Analysis of genetic diversity and antibiotic options for clinical *Listeria monocytogenes* infections in China

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ABSTRACT

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, trimethoprim/sulfamethoxazole were determined using the time-kill assay.

RESULTS: Four sequence types (STs) were identified (ST1, ST3, ST87, ST451). Multi-virulence-locus sequence typing (MVLST) 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 six isolates. Seven genes associated with haemolysin 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 five other isolates. Even all isolates were susceptible to vancomycin, the *in vitro* time-kill assay showed vancomycin monotherapy resulted in less than 2 log₁₀ CFU/mL compared with the initial count. Trimethoprim/sulfamethoxazole at serum or cerebrospinal fluid concentrations had bactericidal effect against tested Lm strains at 24 h.

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: resistance mechanism; virulent factors; trimethoprim/sulfamethoxazole; bactericidal effect
1. Background

*Listeria monocytogenes* (Lm) is one of the most serious foodborne diseases, including a non-invasive type and an invasive type of listeriosis. According to the World Health Organization (WHO) 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, Centers for Disease Control and Prevention (CDC) surveillance data showed 23% 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.

The key to the pathogenesis of Lm is associated with virulence factors [5]. The therapeutic guidelines for Lm are not evidence based on randomized clinical trials due to scatter 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 the favorable effect on Lm infections as well [9-11]. Unfortunately, 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.

2. Methods

2.1 Collection of bacterial strains

Six Lm isolates (23949, 26530, 34096, 112555, 115423, 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).

2.2 Antibiotic susceptibility test

The minimum inhibitory concentrations (MICs) for erythromycin, levofoxacin, moxifloxacin, tetracycline, rifampin, amikacin, clindamycin, fosfomycin, PEN, MEM, LNZ, VAN, 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 strains *Streptococcus pneumoniae* ATCC 49619 was included. 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].

2.3. Genome sequencing and data analysis

Genomic DNA was extracted by QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Whole-genome sequencing (WGS) 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 (http://soap.genomics.org.cn/soapdenovo.html) into a number of 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 accession of WJRX00000000, WJRY00000000, WJRZ00000000, JACXAW00000000, JACXAX000000000, JACXAY000000000 (the data will be released after publication).

2.4 Multi-virulence-locus sequence typing (MVLST) comparisons

Six Lm in the present study and 40 publicly available clinical Lm genomes (Supplementary Table 1) from China were analyzed MVLST using six 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].

2.5 Anti-biofilm formation testing

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

2.6 Time-kill assays

The bactericidal activity of five drugs (MEM, LNZ, PEN, VAN, TMP/SMX) against six 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], 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].

3. Results

3.1 Antimicrobial susceptibility and STs

Antimicrobial susceptibility tests demonstrated six 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 one strain 26530 was isolated from CSF. Multi-locus sequence typing (MLST) revealed four different STs, ST87 for isolate 23949, 34096 and 117437, ST3 for isolate 26530, ST451 for isolate 112555, ST1 for isolate 115423, respectively. The MVLST based on six genes revealed three main clusters supported by bootstrap values of 97, 100, and 100, respectively. Cluster III contained all ST87 isolates (Figure 1).

3.2 Antibiotic resistance mechanism of Lm

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, while all isolates were susceptible to vancomycin. NorB and VgaALC were belong to 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.
3.3 Characteristics of pathogenicity

There are four 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 two more genes cadA (PHI:7386) and cadC (PHI:7387) in isolate 26530 resulted in a reduced-virulence phenotype as well.

There were 80 virulence genes detected and 72 genes were found in all six isolates (Supplementary Table 3). All isolates were positive for 26 genes participating in the structure (flaA-E, flaG, flaK, flaL, fliD-F, fliH, fliI, fliS), biosynthesis (flhA, flhB, flhF, fliP-R) and motor switch (fliG, fliM, lmo0693, lmo0698, lmo0700, motA) of flagella. The other virulence genes were 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, illsB, illsD, illsG, illsH, illsP, illsX, illsY) were found in 26530 isolated from CSF and 115423 isolated from blood, 7 of which were associated with haemolysin.

