Genetic structure characteristics and treatment for *Listeria monocytogenes* infections

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Abstract

Background: Invasive *Listeria monocytogenes* (Lm) carry a high mortality despite antibiotic treatment. The aim of this study was to investigate the mechanism of pathogenicity and resistance. In addition, the effect of existing treatment options against Lm were systematically evaluated as well.

Methods: Three Lm isolates were collected and 15 antibiotics susceptibility tests were done. Subsequently, whole genome sequencing and bioinformatics analysis were performed. Furthermore, the effect of meropenem, linezolid, benzylpenicillin, vancomycin, trimethoprim/sulfamethoxazole were determined using the time-kill assay.

Results: Two sequence types (STs) were identified for isolate 23949 (ST87), 26530 (ST3), 34096 (ST87), respectively. All isolates were resistant to fosfomycin and daptomycin. The resistant genes *fosX*, *mprF*, *norB* and *vgaALC* were identified in all isolates. Furthermore, 80 virulence genes were detected and 72 genes were found in all three isolates. There were 26 virulent genes associated with the structure, biosynthesis, motor switch of flagellum. And other virulent genes were involved in chemotaxis, protease, internalin and metabolism. It is of note that 8 genes (*inlJ*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX*, *llsY*) were only found in 26530 isolated from cerebrospinal fluid (CSF), 7 of which were associated with haemolysin. Further *in vitro* time-kill assay found trimethoprim/sulfamethoxazole at serum or CSF concentrations had bactericidal effect (>3.5 log10 CFU/ml) against three tested Lm strains at 24 h.

Conclusions: The involved virulence factors were mainly associated with bacterial pathogenicity. Notably, trimethoprim/sulfamethoxazole might be greater potential therapeutic option against Lm bloodstream infection or intracranial infection.

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 the countries and regions of the world [1]. The 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 addition, European Centre for Disease Prevention and Control (ECDC) reported that the increasing trend in the number of listeriosis cases in the European Union, probably partly due to the absolute increased population size of the elderly susceptible population [4], making it a significant public health concern.

The key to the pathogenesis of Lm is associated with virulence factors [5]. Antibiotics, as key factors influencing the prognosis, is a vital part of treatment. Ampicillin or benzylpenicillin (PEN) are used as the first choice, however, these antibiotics delayed bactericidal activity *in vitro* at levels that are obtainable in the CSF [6-7]. Moreover, meropenem (MEM), linezolid (LNZ), vancomycin (VAN) and

trimethoprim/sulfamethoxazole (TMP/SMX) had the favorable effect on Lm infections as well [8-10]. Unfortunately, comprehensive evaluation and comparison of therapy data are quite limited. Therefore, the aim of this study was to assess the pathogenicity and examined *in vitro* time-kill assays to improve clinical treatment.

2. Methods

2.1 Collection of bacterial strains

Three Lm isolates (23949, 26530, 34096) were collected from patients hospitalized at The First Affiliated Hospital of Zhejiang University. The bacterial species were identified by both API20 (bioMérieux, Durham, NC, USA) and MALDI-TOF technique (Bruker Diagnostics, Bremen, Germany).

2.2 Antibiotic susceptibility test

The minimum inhibitory concentrations (MICs) for erythromycin, levofloxacin, 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 [11]. 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 [12].

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 PHI (Pathogen Host Interactions) [13]. The resistance genes and virulence genes were identified by VFDB (Virulence Factors Database) and ARDB (Antibiotic Resistance Genes Database) [14-15].

The Whole Genome Shotgun BioProject for the Lm has been deposited at GenBank under the accession of WJRX00000000, WJRY00000000, WJRZ00000000.

