The bacteriostatic effect and mechanism of berberine on Methicillin resistant *Staphylococcus aureus in vitro*

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Research article

Keywords: Berberine, Methicillin resistant Staphylococcus aureus (MRSA), Combination, Antibiotics, Bacteriostatic effect

Posted Date: June 4th, 2020

DOI: https://doi.org/10.21203/rs.2.9640/v3

Abstract

Background: To observe the bacteriostatic effect of berberine (BBR) and BBR combined with gentamicin (GEN), levofloxacin (LEV) and amikacin (AMI) on Methicillin resistant Staphylococcus aureus (MRSA), while also exploring the bacteriostatic mechanism of BBR on MRSA.

Results: The MICs range of BBR on 26 strains of MRSA was 32-256 μg/mL. BBR combined with GEN, LEV and AMI had obvious bacteriostatic effect on MRSA. After co-culturing MRSA with BBR at 512 μg/mL, 64 μg/mL and 8 μg/mL, respectively, the electrical conductivity increased, compared with the control group, by 8.14%, 13.08% and 12.01%, respectively. Using transmission electron microscopy, we found that low concentration of BBR (8 μg/mL; 1/8 MIC) caused no significant damage to MRSA, and the bacterial structure of MRSA remained intact, while high concentration of BBR (512 μg/mL; 8 MIC) induced the destruction and dissolution of MRSA cell wall structure and the leakage of bacterial contents, leading to bacterial lysis. RNA-sequencing results showed that there were 754 differentially expressed genes in the high concentration group compared with the normal control group. Compared with the low concentration group, there were 590 differentially expressed genes in the high concentration group. Compared with the control group, only 19 genes were differentially expressed in the low concentration group. The up-regulated genes are mainly related to the cell wall hydrolysis regulatory genes, while the down-regulated genes are mainly related to the serine protease family.

Conclusions: BBR displayed an excellent bacteriostatic effect on MRSA. BBR combined with GEN and AMI significantly enhanced the bacteriostatic effect on MRSA, while BBR combined with LEV showed no significant change in the bacteriostatic effect on MRSA. BBR inhibited bacteria by destroying and dissolving the structure of MRSA cell wall. RNA-sequencing results further demonstrated that the expression of cell wall hydrolysis genes ssaA, lytM and virulence factor serine protease genes were significantly differentially expressed when high concentration BBR treated on MRSA.

Background

Staphylococcus aureus (*S. aureus*) is one of the most common pathogenic bacteria in pyogenic infections, causing systemic infections such as local pyogenic infections, pneumonia, pseudomembranous enteritis, pericarditis and sepsis[1]. In recent years, the emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistance to most beta-lactam, has severely limited treatment options [2-3]. Vancomycin is commonly used to treat MRSA infections[4]. However, Vancomycin-intermediate *Staphylococcus aureus* (VISA) appeared in the 1990s, and Vancomycin-resistant *Staphylococcus aureus* (VRSA) was first reported in the United States in 2002[5]. The emergence of VISA and VRSA has raised the importance of discovering new treatments for MRSA infections.

Berberine (BBR) is the main component of the traditional Chinese medicine *Coptis chinensis* and *Cortex Phellodendri*, with an isoquinoline alkaloid structure. BBR is one of the commonly used drugs in the treatment of intestinal infections in China[6]. In addition to its broad spectrum bacteriostatic effects[7-9],

BBR is reported to have anti-inflammatory, anti-oxidant, anti-tumor, hypoglycemic and anti-cardiac arrhythmia qualities[10-14]. Studies also reported that BBR had good bacteriostatic effect on *E. coli* and *Bacillus subtilis*[15]. Subsequent studies reported that the MIC of BBR against the MRSA reference strain ATCC33591 was 128 μg/mL. BBR can affect the aggregation of amyloid fibers in PSMs of MRSA biofilm, thus inhibiting the formation of MRSA biofilm and increasing the bactericidal activity of antibiotics[9]. Recent studies have reported that BBR had bacteriostatic effects on MRSA, yet no specific study on its bacteriostatic mechanism has been reported. In our hospital, aminoglycoside antibiotics (GEN, AMI) and quinolone antibiotics (LEV) are routinely used to treat MRSA infection. With the increase of drug resistance, this study sought to investigate the bacteriostatic effect of BBR and BBR combined with GEN, LEV and AMI on clinical strains of MRSA, while further exploring the bacteriostatic mechanism of BBR on MRSA.

Results

The STs of 26 clinical isolated MRSA strains

All 26 clinical MRSA isolates were typed by multilocus sequence typing(MLST). There were 9 distinct STs identified within the 26 isolates (Fig.1), among which the most frequently represented were ST764 (38.5%, 10/26), ST5 (19.2%, 5/26), ST59 (11.5%, 3/26), and ST398 (11.5%, 3/26), while only 1 strain (3.8%) for ST1, ST15, ST152, ST338 and ST630.

Antimicrobial susceptibility profiles

Reference strains USA300 and 26 clinical isolates of MRSA strains were identified by automatic identification and drug susceptibility system (VITEK 2 compact microbiology analysis system, BioMérieux). According to the 2018 version of the Clinical and Laboratory Standards Institute (CLSI)[16], all strains were identified as MRSA. Drug susceptibility test showed that 26 MRSAs were all resistant to oxacillin, 5 to gentamicin, 17 to levofloxacin, 6 to amikacin, 22 to clindamycin, 19 to ciprofloxacin, 21 to erythromycin, 18 to moxifloxacin, 1 to Trimethoprim-sulfamethoxazole(SMZ-TMP), and 17 to tetracycline. All 26 strains were sensitive to rifampicin, linezolid and vancomycin(Table 1). In addition, In addition, 26 clinical isolated strains were identified as MRSA by *mecA* gene using PCR.

Bacteriostatic effect of BBR, GEN, LEV and AMI on MRSA

The MIC of BBR on reference strain USA300 is 128 μg/mL, while the MIC of BBR on 26 clinical isolated MRSA strains is 32-256 μg/mL, indicating that BBR has strong bacteriostatic effect both on USA300 and clinical isolated strains, which is consistent with the previous research results of Chu, M. etc.[9], who reported that the MIC of BBR on reference strain ATCC33591 was 128 μg/mL (Table 2).