3.4 Biofilm formation

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

3.5 Bacterial time-kill effect

The growth and kill patterns of six Lm isolates cultured with five antibiotics at serum and CSF concentrations are shown in Figure 3. TMP/SMX can decrease the bacterial load >3.5 log_{10} CFU/ml compared with the initial count at both serum and CSF concentrations, showed bactericidal activity against the six isolates at 24 h. Of note, for PEN, VAN, LNZ, MEM monotherapy at CSF concentrations against isolate 26530, re-growth was observed after 12 hours (Figure 3b, 3e). Expect of 117437, the antibacterial effects of PEN, VAN, LNZ, 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 four strains (23949, 34096, 112555, 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 others antibiotics.

4. Discussion

Lm 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 as a significant public health concern [27]. Unfortunately, few randomized clinical trials focus on the system assessment for treatment options. In present experiments, we found 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, affecting antibacterial effect as well. Fortunately, 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 study showed the three most frequent STs among the human in Austria were ST1, ST155, and ST451, while ST87 were the most common in China [29-30].

Usually, virulence factors were the main pathogenicity for Lm infetions. There were 72 virulence genes were found in six isolates, participating in different stages of pathogenesis. Lm 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-cerebrospinal fluid barrier [32]. However, in our study, six isolates were only identified inlB gene. This might have been due to different pathogenesis by different signaling pathway. In addition to internalin, many other virulence factors are also involved in the Lm infections cycle. A feature of highly virulent strains is their ability to lyse red blood cells (RBCs) by secreting hemolysins [33]. Yin et al [34] reported a hybrid sub-lineage of Lm comprising hypervirulent isolates, harbouring both the Lm Pathogenicity Island (LIPI)-1 and a truncated LIPI-2 locus. Eight genes (inlJ, llsB, llsD, llsG, llsH, llsP,
llsX, llsY) were found in 26530 isolated from CSF and 115423 isolated from blood, 7 of which were associated with haemolysin. However, the biofilm formation ability of isolate 115423 were less than other five isolates. Further genomic islands analysis found isolate 115423 was lack of one genomic island including virulence genes related to flagellar synthesis. Previous studies demonstrated 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 clinic 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. Lm are intrinsic resistant to cephalosporins and fosfomycin [37]. FosX, as the fosfomycin resistance protein, catalyzes the hydration of fosfomycin. Previous studies showed fosX-mediated resistance could be suppressed by hpt and prfA [38]. In addition, Scortti et al [31] suggested that Lm isolates could become susceptible to fosfomycin despite 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]. Notably, mprF is the most frequently described mutation in clinical isolates including our present study [41]. In addition, norB and vgaALC genes were identified, resulted in the resistance by the action of efflux pumps that actively export the antibiotics. Thus, additional researches 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]. Lm is highly susceptible to MEM in vitro, but data on the efficacy of MEM in clinical cases of listeriosis are scarce. And MEM therapy failure in Lm has been reported [43]. Furthermore, a observational study showed definitive therapy with MEM against Lm were associated with significantly higher 30-day mortality [44]. Similarly, 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 the bactericidal activity of VAN was less than $2 \log_{10}$ CFU/ml, those may be related to vanZ. However, the gene vanZ showed no effect on the phenotype of VAN resistance.

TMP/SMX is thought to be the best alternative single agent for patients intolerant of PEN as well. Our in vitro data showed TMP/SMX had more antibacterial activity than PEN, VAN, LNZ, MEM at both serum and CSF concentrations. Appleman et al [42] found PEN, VAN, ampicillin, 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, MEM monotherapy at CSF concentrations showed re-growth after 12 hours. This is probably because gene recombination was found in the upstream of fosX in 26530. There are many worthy of further research and exploration of this finding. Interestingly, the concentrations for TMP/SMX depended on the clinical therapeutic dose, lower than previous studies, could also achieve durable bactericidal effect. In addition, clinical studies reported ten patients were treated with TMP/SMX alone and only one 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, in order to improve the prognosis of Lm infections.

5. Conclusions

In conclusion, clinical Lm infections remained sporadic in China. Virulence factors, associated with flagellar synthesis, could influence biofilm formation. VAN has not yet shown promising antibacterial effect against VAN-sensitive LM. The most interesting observation is 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.
Abbreviations

Lm, *Listeria monocytogenes*

STs, sequence types

MVLST, multi-virulence-locus sequence typing

CSF, cerebrospinal fluid

WHO, World Health Organization

CDC, Centers for Disease Control and Prevention

PEN, penicillin

MEM, meropenem

LNZ, linezolid

VAN, vancomycin

TMP/SMX, trimethoprim/sulfamethoxazole

MICs, minimum inhibitory concentrations

CLSI, Clinical and Laboratory Standards Institute

EUCAST, European Committee on Antimicrobial Susceptibility Testing

MLST, Multi-locus sequence typing
Declarations

Consent for Publication. All authors have seen and approved the content and fulfil the journal’s requirements for authorship.