2.4 Time-kill assays

The bactericidal activity of five drugs (MEM, LNZ, PEN, VAN, TMP/SMX) against three isolates was determined using the time-kill method described in the CLSI guidelines [16]. The following concentrations referring to human body pharmacokinetics (Supplemental Table 1) were used for serum and CSF concentrations: MEM 14.6 mg/L and 1.1 mg/L [17-18], LNZ 4 mg/L and 1.8 mg/L [18-19], PEN 21 mg/L

and 0.56 mg/L [20], VAN 13.32 mg/L and 10.64 mg/L [21], TMP/SMX 1.3/48.3 mg/L and 0.2/5.9 mg/L [18,22]. The time-kill assays were done and interpreted as described previously [18].

3. Results

3.1 Antimicrobial susceptibility and STs

Antimicrobial susceptibility tests demonstrated that the three isolates were widely susceptible to clinically-relevant antibiotics against Gram-positive bacteria, except for fosfomycin (MIC 512 mg/L, 128 mg/L, 512 mg/L) and daptomycin (MIC 8 mg/L) (Table 1). However, the MICs of MEM (0.25 mg/L), TMP/SMX (0.0625/1.1875 mg/L), clindamycin (0.5 mg/L) were close to the resistance breakpoints.

Two strains (23949 and 34096) were isolated from blood and one strain 26530 was isolated from CSF. Multi-locus sequence typing (MLST) revealed two different STs for isolate 23949 (ST87), 26530 (ST3), 34096 (ST87), respectively.

3.2 Antibiotic resistance mechanism of Lm

The resistant genes *fosX*, *mprF*, *norB* and *vgaALC* were identified in all isolates. MprF was linked to daptomycin resistance. The gene *fosX* confered intrinsic resistance to fosfomycin in Lm. NorB and VgaALC were belong to efflux pump complex, resulted in antibiotics resistance. It is of note that the genes *fosX* and *mprF* were in the same contigs. In addition, *mprF* was a downstream gene of *fosX* (Figure 1).

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. 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 three isolates (Supplemental Table 2). Three isolates were all 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 flagellum. The other virulence genes were primarily involved with chemotaxis, protease, internalin and metabolism, playing an important role in adhesion and invasion, inhibition of innate immune response, autophagy evasion. It is of note that 8 genes (*inlJ*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX*, *llsY*) were only found in 26530 isolated from CSF, 7 of which were associated with haemolysin.

3.4 Bacterial time-kill effect

The growth and kill patterns of three Lm isolates cultured with five antibiotics at serum and CSF concentrations are shown in Figure 2. TMP/SMX can decrease the bacterial load >3.5 log10 CFU/ml compared with the initial count at both serum and CSF concentrations, showed bactericidal activity

against the three 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 2c, 2d). For isolates 23949 and 34096, 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 log10 CFU/ml) against the two strains. However, this effect has not been achieved at CSF concentration. Thus, TMP/SMX showed more antibacterial activity than others antibiotics.

4. Discussion

Lm can cause severe infections, such as septicemia and meningitis [2]. Although the number of cases of listeriosis is small, the high mortality rate associated with this infection makes it a significant public health concern [24]. Unfortunately, few studies focus on the system assessment for treatment options. In present experiments, we found that virulence factors were the main pathogenicity for Lm, especially for 26530 isolated from CSF. In addition, the antibacterial effect of TMP/SMX was more distinctive than others antibiotics.

The lipopeptide antibiotic daptomycin is a frequently used treatment option for gram-positive microorganisms. However, a high daptomycin MIC, as reported previously, was observed in all three isolates [25-26]. 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 [27]. Notably, *mprF* is the most frequently described mutation in clinical isolates including our present study [28-29]. In addition, *norB* and *vgaALC* genes were identified, resulted in the resistance by the action of efflux pumps that actively export the antibiotics.

Usually, Lm are intrinsic resistant to cephalosporins and fosfomycin [30]. FosX, as the fosfomycin resistance protein, catalyzes the hydration of fosfomycin. There is also evidence that FosX-mediated resistance could be suppressed by *hpt* and *prfA* [31]. The regulon induced increased fosfomycin influx into the bacterial cell upon activation by host signals [31]. Therefore, 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 in our studies as well, they were still resistant to fosfomycin *in vitro*. Thus, additional researches would be needed to assess the clinical efficacy and safety of intrinsic antibiotics.