Synergy studies

GEN, LEV and AMI are common drugs in the treatment of MRSA infection in our hospital. According to Table 1, among the 26 clinical isolates of MRSA strains, 5 were resistant to GEN, 17 to LEV and 6 to AMI. With the increase of antibiotic resistance rate, the treatment choice of MRSA is becoming more and more limited. BBR combined with 3 kinds of antibiotics to which MRSA is resistant were tested. In order to further understand the bacteriostatic effect of BBR combined with antibiotics, FICI analysis of chessboard method was carried out. Table 3 showed MIC, FICI and bacteriostatic effect of BBR combined with 3 antibiotics. After the combined action of BBR and GEN on GEN-resistant MRSA strains, 4 strains (80%) displayed an additive effect. After the combined action of BBR and LEV on LEV-resistant MRSA strains, only 3 strains (17.64%) displayed an additive effect. After the combined action of BBR and AMI on AMI-resistant MRSA strains, 4 strains (66.67%) shown synergistic effect, and 1 (16.67%) was additive. (Table 3, Supplementary Table 1-3)

BBR combined with antibiotics time-kill analysis

Of the 26 clinical MRSA strains, strain MRSA02, which was resistant to GEN, LEV, and AMI, was selected to assess the bacteriostatic effect of BBR combined with these 3 antibiotics. Time-kill analysis was conducted according to CLSI guidelines. The initial inoculation amount was 1×10^6CFU/mL. We combined 0.5 MIC of BBR with 0.5 MIC of different kinds of antibiotics for time-kill experiment. According to Fig.2, when 0.5 MIC of BBR acted alone on MRSA02, the activity of bacteria did not change obviously, and the bacteriostatic effect was not significant. When 0.5 MIC of BBR combined with GEN, LEV and AMI for 8 h, the activity of bacteria began to decrease; After 12 h, the activity of bacteria decreased significantly. BBR obviously enhanced the bacteriostatic ability of these 3 antibiotics. (Fig.2)

BBR increased membrane permeability of MRSA

In bacteria, cell membrane is a selective barrier. Bacterial cell membranes protect bacteria from harmful compounds such as drugs, toxins, detergents and degrading enzymes, and allow nutrients to penetrate to promote bacterial growth[17].The integrity of cell membrane will affect the life activity of bacteria. In this study, the results showed that the conductivity of bacterial solution increased rapidly after BBR treatment. Therefore, BBR can increase the permeability of bacterial cell membrane and induce cytoplasmic leakage in a short time. MRSA02 strain, which was resistant to 3 antibiotics, was selected to observe the changes in conductivity of the culture medium after BBR treatment. The effect of BBR solution on the conductivity of MRSA02 medium is shown in Fig. 3. After treated with 512 μg/mL (8 MIC), 64 μg/mL (1 MIC) and 8 μg/mL (1/8 MIC) BBR for 4 hours, the conductivity increased by 8.14%, 13.08%, and 12.01%, respectively

(Fig. 3A). When 512 μg/mL of BBR was applied to the MRSA02 for 0.5 h, the conductivity of the culture medium was almost unchanged. After 64 μg/mL of BBR was applied to the MRSA02, the conductivity increased significantly and increased with time within a 3.5 hours period. After 8 μg/mL of BBR was applied to the MRSA02, the conductivity also increased significantly and increased with time within a 3.5 hours period (Fig. 3B).

Effect of BBR on cell wall of MRSA

Among the 26 clinical MRSA strains, the MRSA02 strain, which was resistant to 3 antibiotics, was selected to observe cell wall damage after BBR treatment. The cell wall of the MRSA02 strain was damaged to different degrees after being cultured in low (8 μg/mL; 1/8 MIC), and high (512 μg/mL; 8 MIC) BBR solutions (Fig. 4A-D). Low concentration (8 μg/mL) BBR did not cause any obvious damage to MRSA02 strain. MRSA02 kept an intact structure, with BBR crystals around the cell wall (Fig.4A-B). High concentration (512 μg/mL) of BBR induced severe destruction and dissolution of cell wall structure of MRSA02 strain and large amount of leakage of cell contents, leading to bacterial lysis and death, while BBR crystal around the cell was significantly reduced(Fig.4C-D). The results of TEM were consistent with the results of conductivity.

Analysis of RNA-seq results

RNA sequencing was performed after USA300 was exposed to BBR of high concentration (64 μg/mL) and low concentration (16 μg/mL) for 3 hours. Sequencing results showed that there were 754 differentially expressed genes in the high concentration group compared with the normal control group, of which 561 genes were up-regulated and 193 genes were down-regulated. Compared with the control group, only 19 genes were differentially expressed in the low concentration group, of which 11 genes were up-regulated and 8 genes were down-regulated. Compared with the low concentration group, there were 590 differentially expressed genes in the high concentration group, of which 402 genes were up-regulated and 188 genes were down-regulated(Fig. 5A-B,6A-C). Among them, *ssaA and lytM* were significantly up-regulated. The down-regulated genes were mainly serine protease family genes (**Table 4**). There are 14 overlaps in the differential regulation genes of USA300 at high and low concentrations, among which 5 are up-regulated overlaps, including *speG*, phage integrase family protein, *scpB* and some proteins with unknown functions, while 6 are down-regulated overlaps, including putative membrane protein, staphylococcal accessory regulator R, *choline dehydrogenase* and some proteins with unknown functions. How BBR regulates the function of these proteins will be further explored. The expression of differentially expressed genes in reference strain USA300 was significantly increased by high concentration BBR, while the expression of differentially expressed genes in low concentration BBR group was not significantly changed.

Discussion

In this study, we found that BBR had obvious bacteriostatic effect on MRSA, and its combination with GEN, LEV and AMI significantly enhanced the bactericidal effect. According to the time-kill analysis, BBR combined with AMI showed the best bacteriostatic effect on MRSA. This study found that the single components of traditional Chinese medicine could be excellent bacteriostatic drugs. Our previous study found that *Phellodendron amurense* also showed a good bacteriostatic effect on MRSA[18], laying a foundation for the treatment of drug-resistant bacteria with Chinese medicine alone or in combination with antibiotics. AMI is a kind of aminoglycoside antibiotics and is mainly used for Gram-negative bacilli infection treatment. The bacteriostatic mechanism is that the antibacterial drugs enter the bacteria through the cell wall of Gram-negative bacilli, irreversibly binding to the 30S ribosomal subunit. This results in inhibition of the synthesis of bacterial proteins thus achieving a bacteriostatic effect. However, MRSA are Gram-positive cocci with thicker cell walls. How does AMI enter MRSA through the cell wall? Why does BBR combined with AMI enhance the bacteriostatic effect of AMI on MRSA? How does BBR play a role in this process? For these questions, we performed conductivity tests and TEM examination, to further investigate the possible bacteriostatic mechanism of BBR.

MRSA is a Gram-positive coccus, its cell wall is mainly composed of peptidoglycan and teichoic acid. Cell wall inhibitors such as β-lactam and vancomycin are beneficial to bacterial uptake of AMI[19,20]. Our study further confirmed that BBR can inhibit the synthesis of MRSA cell wall. High concentration BBR destroyed the structure of the MRSA cell wall, resulting in thinning or even lysis of the bacterial cell wall, enabling AMI to penetrate the cell wall more easily, with consequent action on the synthesis of DNA in bacteria. This resulted in further inhibition of synthesis of bacterial protein, leading to bacterial death.

