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MAJOR ARTICLE

Analysis of Genetic Diversity and Antibiotic Options for Clinical *Listeria monocytogenes* Infections in China

Wei Yu,[1] Yicheng Huang,[2] Chaoqun Ying,[1] Yanzi Zhou,[1] Li Zhang,[1] Jiajie Zhang,[2] Yingsha Chen,[1] and Yunqing Qiu[1]

[1]State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang Provincial Key Laboratory for Drug Clinical Research and Evaluation, The First Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, China, [2]Department of Infectious Diseases, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, China

Background. The aim of this study was to investigate the mechanism of *Listeria monocytogenes* (Lm) pathogenicity and resistance. In addition, the effect of existing treatment options against Lm were systematically evaluated.

Methods. Six Lm isolates were collected and antimicrobial susceptibility testing of 15 antibiotics were done. Subsequently, whole genome sequencing and bioinformatics analysis were performed. Biofilm formation was evaluated by crystal violet staining. Furthermore, the effect of meropenem, linezolid, penicillin, vancomycin, and trimethoprim/sulfamethoxazole were determined using the time-kill assay.

Results. Four sequence types (STs) were identified (ST1, ST3, ST87, ST451). Multivirulence-locus sequence typing results classified ST87 isolates into cluster. All isolates were resistant to fosfomycin and daptomycin with *fosX* and *mprF*. In addition, a total of 80 virulence genes were detected and 72 genes were found in all 6 isolates. Seven genes associated with hemolysin were found in 26530 and 115423. However, due to lack of one genomic island including virulence genes related to flagellar synthesis, isolate 115423 produced less biofilm than 5 other isolates. Although all isolates were susceptible to vancomycin, the in vitro time-kill assay showed that vancomycin monotherapy resulted in less than 2 $\log_{10}$ cerebrospinal fluid (CFU)/mL compared with the initial count. Trimethoprim/sulfamethoxazole at serum or CFU concentrations had bactericidal effect against tested Lm strains at 24 hours.

Conclusions. ST87 clone was a typical prevalent ST in clinical Lm isolates in China. Trimethoprim/sulfamethoxazole might be greater potential therapeutic option against Lm infections.

Keywords. bactericidal effect; resistance mechanism; trimethoprim/sulfamethoxazole; virulent factors.

Listeria monocytogenes (Lm) is one of the most serious foodborne diseases, including a noninvasive type and an invasive type of listeriosis. According to the World Health Organization data, the incidence of Lm infections is 0.1 to 10 cases per 1 million people per year depending on different countries and regions of the world [1]. Recent largest outbreak of listeriosis was reported in South Africa from January 2017 to March 2018 [2]. In 2014, the Centers for Disease Control and Prevention (CDC) surveillance data showed that 23% of patients with invasive listeriosis died, and most isolates were from blood (81%) or cerebrospinal fluid (CSF) (13%) [3]. In China, no outbreak of listeriosis have been reported so far [4]. Therefore, the information on Lm infections is limited among the Chinese population.

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Correspondence: YQ Qiu, MD, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, National Clinical Research Center for Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Zhejiang Provincial Key Laboratory for Drug Clinical Research and Evaluation, The First Affiliated Hospital, Zhejiang University School of Medicine, No.79 Qingchun Road, Hangzhou, China (qiuyq@zju.edu.cn).

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The key to the pathogenesis of Lm is associated with virulence factors [5]. The therapeutic guidelines for Lm are not evident based on randomized clinical trials due to scattered cases in clinics. Antibiotics, as key factors influencing the prognosis, is a vital part of treatment. Ampicillin or penicillin (PEN) along with aminoglycosides are used as the first choice; however, these antibiotics delayed bactericidal activity in vitro at levels that are obtainable in the CSF [6–8]. Moreover, meropenem (MEM), linezolid (LNZ), vancomycin (VAN), and trimethoprim/sulfamethoxazole (TMP/SMX) had a favorable effect on Lm infections as well [9–11]. It is unfortunate that comprehensive evaluation and comparison of therapy data are quite limited. Therefore, the aim of this study was to assess the genomic profiles of Lm and examined in vitro time-kill assays to assess antibacterial effect.