There were 72 virulence genes were found in all three isolates, participating in the adhesion and invasion, inhibition of innate immune response, autophagy evasion. 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 [32]. Based on *in vitro* studies, InlA and InlB are needed for crossing the blood-cerebrospinal fluid barrier [33]. Gessain *et al* [34] has demonstrated that InlA-dependent entry required PI3K activity but did not activate PI3K/AKT signaling, whereas the interaction of InlB with Met receptor activated the PI3K/AKT signal transduction cascade. However, in our study, three isolates were only identified inlB gene, one of which isolated from CSF. This might have been due to different pathogenesis by different signaling pathway.

In addition to internalin, many other virulence factors are involved in the Lm infections cycle. A feature of highly virulent strains is their ability to lyse red blood cells (RBCs) by secreting hemolysins [35]. Eight genes (*inlJ*, *llsB*, *llsD*, *llsG*, *llsH*, *llsP*, *llsX*, *llsY*) were only found in 26530 isolated from CSF, 7 of which were associated with haemolysin. Yin *et al* [36] reported a hybrid sub-lineage of Lm comprising hypervirulent isolates, harbouring both the Lm Pathogenicity Island (LIPI)-1 and a truncated LIPI-2 locus. They could encode sphingomyelinase (SmcL), a virulence factor required for invasion and bacterial translocation from the gut. The gene *prfA*, as an important switch regulon, was identified in all isolates. It has been proved that PrfA regulated LIPI-1 genes and several genes secreted internalins tightly and regulated motility-associated genes *motA* and *flaA* negatively [37]. It can be seen from this that PrfA regulon were not only involved in resistance but also virulence.

In the present study, except for fosfomycin and daptomycin, clinical isolates of Lm resistant to antibiotics remains low. In general, PEN is generally considered the preferred agent for treatment of listeriosis [6]. However, PEN, VAN, and imipenem have demonstrated delayed in vitro bactericidal activity at levels that are obtainable in the CSF [7,38]. Lm is highly susceptible to MEM in vitro, but data on the efficacy of MEM in clinical cases of listeriosis are scarce. It is of note that MEM therapy failure in Lm has been reported [39]. Furthermore, one observational study showed definitive therapy with MEM against Lm were associated with significantly higher 30-day mortality [40]. Similarly, VAN has been used successfully in a few patients with listeriosis who are allergic to PEN, but other patients have developed listerial meningitis [41-43].

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* [38] 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. 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 [8,44]. Together with previous studies, we suggest that 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, this is a system evaluation for treatment options, which is valuable to study more.

5. Conclusions

In conclusion, virulence factors were the main pathogenicity for Lm infetions. Whether bloodstream infection or intracranial infection, TMP/SMX shows great potential as a therapeutic option for Lm infections. In addition, further investigations and prospective randomized clinical trials will be required to evaluate the clinical cure rates.

Abbreviations

Lm, *Listeria monocytogenes*

STs, sequence types

CSF, cerebrospinal fluid

WHO, World Health Organization

CDC, Centers for Disease Control and Prevention

ECDC, European Centre for Disease Prevention and Control

PEN, benzylpenicillin

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

Ethics approval and consent to participate. The work was in accordance with the Declaration of Helsinki. This study was approved by the recommendations of the Ethics Committee of the First Affiliated Hospital, College of Medicine, Zhejiang University. Informed consent was waived by the Clinical Research Ethics Committee because no intervention was involved and no patient identifying information was included.

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, WJRZ00000000.

Competing interests. None.

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Table

Table 1. Minimum inhibitory concentrations of 15 antimicrobial agents against 3 Lm

Antibiotics	23949	26530	34096
Benzylpenicillin [a]	0.5	0.5	0.5
Meropenem [a]	0.25	0.25	0.25
Erythromycin [a]	0.125	0.125	0.125
Levofloxacin [b]	1	0.5	1
Moxifloxacin [b]	0.5	0.25	0.5
Tetracycline [b]	0.5	0.5	0.5
Linezolid [b]	1	1	1
Vancomycin [a]	1	1	1
Rifampin [b]	0.03	0.03	0.03
Daptomycin [b]	8	8	8
Tigecycline [b]	0.25	0.25	0.25
Amikacin [b]	2	2	2
Trimethoprim-sulfamethoxazole [a]	0.0625/1.1875	0.0625/1.1875	0.0625/1.1875
Clindamycin [b]	0.5	0.5	0.5

a Breakpoints for Lm.