In this study, we found that the expression of *ssaA* and *lytM* genes in the up-regulated genes of high concentration group was significantly different. According to the literature, these genes were all related to cell wall hydrolysis[21]. *SsaA* and *lytM* have potential WalKR binding sites. WalKR system directly regulates the hydrolysis of bacterial cell wall[22]. It is speculated that high concentration of BBR increases the binding sites of *ssaA* and *lytM* to WalKR, and enhances the ability of WalKR system to hydrolyze bacterial cell wall, thus causing bacterial cell wall lysis and bacterial death. In the high concentration group, the expression of *splB~splF* gene of serine protease family was significantly down-regulated. The serine protease family plays a hydrolytic role in protein metabolism, breaking the peptide bonds of macromolecules and forming small-molecule propeptides[23], which are important component of bacterial cell wall[24]. Lack of small-molecule propeptides would affect the synthesis of bacterial cell wall, resulting in thinning and dissolution of bacterial cell wall. High concentration BBR increased the expression of *ssaA* and *lytM* genes in USA300 and down-regulated serine protease family genes, thus enhancing the damage of BBR to bacterial cell wall. However, the expression of *ssaA*, *lytM* and serine protease family genes in USA300 was not changed by low concentration of BBR. These results further validated the results of TEM and conductivity: high concentration of BBR induced cell wall lysis and change of cell wall permeability. The regulation mechanism of BBR on *ssaA, lytM* and serine protease genes will be further explored in our future study. BBR showed an excellent bacteriostatic effect on MRSA.

The combination of BBR and aminoglycoside antibiotics significantly reduced the resistance of aminoglycoside antibiotics. In addition, BBR enhanced antibiotic activity by inhibiting the synthesis of MRSA cell wall. We will study further on the bacteriostatic mechanism of BBR against bacteria.

Conclusions

BBR is an effective monomer of many traditional Chinese medicines. It displayed an excellent bacteriostatic effect on MRSA in vitro, with a MIC of 32-256 μg/mL. BBR combined with aminoglycoside antibiotics GEN and AMI significantly enhanced the bacteriostatic effect on MRSA, while BBR combined with quinolone antibiotics LEV showed no significant change in the bacteriostatic effect on MRSA. According to the time-kill analysis, after BBR combined with antibiotics for 8 h, its bacteriostatic effect was significantly enhanced. Further study confirmed that BBR destroyed and dissolved the structure of MRSA cell wall by regulating the expression of *ssaA, lytM* and serine protease genes, and thus achieving the bacteriostatic effect. In conclusion, our study suggested that BBR had an obvious bacteriostatic effect on MRSA, and its bacteriostatic mechanism will be further studied in the future.

Methods

Source of strains

S. aureus reference strain USA300 was a gift from Prof. Lan of Shanghai Institute of Materia Medica, Chinese Academy of Sciences. All 26 strains of MRSA were isolated from inpatient specimens of Shanghai Eighth People's hospital from October 2016 to March 2017. All strains were identified using the VITEK 2 compact microbiology analysis system (BioMérieux Industry, France). In addition, we confirmed the strains were MRSA by PCR for detection of the *mecA* gene.

(*mecA* primer: *mecA*-R:GTAGAAATGACTGAACGTCCGATAA,

mecA-F:TTACAGAGTTAACTGTTACC, with a size of 310 bp) The 26 isolated strains of MRSA were numbered as MRSA01~MRSA26.

BBR and antibiotics

BBR was purchased from Tianzheng Pharmaceutical Co., LTD (Northeast pharmaceutical group, batch number: 0361611030) and was prepared with dimethyl sulfoxide (DMSO) to 32 mg/mL solution. After filtration and sterilization with 0.22 μm filter membrane, BBR solution was separated into sterilized EP tubes and stored at 4°C for reserve. GEN (Xinchen Pharmaceutical Co., LTD, batch number: 1607252211), AMI (Shanghai Xinyijinzhu Pharmaceutical Co., LTD, batch number: 1611107), and LEV (Yangtze River Pharmaceutical Co., LTD, batch number: 16080231) were all purchased from the pharmacy of Shanghai

Eighth People's hospital. These antibiotics were freshly prepared with sterile water to a concentration of 1024 μg/mL.

Multilocus sequence typing (MLST)

Isolates were screened using a previously described method [25] to detect the following seven housekeeping genes: *carbamate kinase (arcC), shikimate dehydrogenase (aroE), glycerol kinase (glp), guanylate kinase (gmk),phosphate acetyltransferase (pta), triosephosphate isomerase (tpi)*, and *acetyl coenzyme A acetyltransferase(yqiL)*. The sequences of the PCR products were compared with the existing sequences available from the MLST website (http://www.mlst.net) for *S. aureus* [26],and the allelic number was determined for each sequence.

Susceptibility testing of the BBR and antimicrobial agents

According to the 2018 standard of the Clinical and Laboratory Standards Institute (CLSI)[16], the MIC of GEN, LEV, AMI and BBR on MRSA was determined by broth microdilution. GEN, LEV, AMI, and BBR solutions were serially diluted to a final concentration of 8, 16, 32, 64, 128, 256, 512 and 1024 μg/mL with M-H broth. 50 μL different concentration of antibiotics or BBR solution was added to each well of a 96-well plate. The bacterial suspension was prepared with 0.5 McFarland standard, then diluted to 1:1000 with M-H broth. 50 μL bacterial suspension was added to each well of the 96-well plate and incubated in a Heal Force CO_2 incubator (Likang, Shanghai, China) at 37^{o}C for 24 h. The minimum drug concentration without bacterial growth was MIC.

Synergy Testing

5 strains (MRSA01~MRSA05) of MRSA resistant to GEN, 17 strains (MRSA01~MRSA04, MRSA06~MRSA18) resistant to LEV and 6 strains (MRSA02, MRSA04, MRSA07~MRSA10) resistant to AMI were selected for the combined bacteriostatic test. BBR solution was diluted to 4 dilutions (32, 64, 128, and 256 μg/mL) with M-H broth. GEN, LEV and AMI were diluted with M-H broth to 8 increasing concentrations (8, 16, 32, 64, 128, 256, 512, and 1024 μg/mL). Bacterial suspensions were prepared with 0.5 McFarland standard, then diluted to 1:1000 with M-H broth. 50 μL of BBR solution (32, 64, 128, and 256 μg/mL) and GEN, LEV, AMI solution (8, 16, 32, 64, 128, 256, 512, and 1024 μg/mL) were arranged in a 96-well plate, and 100 μL bacterial suspension was added to the sterile microporous plate. The final concentrations of BBR were 8, 16, 32, and 64 μg/mL, respectively. The final concentrations of GEN, LEV and AMI were 2, 4, 8, 16, 32, 64, 128, and 256 μg/mL, respectively. All plates were incubated at 37^{o}C for 24 h. After 24 h incubation, the minimum drug concentration without bacterial growth was considered to

be the MIC. The interaction was judged by calculating the fractional inhibitory concentration (FICIs). These were calculated as follows:

[Please see the supplementary files section to view the equation.]