METHODS

Collection of Bacterial Strains

Six Lm isolates (23949, 26530, 34096, 112555, 115423, and 117437) were collected from patients hospitalized at The First Affiliated Hospital, Zhejiang University School of Medicine. The bacterial species were identified with API Listeria (BioMérieux, Marcy l'Etoile, France).

Antibiotic Susceptibility Test

The minimum inhibitory concentrations (MICs) for erythromycin, levofloxacin, moxifloxacin, tetracycline, rifampin, amikacin, clindamycin, fosfomycin, PEN, MEM, LNZ, VAN, and TMP/SMX were determined by agar dilution method, and the susceptibility to tigecycline and daptomycin was tested by the broth dilution according to Clinical and Laboratory Standards Institute (CLSI) recommendations [12]. The control strain *Streptococcus pneumoniae* ATCC 49619 was included. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoint recommendations were chosen for erythromycin, PEN, MEM, VAN, and TMP/SMX. The results for other antibiotics were interpreted according to *Staphylococcus* spp by EUCAST criteria [13].

Genome Sequencing and Data Analysis

Genomic deoxyribonucleic acid (DNA) was extracted by QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). Whole genome sequencing was performed on the Illumina HiSeq PE150 platform at the Beijing Novogene Bioinformatics Technology Co., Ltd. All good-quality paired reads were assembled using the SOAP de novo into several scaffolds. The pathogenicity was performed using Pathogen Host Interactions (PHI) [14]. The resistance genes and virulence genes were identified by VFDB (Virulence Factors Database) and ARDB (Antibiotic Resistance Genes Database) [15, 16]. The genomic island analysis was carried out using IslandPath-DIOMB (https://github.com/brinkmanlab/islandpath). The sequencing data for Lm has been deposited at GenBank under the accessions numbers WJRX00000000, WJRY00000000, WJRZ00000000, JACXAW000000000, JACXAX000000000, and JACXAY000000000 (the data will be released after publication).

Multivirulence-Locus Sequence Typing Comparisons

Six Lm in the present study and 40 publicly available clinical Lm genomes (Supplementary Table 1) from China were analyzed by multivirulence-locus sequence typing (MVLST) using 6 genes (*prfA*, *inlB*, *inlC*, *dal*, *clpP*, and *lisR*) [17]. Multiple sequence alignments were performed using MEGA6 [18]. The resulting consensus tree was visualized and edited using the Interactive Tree of Life (iTOL) [19].

Anti-Biofilm Formation Testing

All isolates were inoculated into 96-well polystyrene microtiter plates containing brain-heart infusion medium and 3% (v/v) glucose for 24 hours, 48 hours, and 72 hours. After static incubation, plates were washed with 0.9% saline and stained with 1% crystal violet (CV) for 20 minutes. The CV was then dissolved in absolute alcohol, and the absorbance was tested using a plate reader at 570 nm.

Time-Kill Assays

The bactericidal activity of 5 drugs (MEM, LNZ, PEN, VAN, and TMP/SMX) against 6 isolates was determined using the time-kill method described in the CLSI guidelines [20]. The following concentrations referring to human body pharmacokinetics (Supplementary Table 2) were used for serum and CSF concentrations: MEM 14.6 mg/L and 1.1 mg/L [21, 22], LNZ 4 mg/L and 1.8 mg/L [23], PEN 21 mg/L and 0.56 mg/L [24], VAN 13.32 mg/L and 10.64 mg/L [25], and TMP/SMX 1.3/48.3 mg/L and 0.2/5.9 mg/L [26]. The time-kill assays were done and interpreted as described previously [22].