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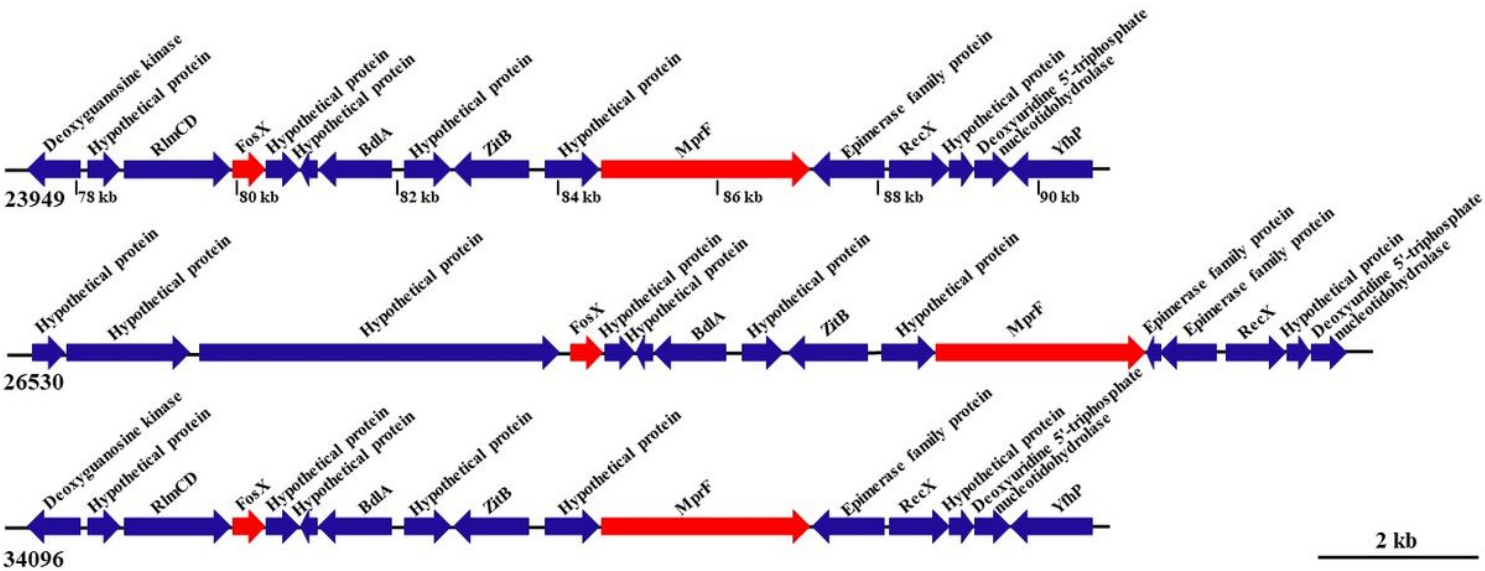
Figures