FICI ≤ 0. 5, 0.5 < FICI ≤1, 1 < FICI ≤2, 2 ≤ FICI represents synergy, additivity, indifference, and antagonism, respectively[27].

Time-kill curve studies

MRSA02 strain, which was resistant to all 3 antibiotics, was selected for the evaluation of its bacteriostatic activity kinetics in vitro. The frozen MRSA02 strain was inoculated on LB agar plate and incubated overnight at 37^{o}C. Several well separated colonies were collected and suspended in M-H broth. The turbidity was adjusted to 0.5 McFarland standard (approximately 10^8 CFU/mL). The initial inoculum was prepared by inoculating 10 μL MRSA02 bacterial suspension into 20 mL M-H supplemented with 0.1% BSA. After being placed in a shaking incubator (37°C, 200 rpm) for 1 h, 2.7 mL of culture solution was equally distributed to 6 tubes, and 0.03 mL of BBR solution(concentration was 102.4mg/mL, 51.2mg/mL, 25.6mg/mL, 12.8mg/mL, 6.4mg/mL, and 3.2mg/mL, respectively) were added. 0.03 mL aliquots were immediately removed to determine the initial CFU (0 hour). The incubation continued and 0.02 mL aliquots were taken at 1, 2, 4, 8 and 24 h, respectively. After sampling, the culture was diluted continuously in sterile saline and inoculated on TSA agar. CFU was measured after incubation overnight. According to the bacterial survival rate in the sampling interval, the time-kill curve was drawn[28]. According to the above-mentioned method, the bacteriostatic activity kinetics of BBR combined with three antibiotics in vitro was also evaluated.

Conductivity test

MRSA02 strain was selected for the conductivity test. Measuring the conductivity of culture medium with a conductivity meter is to measure the ionic concentration of culture medium. The higher the ion concentration is, the greater the conductivity. BBR solution at 16 μg/mL, 128 μg/mL, and 1024 μg/mL were added to the bacterial suspension culture at logarithmic phase. The final concentrations of BBR solution were 8 μg/mL, 64 μg/mL, and 512 μg/mL, respectively. 5 mL of suspension was taken and centrifuged at 2800×g for 10 min at 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4 h, respectively. Conductivity of the supernatant was measured by DDS-11A conductivity meter (Leici, Shanghai) after a 20-time dilution of the supernatant. Absolute ethanol was taken as the control group. The test was repeated 3 times, and the average value was obtained.

TEM examination

MRSA02 strain was selected for the TEM examination. Bacterial suspensions were prepared with 0.5 McFarland standard. After culture with 8 μg/mL and 512 μg/mL BBR solution at 37^{o}C for 24 h, 10 mL solution was taken and centrifuged at 12000×g for 10 min, and the supernatant was removed to collect 0.5 mL sediment. After fixation with 2% glutaraldehyde PBS fixing solution at 4^{o}C for 2 h, the sediment was washed twice with PBS. After fixed with 1% osmium acid-PBS fixing solution at 4^{o}C for 2 h, cells were washed twice with PBS. Bacteria were dehydrated step by step with ethanol, replaced with propylene oxide and immersed in epoxy resin. They were then sliced with a LKB V ultrathin section machine and stained with lead citrate. The changes in MRSA cell wall were observed by an H-7650 transmission electron microscopy (HITACH, Japan).

RNA isolation, mRNA enrichment and sequencing

After culturing *S. aureus* USA300 strain to logarithmic phase, USA300 was cultured with BBR of different concentration for 3 h, and total RNA was extracted. The samples were divided into 3 groups: normal control group (group A), high concentration group (group B, 1/2 MIC, 64 μg/mL), and low concentration group (group C, 1/8 MIC, 16 μg/mL). Each group had 3 repetitive samples. Total RNA was extracted from bacterial cells using RNeasy Mini kit (Qiagen)[29]. Qubit 2.0 RNA detection kit was used to quantify total RNA accurately to determine the amount of total RNA added to the library. rRNA was removed by kit and fragmentation buffer was added to the obtained mRNA to make the fragments short. The fragmented RNA was used as template to synthesize the first strand of the DNA with random hexamers, and the second strand was synthesized by adding buffer, dNTPs, RNase H and DNA polymerase I. The product was purified by QiaQuick PCR kit and eluted by EB buffer. After terminal repair, base A and sequencing connector were added, the target fragments were collected by agarose gel electrophoresis, and amplified by PCR. The whole library was prepared and the library was sequenced by Illumina HiSeq2500[30].

Abbreviations

BBR:berberine; GEN:gentamicin; LEV:levofloxacin AMI:amikacin; MRSA:Methicillin resistant *Staphylococcus aureus*; VISA:Vancomycin-intermediate *Staphylococcus aureus*; VRSA:Vancomycin-resistant *Staphylococcus aureus*; CLSI:Clinical and Laboratory Standards Institute; FICI:fractional inhibitory concentration; MIC:minimum inhibitory concentration; CFU:colony-forming units; TEM: transmission electron microscopy; DNA:deoxyribonucleic acid; RNA: ribonucleic acid; PCR: polymerase chain reaction; MLST:multilocus sequence typing

Declarations

Acknowledgements

We are grateful to the responsible authorities of Shanghai Eighth People's Hospital and Shanghai Pharmaceutical Institute for kindly supporting the progress of this study.

Authors' contributions

L.W conceived and designed the study. F.Z, W.G, M.L and S.J performed the experiments. F.Z and M.X analyzed the data and wrote the manuscript. L.W, X.Z and P.L reviewed and edited the manuscript. All authors read and approved the manuscript.

Funding

No specific funding was obtained for the purpose of this study.

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval and consent to participate

The competent Ethics Committee (The Ethics Committee of Shanghai Eighth People's Hospital) approved this study (No.2020-002) and all patients have provided written informed consent.

Consent for publication

Publication has been approved by all authors and the responsible authorities at the institution where the work is carried out. The authors confirm that the work described has not been published before and it is not under consideration for publication elsewhere.

Competing interests

The authors report no conflicts of interest in this work.