RESULTS

Antimicrobial Susceptibility and Sequence Types

Antimicrobial susceptibility tests demonstrated that 6 isolates were widely susceptible to clinically relevant antibiotics against Gram-positive bacteria, except for fosfomycin (MIC >128 mg/L) and daptomycin (MIC = 8 mg/L) (Table 1).

Five strains were isolated from blood and 1 strain, 26530, was isolated from CSF. Multilocus sequence typing revealed 4 different sequence types (STs): (1) ST87 for isolates 23949, 34096, and 117437, (2) ST3 for isolate 26530, (3) ST451 for isolate 112555, and (4) ST1 for isolate 115423, respectively. The MVLST based on 6 genes revealed 3 main clusters supported by bootstrap values of 97, 100, and 100, respectively. Cluster III contained all ST87 isolates (Figure 1).

Antibiotic Resistance Mechanism of *Listeria monocytogenes*

The resistant genes *fosX*, *mprF*, *vanZ*, *norB*, and *vgaALC* were identified in all isolates. The gene *fosX* conferred intrinsic resistance to fosfomycin in Lm. MprF was linked to daptomycin resistance. VanZ was associated with glycopeptide antibiotics resistance, whereas all isolates were susceptible to vancomycin. NorB and VgaALC belonged to the efflux pump complex. The genes *fosX*, *mprF*, and *vanZ* were in the same contig. In addition, *vanZ* and *mprF* were downstream genes of *fosX* (Figure 2). Furthermore, site-specific DNA recombinase and gene related to DUF3883 domain-containing protein were found in the upstream of *fosX* in 26530.

Characteristics of Pathogenicity

There are 4 PHI phenotypes, including hypervirulence, loss of pathogenicity, reduced virulence, and unaffected pathogenicity. The gene *gshF* (PHI:3652) mutant led to a loss of pathogenicity phenotype in 23949, 26530, 34096, and 112555. The majority of phenotypes are reduced virulence. In addition, deletion of 2 more genes, *cadA* (PHI:7386) and *cadC* (PHI:7387), in isolate 26530 resulted in a reduced-virulence phenotype as well.

Table 1. Minimum Inhibitory Concentrations of 15 Antimicrobial Agents Against 6 Lm

Antibiotics	23949	26530	34096	112555	115423	117437
Penicillin[a]	0.5	0.5	0.5	2	0.5	0.5
Meropenem[a]	0.25	0.25	0.25	0.25	0.5	0.5
Erythromycin[a]	0.125	0.125	0.125	0.25	0.125	0.125
Levofloxacin[b]	1	0.5	1	1	1	1
Moxifloxacin[b]	0.5	0.25	0.5	0.5	0.5	0.5
Tetracycline[b]	0.5	0.5	0.5	0.5	0.25	0.25
Linezolid[b]	1	1	1	2	0.5	1
Vancomycin[a]	1	1	1	0.5	0.25	1
Rifampin[b]	0.03	0.03	0.03	0.125	0.125	0.125
Daptomycin[b]	8	8	8	8	8	8
Tigecycline[b]	0.25	0.25	0.25	0.5	0.25	0.25
Amikacin[b]	2	2	2	2	2	8
Trimethoprim-sulfamethoxazole[a]	0.0625/1.1875	0.0625/1.1875	0.0625/1.1875	0.016/0.304	0.032/0.608	0.008/0.152
Clindamycin[b]	0.5	0.5	0.5	0.25	0.25	2

Abbreviations: Lm, *Listeria monocytogenes*.

[a]Breakpoints for Lm.

[b]Breakpoints for *Staphylococcus* spp due to missing breakpoints for Lm.