Availability of data and material. The Whole Genome Shotgun BioProject has been deposited at GenBank under the accession WJRX00000000, WJRY00000000, WJRX00000000, JACXAW000000000, JACXAX000000000, JACXAY000000000 (the data will be released after publication).

Conflicts of Interest. None.

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Authors' contributions. WY and YQQ developed the concept and designed the experiments. YCH, CQY and YZZ isolated bacteria. WY, YCH, LZ, and JJZ performed the laboratory measurements. WY, YCH and YSC analyzed the data. YQQ gave conceptual advice. WY wrote the paper. All authors discussed the results and implications and commented on the manuscript at all stages.

Patient Consent Statement. We declare no ethical competing interest. In our study, we did not perform any experiments with animals or higher invertebrates, neither performed experiments on humans nor the use of human tissue samples. Our data have been originated from bacteria, not linked to clinical information.

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Figures Legend

**Figure 1.** Unrooted maximum likelihood tree of 46 clinical Lm isolates from China based on multi-virulence-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.

**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 five antibiotics at serum and CSF concentrations against isolate 23949 respectively; (b) and (e) The five antibiotics at serum and CSF concentrations against isolate 26530 respectively; (c) and (f) The five antibiotics at serum and CSF concentrations against isolate 34096 respectively; (g) and (j) The five antibiotics at serum and CSF concentrations against isolate 112555 respectively; (h) and (k) The five antibiotics at serum and CSF concentrations against isolate 115423 respectively; (i) and (l) The five antibiotics at serum and CSF concentrations against isolate 117437 respectively. MEM, meropenem; LNZ, linezolid; PEN, penicillin; VAN, vancomycin; TMP/SMX, trimethoprim/sulfamethoxazole. Antibiotic concentrations are denoted by different symbols.
### Table 1. Minimum inhibitory concentrations of 15 antimicrobial agents against six Lm

| Antibiotics               | 23949 | 26530 | 34096 | 112555 | 115423 | 117437 |
|---------------------------|-------|-------|-------|--------|--------|--------|
| Penicillin<sup>a</sup>    | 0.5   | 0.5   | 0.5   | 2      | 0.5    | 0.5    |
| Meropenem<sup>a</sup>     | 0.25  | 0.25  | 0.25  | 0.25   | 0.5    | 0.5    |
| Erythromycin<sup>a</sup>  | 0.125 | 0.125 | 0.125 | 0.25   | 0.125  | 0.125  |
| Levofloxacin<sup>b</sup>  | 1     | 0.5   | 1     | 1      | 1      | 1      |
| Moxifloxacin<sup>b</sup>  | 0.5   | 0.25  | 0.5   | 0.5    | 0.5    | 0.5    |
| Tetracycline<sup>b</sup>  | 0.5   | 0.5   | 0.5   | 0.25   | 0.25   | 0.25   |
| Linezolid<sup>b</sup>     | 1     | 1     | 1     | 2      | 0.5    | 1      |
| Vancomycin<sup>a</sup>    | 1     | 1     | 1     | 0.5    | 0.25   | 1      |
| Rifampin<sup>b</sup>      | 0.03  | 0.03  | 0.03  | 0.125  | 0.125  | 0.125  |
| Daptomycin<sup>b</sup>    | 8     | 8     | 8     | 8      | 8      | 8      |
| Tigecycline<sup>b</sup>   | 0.25  | 0.25  | 0.25  | 0.5    | 0.25   | 0.25   |
| Amikacin<sup>b</sup>      | 2     | 2     | 2     | 2      | 2      | 2      |
| Trimethoprim-sulfamethoxazole<sup>a</sup> | 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<sup>b</sup>   | 0.5   | 0.5   | 0.5   | 0.25   | 0.25   | 2      |

<sup>a</sup> Breakpoints for Lm.

<sup>b</sup> Breakpoints for *Staphylococcus spp.* due to missing breakpoints for Lm.
Figure 2