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Tables

Table 1. Drug sensitivity analysis of 26 clinical isolates of MRSA strains

ID	OXA	GEN	LEV	AMI	CLI	CIP	ERY	LZD	MXF	RFP	SMZ	TET	VAN
MRSA01	R	R	R	S	R	R	R	S	R	S	S	R	S
MRSA02	R	R	R	R	R	R	R	S	R	S	S	R	S
MRSA03	R	R	R	S	S	R	S	S	I	S	S	S	S
MRSA04	R	R	R	R	R	R	R	S	R	S	S	R	S
MRSA05	R	R	S	S	R	R	S	S	R	S	S	S	S
MRSA06	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA07	R	S	R	R	R	R	R	S	R	S	S	R	S
MRSA08	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA09	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA10	R	S	R	R	R	R	R	S	R	S	S	S	S
MRSA11	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA12	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA13	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA14	R	S	R	S	R	R	R	S	R	S	R	R	S
MRSA15	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA16	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA17	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA18	R	S	R	S	R	R	R	S	R	S	S	R	S
MRSA19	R	S	S	S	S	S	S	S	S	S	S	S	S
MRSA20	R	S	S	S	S	S	S	S	S	S	S	S	S
MRSA21	R	S	S	S	S	S	S	S	S	S	S	R	S
MRSA22	R	S	S	S	R	S	R	S	S	S	S	I	S
MRSA23	R	S	S	S	R	R	R	S	R	S	S	R	S
MRSA24	R	S	S	S	R	S	R	S	S	S	S	S	S
MRSA25	R	S	S	S	R	S	R	S	S	S	S	S	S
MRSA26	R	S	S	S	R	S	R	S	S	S	S	S	S
Sum of R	26	5	17	4	22	19	21	0	18	0	1	17	0

Note：S: sensitive; R: resistant; I: intermediate; OXA: oxacillin; GEN: gentamicin; LEV: levofloxacin; AMI: amikacin; CLI: clindamycin; CIP: ciprofloxacin; ERY: erythromycin; LZD: linezolide; MXF: moxifloxacin; RFP: rifampicin; SMZ: sulfamethoxazole; TET: tetracycline; VAN: vancomycin

Table 2. Bacteriostatic effect of BBR and 3 antibiotics on MRSA

Antimicrobials	MIC of control strains (μg/mL)	MIC of 26 clinical isolates (μg/mL)				[a]Resistant/total (%Resistant)
	USA300	Range		50%	90%	
		[a]S	[a]R			
BBR	128	32-256		64	128	[b]N
GEN	>512	0.125-4	16-64	0.5	32	5/26(84.6%)
LEV	>512	0.125-0.5	8-256	16	256	17/26(34.6%)
AMI	2	0.125-2	64-256	0.5	128	6/26（76.9%）

Note: [a] According to the 2017 version of CLSI: GEN: ≥ 16μg/mL is Resistant; LEV: ≥ 4 μg/mL is resistant; AMI: ≥ 64 μg/mL is resistant; S: sensitive strains; R: resistant strains. [b] N: not applicable.

Table 3. Bacteriostatic effect of BBR combined with antibiotics on MRSA

crobial ination	Range of FICI	Median of FICI	Checkerboard effect/strain			
			synergistic	additive	indifferent	antagonistic
-GEN	0.53-1.06	0.56	0	4	1	0
-LEV	0.62-1.5	1.125	0	3	14	0
-AMI	0.16-1.25	0.26	4	1	1	0

Note: FICI ≤ 0.5, 0.5 < FICI ≤1, 1 < FICI ≤2, 2 ≤ FICI represented synergistic, additive, indifferent and antagonistic effect, respectively.

Table 4. Effect of BBR on USA300 at different concentrations and analysis of differential genes

	gene symbol	log2FoldChange (B vs A)	log2FoldChange (B vs C)	p-value (B vs A)	p-value (B vs C)	Description
2249	*ssaA*	3.42	3.45	5.86E-16	9.62E-15	secretory antigen precursor *ssaA*
0270	*lytM*	1.66	1.45	1.05E-06	8.34E-06	peptidoglycan hydrolase
1753	*splF*	-2.73	-3.16	7.71E-28	4.34E-39	serine protease *SplF*
1754	*splE*	-2.63	-3.03	1.75E-25	1.16E-30	serine protease *SplE*
1755	*splD*	-3.16	-3.62	1.29E-21	9.67E-21	serine protease *SplD*
1756	*splC*	-3.16	-3.60	2.11E-34	2.44E-50	serine protease *SplC*
1757	*splB*	-2.93	-3.01	3.87E-23	4.03E-21	serine protease *SplB*

Note: A: Normal control group, B: High concentration group (1/2 MIC, 64 μg/mL)，C: Low concentration group (1/8 MIC, 16 μg/mL).

Supplementary Table 1. MIC of BBR combined with GEN on MRSA

ID	BBR MIC（ug/mL）		GEN MIC (ug/mL)		FIC	Effect
	S MIC	C MIC	S MIC	C MIC		
MRSA02	128	64	64	32	1	additive
MRSA11	256	16	16	8	0.56	additive
MRSA13	256	8	16	8	0.53	additive
MRSA15	256	8	32	16	0.53	additive
MRSA18	64	64	32	2	1.06	indifferent

Note: S MIC: single MIC, C MIC: combined MIC

Supplementary Table 2. MIC of BBR combined with LEV on MRSA

ID	BBR MIC（ug/mL）		LEV MIC（ug/mL）		FIC	Effect
	S MIC	C MIC	S MIC	C MIC		
MRSA02	256	32	256	256	1.125	indifferent
MRSA03	64	32	16	16	1.5	indifferent
MRSA04	64	32	16	16	1.5	indifferent
MRSA05	128	32	16	16	1.25	indifferent
MRSA06	64	32	16	16	1.5	indifferent
MRSA07	128	32	256	256	1.25	indifferent
MRSA10	128	32	256	256	1.25	indifferent
MRSA11	256	32	256	256	1.125	indifferent
MRSA15	128	16	256	256	1.125	indifferent
MRSA16	64	16	32	2	0.75	additive
MRSA17	128	16	256	128	0.62	additive
MRSA18	64	16	256	256	1.25	indifferent
MRSA19	64	16	256	128	0.75	additive
MRSA20	64	16	32	32	1.25	indifferent
MRSA21	128	16	256	256	1.125	indifferent
MRSA22	128	16	256	256	1.125	indifferent
MRSA24	128	2	8	8	1.02	indifferent

Note: S MIC: single MIC, C MIC: combined MIC

Supplementary Table 3. MIC of BBR combined with AMI on MRSA

ID	BBR MIC(ug/mL)		AMI MIC（ug/mL）		FIC	Effect
	S MIC	C MIC	S MIC	C MIC		
MRSA02	256	4	128	2	0.26	synergistic
MRSA03	32	4	64	1	0.25	synergistic
MRSA05	32	4	256	1	0.16	synergistic
MRSA15	128	16	64	2	0.62	additive
MRSA18	64	16	64	64	1.25	indifferent
MRSA19	64	16	128	1	0.31	synergistic