There were 80 virulence genes detected and 72 genes were found in all 6 isolates (Supplementary Table 3). All isolates were positive for 26 genes participating in the structure (*flaA-E*, *flaG*, *flaK*, *flaL*, *fliD-F*, *fliH*, *fliI*, and *fliS*), biosynthesis (*flhA*, *flhB*, *flhF*, and *fliP-R*), and motor switch (*fliG*, *fliM*, *lmo0693*, *lmo0698*, *lmo0700*, and *motA*) of flagella. The other virulence genes were

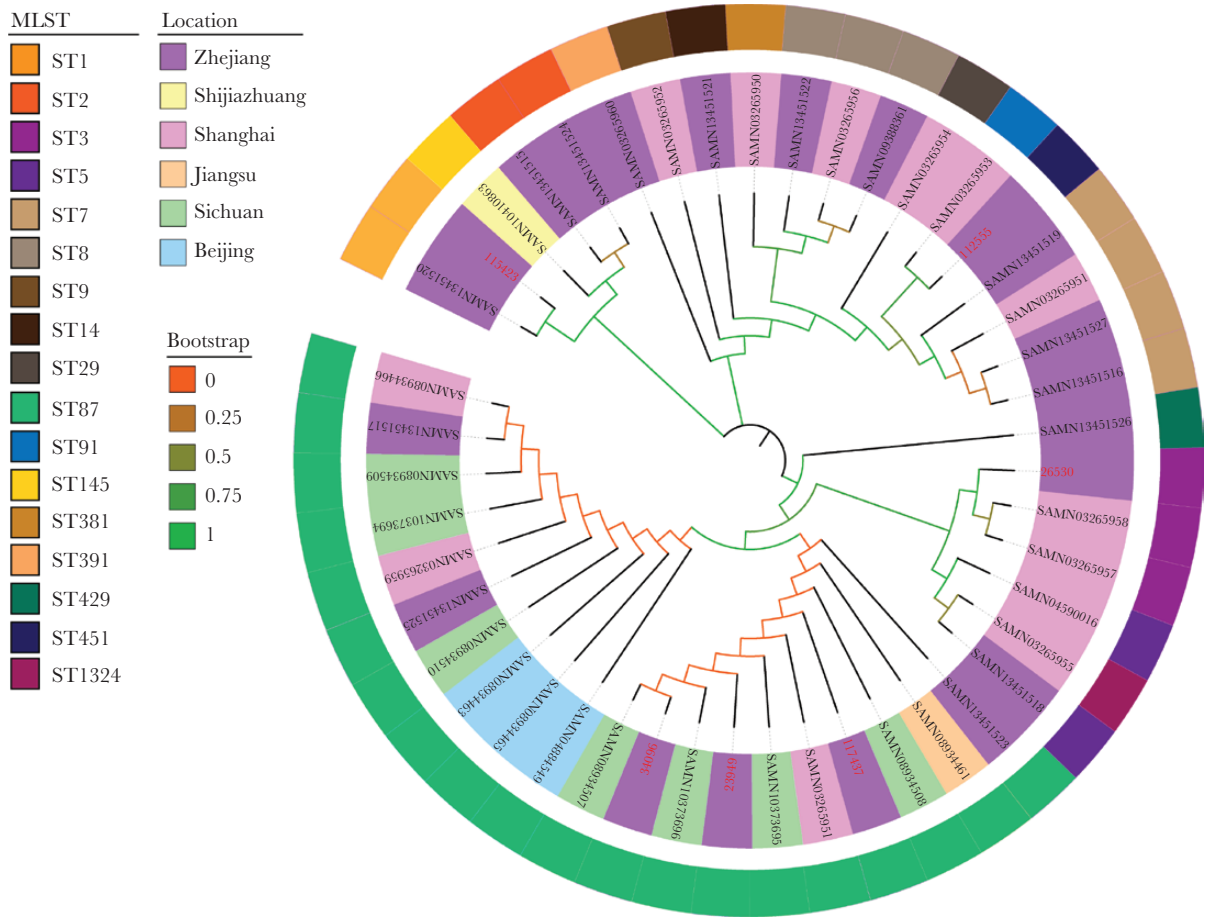

Figure 1. Unrooted maximum likelihood tree of 46 clinical Listeria monocytogenes isolates from China based on multivirulence-locus sequence typing comparisons.

