Microbiol. 2013 Feb;164(2):181–188. https://doi.org/10.1016/j.resmic.2012.10.007

Pule CM, Sampson SL, Warren RM, Black PA, van Helden PD, Victor TC, Louw GE. Efflux pump inhibitors: targeting mycobacterial efflux systems to enhance TB therapy. J Antimicrob Chemother. 2016 Jan;71(1):17–26. https://doi.org/10.1093/jac/dkv316

Que YA, Haefliger JA, Piroth L, François P, Widmer E, Entenza JM, Sinha B, Herrmann M, Francioli P, Vaudaux P, et al. Fibrinogen and fibronectin binding cooperate for valve infection and invasion in *Staphylococcus aureus* experimental endocarditis. J Exp Med. 2005 May 16;201(10):1627–1635. https://doi.org/10.1084/jem.20050125

Rudilla H, Fusté E, Cajal Y, Rabanal F, Vinuesa T, Viñas M. Synergistic antipseudomonal effects of synthetic peptide AMP38 and carbapenems. Molecules. 2016 Sep 12;21(9):1223–1234. https://doi.org/10.3390/molecules21091223

Tegos G, Mylonakis E. Antimicrobial drug discovery: emerging strategies. Wallingford (UK): CABI; 2012.

Trigo G, Dinis M, França A, Bonifácio Andrade E, Gil da Costa RM, Ferreira P, Tavares D. Leukocyte populations and cytokine expression in the mammary gland in a mouse model of *Streptococcus agalactiae* mastitis. J Med Microbiol. 2009 Jul 01;58 (7):951–958. https://doi.org/10.1099/jmm.0.007385-0

Tse BN, Adalja AA, Houchens C, Larsen J, Inglesby TV, Hatchett R. Challenges and opportunities of nontraditional approaches to treating bacterial infections. Clin Infect Dis. 2017 Aug 01; 65(3): 495–500. https://doi.org/10.1093/cid/cix320

Vermote A, Van Calenbergh S. Small-molecule potentiators for conventional antibiotics against *Staphylococcus aureus*. ACS Infect Dis. 2017 Nov 10;3(11):780–796. https://doi.org/10.1021/acsinfecdis.7b00084

Wax RG. Bacterial resistance to antimicrobials. Boca Raton (USA): CRC Press; 2008.

Wei W, Dejie L, Xiaojing S, Tiancheng W, Yongguo C, Zhengtao Y, Naisheng Z. Magnolol inhibits the inflammatory response in mouse mammary epithelial cells and a mouse mastitis model. Inflammation. 2015 Feb;38(1):16–26. https://doi.org/10.1007/s10753-014-0003-2

Zhang JM, An J. Cytokines, inflammation, and pain. Int Anesthesiol Clin. 2007 1;45(2):27–37. https://doi.org/10.1097/AIA.0b013e318034194e

Zore GB, Thakre AD, Jadhav S, Karuppayil SM. Terpenoids inhibit *Candida albicans* growth by affecting membrane integrity and arrest of cell cycle. Phytomedicine. 2011 Oct;18(13):1181–1190. https://doi.org/10.1016/j.phymed.2011.03.008