Note: S MIC: single MIC, C MIC: combined MIC

Figures

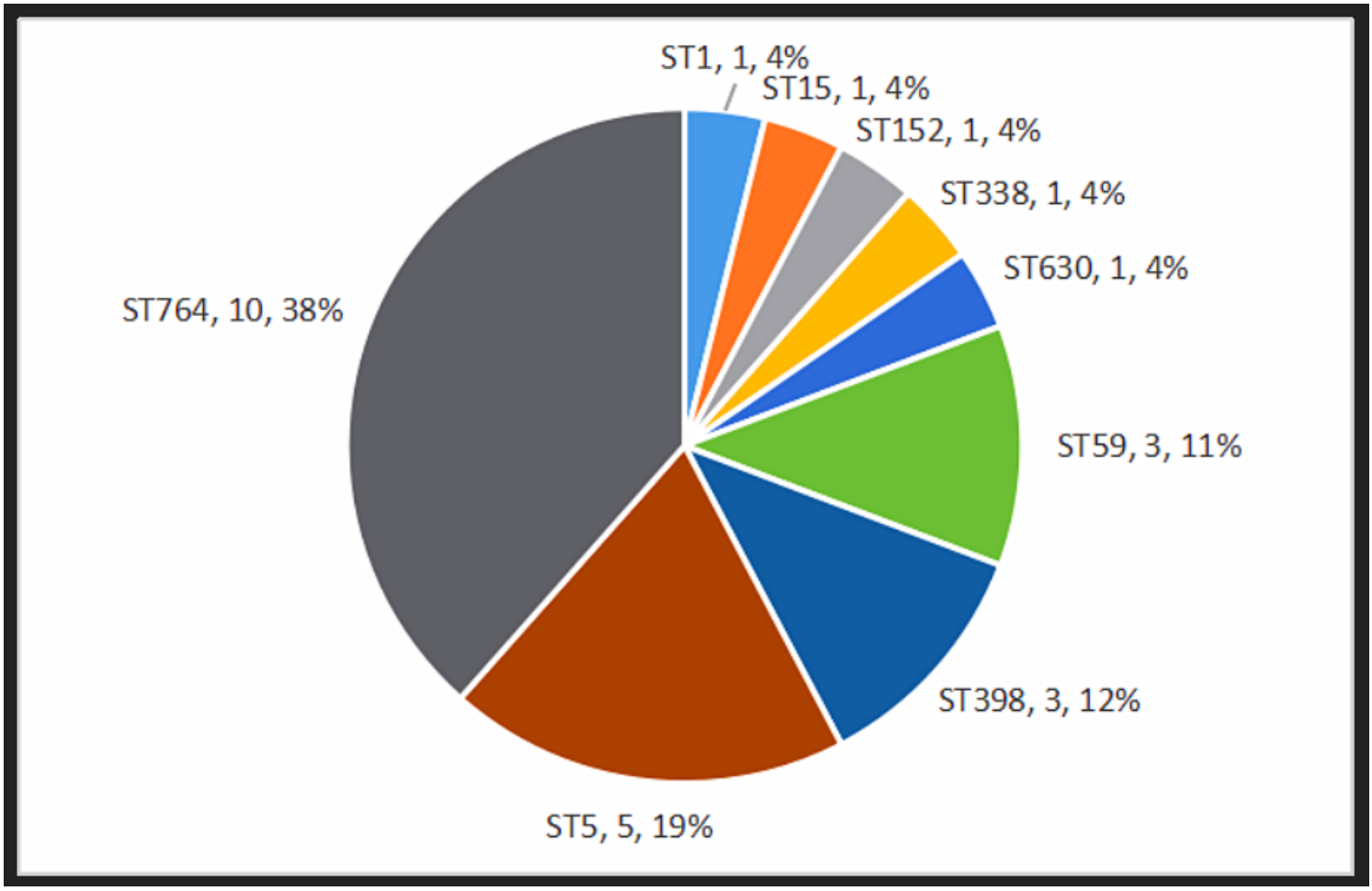

Figure 1

All clinical isolated MRSA were classified by MLST. Totally 9 different ST strains were identified in 26 isolates (Fig.1). The most common was ST764 (38.5%, 10/26), followed by ST5 (19.2%, 5/26), ST59 (11.5%, 3/26), ST398 (11.5%, 3/26), and one strain (3.8%) for ST1, ST15, ST152, ST338 and ST630.

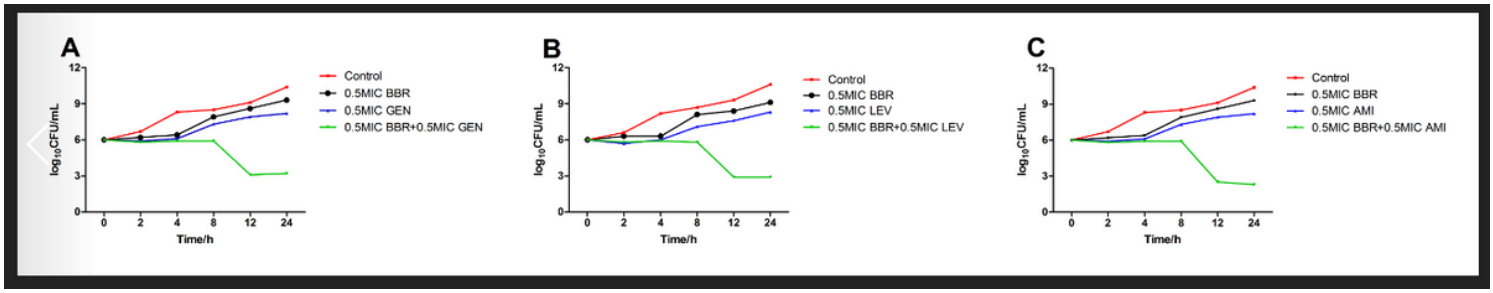

Figure 2

Time-kill curve of BBR combined with 3 antibiotics on MRSA02; A: bacteriostatic effect of BBR combined with GEN on MRSA02 (MIC of BBR = 64 μg/mL, MIC of GEN = 32 μg/mL). B: bacteriostatic effect of BBR

combined with LEV on MRSA02 (MIC of BBR = 128 μg/mL, MIC of LEV = 128 μg/mL); C: bacteriostatic effect of BBR combined with AMI on MRSA02 (MIC of BBR = 128 μg/mL, MIC of AMI = 128 μg/mL).