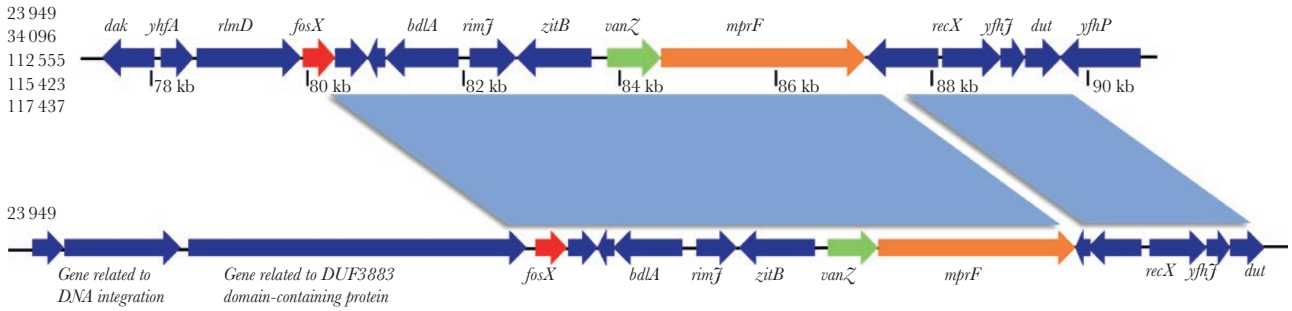

Figure 2. Schematic diagram of the genetic environment of the *fosX* and *mprF* gene in this study. The arrows represent the positions and direction of the elements.

primarily involved with chemotaxis, protease, internalin, and metabolism, playing an important role in adhesion, invasion, inhibition of innate immune response, and autophagy evasion. It is of note that 8 genes (*inlJ*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX*, and *llsY*) were found in 26530 isolated from CSF and 115423 isolated from blood, 7 of which were associated with hemolysin.

Biofilm Formation

The tendency of biofilm formation increased with the time in all isolates (Supplementary Figure 1). However, isolate 115423 produced less biofilm than 5 other isolates, especially at 48 hours and 72 hours, perhaps owing to lack of 1 genomic island including virulence genes (*flgB*, *flgC*, *flgL*, *flgK*, *fliD-H*, *flil*, and *fliS*) related to flagellar formation (Supplementary Figure 2).

Bacterial Time-Kill Effect

The growth and kill patterns of 6 Lm isolates cultured with 5 antibiotics at serum and CSF concentrations are shown in Figure 3. Trimethoprim/sulfamethoxazole can decrease the bacterial load >3.5 $\log_{10}$ colony-forming units (CFU)/mL compared with the initial count at both serum and CSF concentrations and showed bactericidal activity against the 6 isolates at 24 hours. Of note, for PEN, VAN, LNZ, and MEM monotherapy at CSF concentrations against isolate 26530, regrowth was observed after 12 hours (Figure 3b and e). Except for 117437, the antibacterial effects of PEN, VAN, LNZ, and MEM at serum concentrations were better than these drugs at CSF concentrations. In addition, PEN at serum concentration showed bactericidal activity (>3 $\log_{10}$ CFU/mL) against the 4 strains (23949, 34096, 112555, and 115423). However, this effect has not been achieved at CSF concentration. In addition, although all isolates were susceptible to VAN, VAN monotherapy resulted in less than 2 $\log_{10}$ CFU/mL compared with the initial count. Thus, TMP/SMX showed more antibacterial activity than other antibiotics.