Figure 1

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.
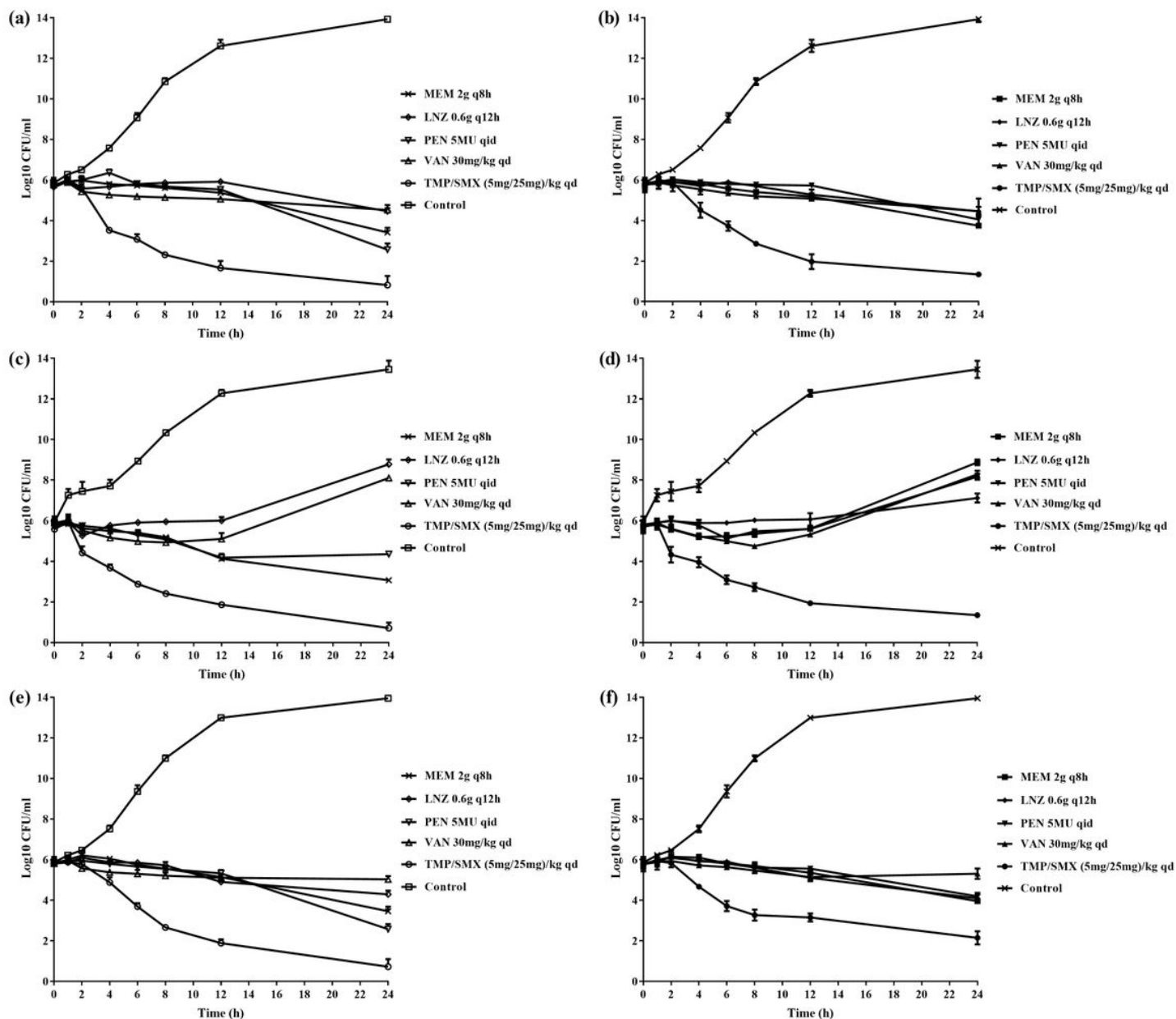
Figure 2

In vitro time-kill assays using serum and cerebrospinal fluid (CSF) concentrations of meropenem, linezolid, benzylpenicillin, vancomycin, and trimethoprim/sulfamethoxazole. (a) and (b) The five antibiotics at serum and CSF concentrations against isolate 23949 respectively; (c) and (d) The five antibiotics at serum and CSF concentrations against isolate 26530 respectively; (e) and (f) The five antibiotics at serum and CSF concentrations against isolate 34096 respectively. MEM, meropenem; LNZ, linezolid; PEN, benzylpenicillin; VAN, vancomycin; TMP/SMX, trimethoprim/sulfamethoxazole. Antibiotic concentrations are denoted by different symbols.