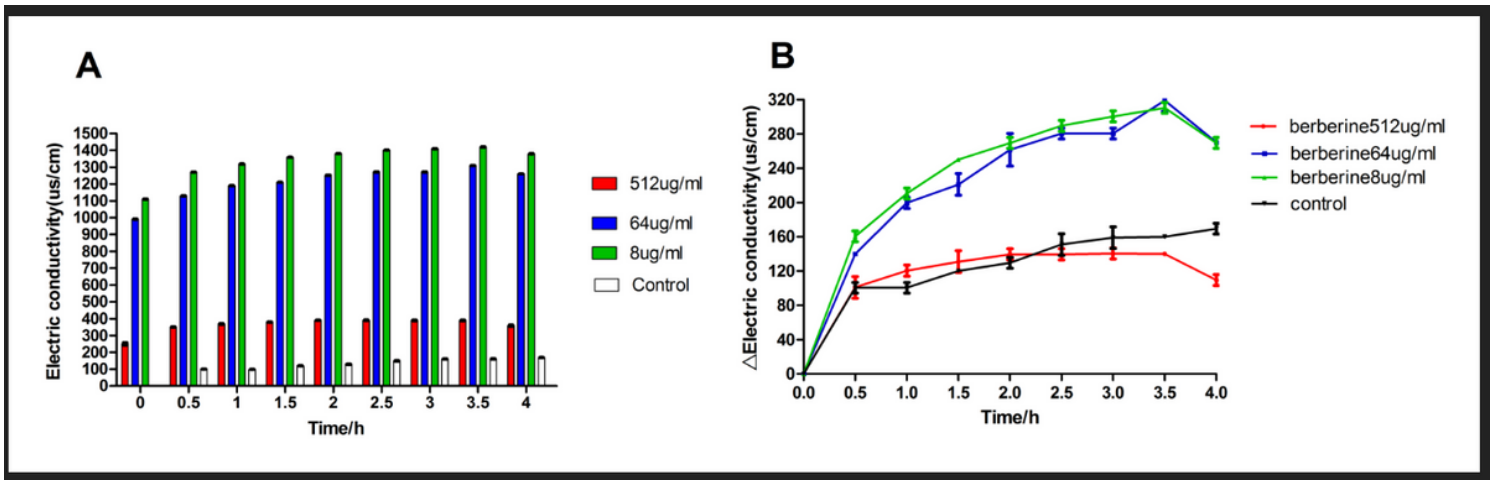

Figure 3

Effect of different concentrations of BBR solution on the conductivity of MRSA02 medium; A: The conductivity of the culture medium increased after the MRSA02 strain was treated with different concentrations of BBR solution; B: After treatment with 512 μg/mL BBR solution for 0.5 h, the conductivity increased by 8.14%, and then did not change significantly with time; C: After treatment with 64 μg/mL BBR for 4 h, the conductivity increased by 13.08%, increasing significantly with time; D: After treatment with 8 μg/mL BBR for 4 hours, the conductivity increased by 12.01%, increasing significantly with time.

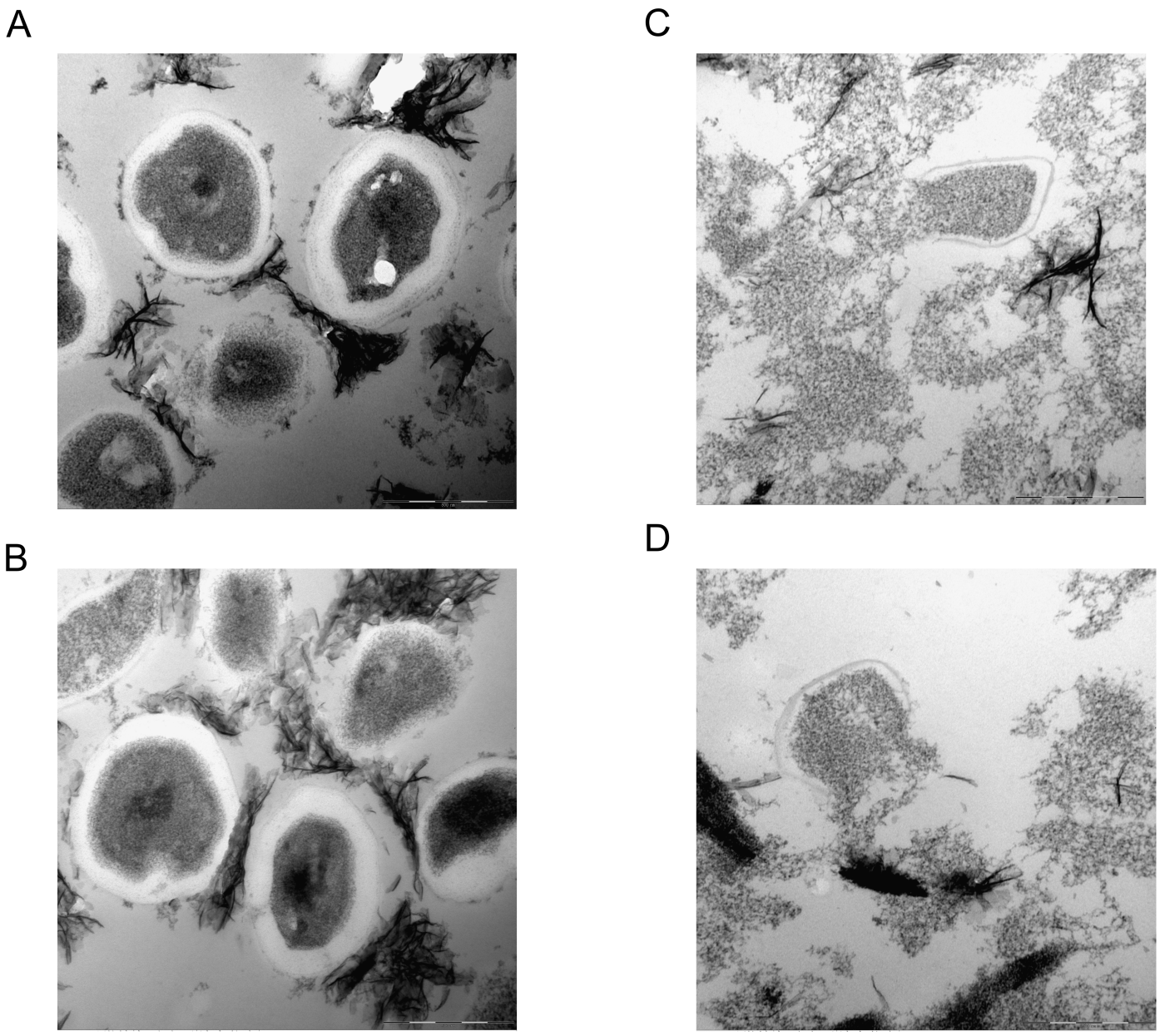

Figure 4

The cell wall of the MRSA02 strain x 80000 times visualized with TEM (Figure 4A-D). A-B: Low concentration (8 μg/mL) of BBR did not significantly damage the cell wall; C-D: High concentration (512 μg/mL) of BBR induced a large amount of cell wall structure destruction, cell lysis, significant leakage of intracellular contents, with consequent bacterial lysis, and a pronounced bactericidal effect. The black substance is BBR crystals.