DISCUSSION

Listeria monocytogenes isolates could cause severe infections, such as septicemia and meningitis [2]. Although listeriosis is rare, the high mortality rate associated with this infection makes it a significant public health concern [27]. It is unfortunate that few randomized clinical trials focus on the system assessment for treatment options. In present experiments, we found that the ST87 clone was a common ST in clinical Lm isolates in China. Although all isolates were susceptible to VAN, the effect of VAN was still unsatisfactory. In addition, the strain 26530 isolated from CSF existed gene recombination phenomena in the upstream of fosX, affecting bactericidal efficacy antibiotics. It is fortunate that the antibacterial effect of TMP/SMX was more distinctive than others antibiotics in vitro.

There are differences in prevalence of Lm clones among different regions and different sources [28]. Based on the MVLST results, ST87 clustered in the same lineage. Previous studies showed that the 3 most frequent STs among humans in Austria were ST1, ST155, and ST451, whereas ST87 was the most common in China [29, 30].

Virulence factors usually were the main pathogenicity for Lm infections. There were 72 virulence genes found in 6 isolates, which participated in different stages of pathogenesis. *Listeria monocytogenes* could enter host cells mediated by binding of the bacterial InlA protein to E-cadherin or InlB protein to MET receptor tyrosine kinase at the host cell plasma membrane at the host cell plasma membrane [31]. Based on in vitro studies, InlA and InlB are needed for crossing the blood-CSF barrier [32]. However, in our study, 6 isolates were only identified in the *inlB* gene. This might have been due to different pathogenesis by a different signaling pathway. In addition to internalin, many other virulence factors are also involved in the Lm infection cycle. A feature of highly virulent strains is their ability to lyse red blood cells by secreting hemolysins [33]. Yin et al [34] reported a hybrid sublineage of Lm comprising hypervirulent isolates, harboring both the Lm Pathogenicity Island (LIPI)-1 and a truncated LIPI-2 locus. Eight genes (*inlJ*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX*, and *llsY*) were found in 26530 isolated from CSF and 115423 isolated from blood, 7 of which were associated with hemolysin. However, the biofilm formation ability of isolate 115423 was less than 5 other isolates. In further genomic islands analysis, researchers found that isolate 115423 lacked 1 genomic

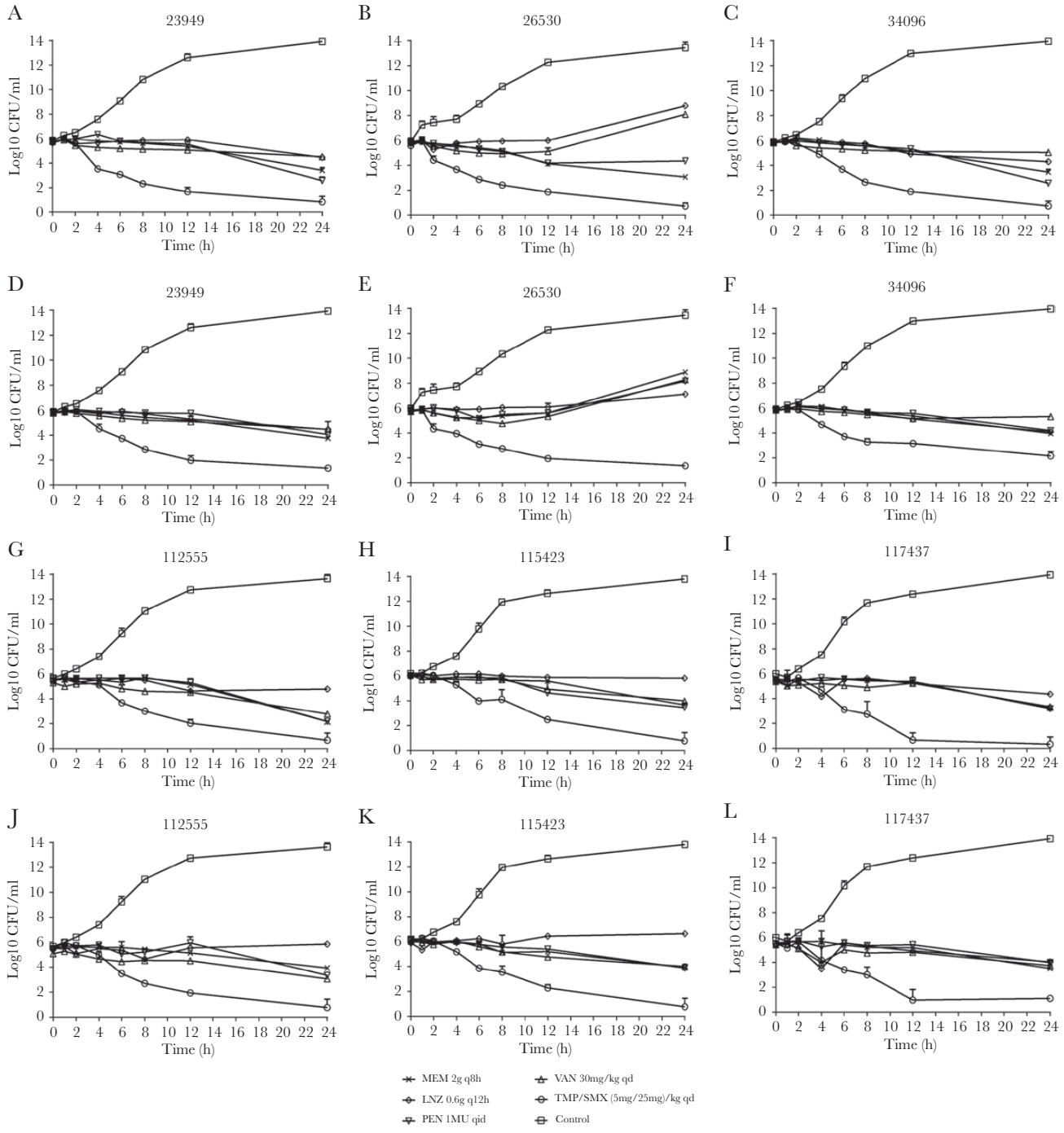

Figure 3. In vitro time-kill assays using serum and cerebrospinal fluid (CSF) concentrations of meropenem, linezolid, penicillin, vancomycin, and trimethoprim/sulfamethoxazole. (a) and (d) The 5 antibiotics at serum and CSF concentrations against isolate 23949, respectively; (b) and (e) the 5 antibiotics at serum and CSF concentrations against isolate 26530, respectively; (c) and (f) the 5 antibiotics at serum and CSF concentrations against isolate 34096, respectively; (g) and (j) the 5 antibiotics at serum and CSF concentrations against isolate 112555, respectively; (h) and (k) the 5 antibiotics at serum and CSF concentrations against isolate 115423, respectively; (i) and (l) the 5 antibiotics at serum and CSF concentrations against isolate 117437, respectively. Antibiotic concentrations are denoted by different symbols. LNZ, linezolid; MEM, meropenem; PEN, penicillin; TMP/SMX, trimethoprim/sulfamethoxazole; VAN, vancomycin.

island, including virulence genes related to flagellar synthesis. Previous studies demonstrated that flagellum-mediated motility could assist adherence to surfaces and differentiation into biofilms [35, 36].

In the present study, except for fosfomycin and daptomycin, the antibiotic resistance of clinical Lm remains low. The resistant genes *fosX*, *mprF*, and *vanZ* in the same contig were identified in all isolates. *vanZ* and *mprF* were downstream genes of *fosX*.

Listeria monocytogenes are intrinsically resistant to cephalosporins and fosfomycin [37]. FosX, as the fosfomycin resistance protein, catalyzes the hydration of fosfomycin. Previous studies showed that *fosX*-mediated resistance could be suppressed by *hpt* and *prfA* [38]. In addition, Scortti et al [38] suggested that Lm isolates could become susceptible to fosfomycin despite that *fosX* confers high-level resistance. Although *hpt* and *prfA* were identified, all isolates in our study were resistant to fosfomycin in vitro.