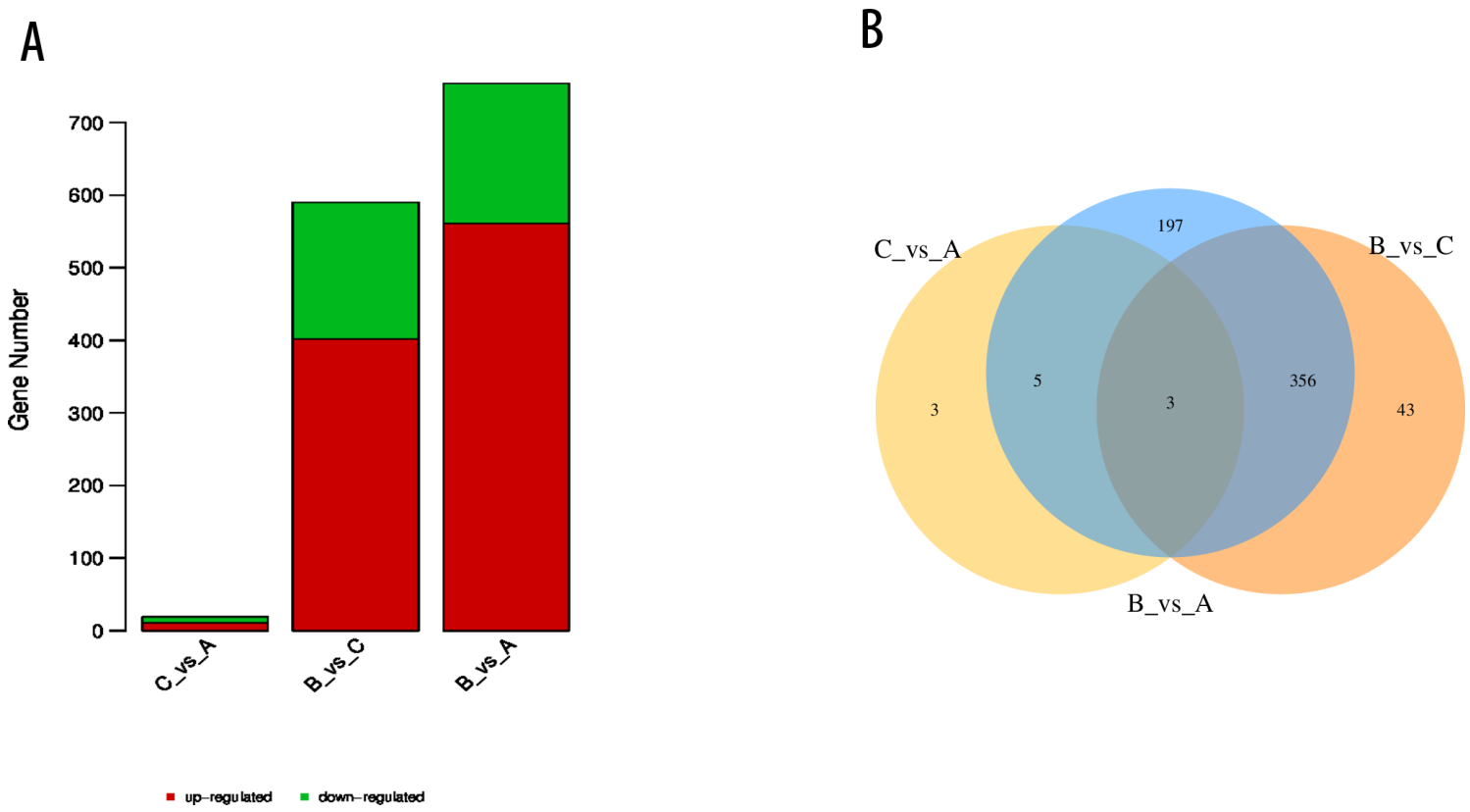

Figure 5

A: Statistical histogram of USA300 expression difference analysis. The horizontal axis is the group name, and the vertical axis is the number of up-down differential genes. Green represents down-regulated gene and red represents up-regulated gene. B: The Wayne Map of differential denes. Different groups are represented by different colors. Figures in the figure represent the number of differentially expressed genes that are specific or common. Overlapping region represents the number of differentially expressed genes shared by different groups, while non-overlapping region represents the number of differentially expressed genes unique to different comparison groups. A: Normal control group, B: High concentration group (1/2 MIC, 64 μg/mL)，C: Low concentration group (1/8 MIC, 16 μg/mL).

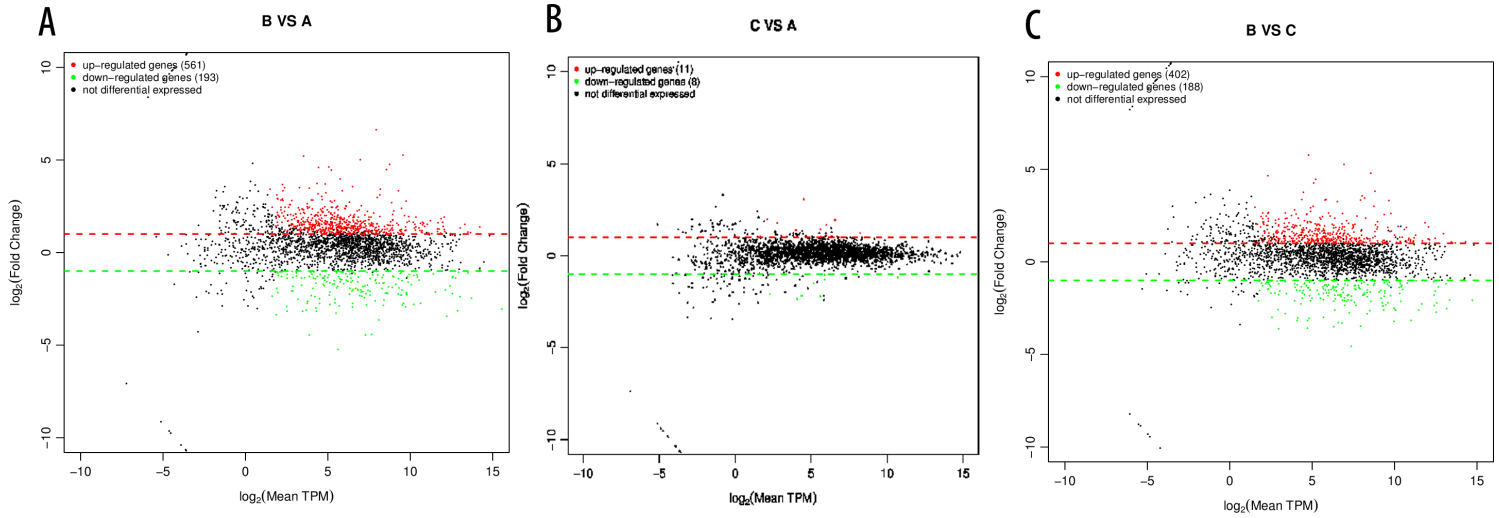

Figure 6

A, B and C groups expressed different MA maps. The horizontal axis is the log (TPM) mean of the two groups of samples, and the vertical axis is the log (Fold change) value. Each point in the graph represents a gene, where red represents an up-regulated gene, green represents a down-regulated gene, and black represents a non-differentiated gene. A: Normal control group, B: High concentration group (1/2 MIC, 64 μg/mL)C: Low concentration group (1/8 MIC, 16 μg/mL).

Supplementary Files

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