As reported previously, a high daptomycin MIC was observed in all isolates [39]. Daptomycin resistance has already been described in *Staphylococcus* spp and *Enterococcus* spp to involve certain genes (*mprF*, *yycG*, *yycH*, *dltABCD*, *rpoB*, *rpoC*, *vraSR*, and *graSR*), acquired mutations that have homologs in Lm [40]. It is notable that *mprF* is the most frequently described mutation in clinical isolates, including in our present study [41]. In addition, two efflux pumps genes (*norB* and *vgaALC*) were identified as well, resulting in the resistance by active export of antibiotics. Thus, additional research would be needed to assess the clinical efficacy and safety of current available antibiotics.

In general, PEN along with aminoglycosides is generally considered the preferred agent for treatment of listeriosis [7]. However, PEN, VAN, and imipenem have demonstrated delayed in vitro bactericidal activity at levels that are obtainable in the CSF [8, 42]. *Listeria monocytogenes* is highly susceptible to MEM in vitro, but data on the efficacy of MEM in clinical cases of listeriosis are scarce. In addition, MEM therapy failure in Lm has been reported [43]. Furthermore, an observational study showed that definitive therapy with MEM against Lm was associated with significantly higher 30-day mortality [44]. Likewise, VAN has been used successfully in a few patients with listeriosis who are allergic to PEN, but other patients have developed listerial meningitis [45–47]. Our study found that the bactericidal activity of VAN was less than 2 $\log_{10}$ CFU/mL, and those may be related to *vanZ*. However, the gene vanZ showed no effect on the phenotype of VAN resistance.

Trimethoprim/sulfamethoxazole is thought to be the best alternative single agent for patients intolerant of PEN as well. Our in vitro data showed that TMP/SMX had more antibacterial activity than PEN, VAN, LNZ, and MEM at both serum and CSF concentrations. Appleman et al [42] found that PEN, VAN, ampicillin, and imipenem with 2 mg/L and 10 mg/L and TMP/SMX with 2/38 mg/L exhibited bactericidal activity for 48 hours. However, for 26530 isolated from CSF, PEN, VAN, LNZ, and MEM monotherapy at CSF concentrations showed regrowth after 12 hours. This is probably because gene recombination was found upstream of *fosX* in 26530. There are many agents that are worthy of further research and exploration of this finding. It is interesting to note that the concentrations for TMP/SMX depended on the clinical therapeutic dose, which was lower than previous studies, and could also achieve durable bactericidal effect. In addition, clinical studies reported that 10 patients were treated with TMP/SMX alone and only 1 died [9, 48]. Together with previous studies, TMP/SMX could be an efficacious and inexpensive therapeutic option.

The study has several limitations, including the relatively small numbers of Lm- and in vitro-relative static time-kill experiments. However, a system evaluation for treatment options is mandatory. Therefore, a further large-scale study is needed for better evaluation of the treatment options, to improve the prognosis of Lm infections.

CONCLUSIONS

In conclusion, clinical Lm infections remained sporadic in China. Virulence factors, associated with flagellar synthesis, could influence biofilm formation. Vancomycin has not yet shown promising antibacterial effect against VAN-sensitive LM. The most interesting observation is that TMP/SMX shows great potential as a therapeutic option for Lm infections. Further investigations and prospective randomized clinical trials will be required to evaluate the clinical cure rates.

Supplementary Data

Supplementary materials are available at Open Forum Infectious Diseases online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Author contributions. W. Y. and Y. Q. developed the concept and designed the experiments. Y. H., C. Y., and Y. Z. isolated bacteria. W. Y., Y. H., L. Z., and J. Z. performed the laboratory measurements. W. Y., Y. H., and Y. C. analyzed the data. Y. Q. gave conceptual advice. W. Y. wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages. All authors have seen and approved the content and fulfill the journal's requirements for authorship.

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