Title

Efficacy of meropenem and amikacin combination therapy against carbapenemase-producing *Klebsiella pneumoniae* mouse model of pneumonia

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20 **Authorship statement**

21 All authors meet the ICMJE authorship criteria.

22

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25

Abstract

Background: The emergence and spread of carbapenem-resistant Enterobacteriaceae (CRE) is a global health problem due to its high mortality and limited treatment options. Combination antimicrobial therapy is reported to be effective against CRE *in vitro*; however, its efficacy *in vivo* has not been thoroughly evaluated. Thus, this study assessed the efficacy of combination therapy of meropenem (MEPM) and amikacin (AMK) in a carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) mouse model of pneumonia.

Materials and Methods: Agar-based bacterial suspension of CR-Kp clinical isolates was inoculated into the trachea of BALB/c mice. Treatment was initiated 6 h post infection, with 100 mg/kg MEPM every 6 h, 100 mg/kg AMK every 12 h, or in combination; survival was evaluated for 7 days. The number of viable bacteria in the lungs, lung histopathology, and neutrophil counts in broncho-alveolar lavage fluid (BALF) were evaluated 42 h after infection.

Results: All mice in the untreated control group died in 48 hours, while all the mice in treatment groups survived past 7 days following infection. The bacterial count in the

lungs (log_{10} CFU/mL, mean ± SEM) in the combination group (2.00 ± 0.00) decreased significantly compared to that in control (10.19 ± 0.11, $p<0.0001$), MEPM (6.38 ± 0.17, $p<0.0001$), and AMK (6.17 ± 0.16, $p<0.0001$) groups. BALF neutrophil count reduced only in the combination therapy group. Combination therapy prevented the progression of lung inflammation, including alveolar neutrophil infiltration and hemorrhage.

Conclusions: This study demonstrates *in vivo* efficacy of MEPM and AMK combination therapy against CR-Kp pneumonia.

Keywords: CPE, CRE, pneumonia mouse model, antibiotic combination therapy, *in vivo*

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Meiji Seika Pharma, Japan Becton, Dickinson and Company, Shionogi & Co. Ltd., and KYORIN Pharmaceutical Co., Ltd.; Lecture fee from Daiichi-Sankyo, MSD K.K., Taisho Toyama Pharmaceutical Co. Ltd., Japan Becton, Dickinson and Company, Astellas Pharma, and bioMérieux Japan Ltd. Other authors declare no conflict of interests associated with this manuscript.

1. Introduction

Emergence of carbapenem-resistant Enterobacteriaceae (CRE) has been considered one of the major global health problems that require immediate and appropriate actions [1,2]. Nevertheless, the global spread of CRE is accelerating. A recent systematic review revealed that the resistance rates to imipenem and meropenem in Enterobacteriaceae are steadily increasing in Asia (from 0.5% and 0.3% in 2000-2004 to 1.9% and 2.4% in 2009-2011, respectively). Among the three most commonly isolated Enterobacteriaceae, *Klebsiella pneumoniae* has demonstrated the highest resistance rates [3].

CRE infections possess a high impact on mortality, which makes CRE an even more urgent problem. A previous study reported that infections caused by CRE have high mortality rates, ranging from 18% to 48% [4]. The mortality rate is higher especially in immunocompromised patients, who are prone to CRE colonization [5]. Therefore, the establishment of appropriate and robust antimicrobial treatment is strongly required. Antibiotic combination therapy is considered to be superior to monotherapy for treatment of CRE infections, due to reduction in use of inappropriate initial antimicrobial therapy, the potential synergistic effects, and suppression of emerging resistance [6]. A recent

clinical observational study revealed that combination therapy with two or more active agents, *in vitro,* was associated with lower mortality than treatment with a single active agent [7]. However, the evidence that supports the effectiveness of anti-CRE agents, colistin and tigecycline, against pneumonia is scarce. In a set of mouse pneumonia experiments, intravenously administered colistin, lacked permeability into lung tissue [8], and has been reported to be less effective against pneumonia than against other types of infection in a clinical study [9]. Tigecycline's clinical effectiveness to pneumonia and safety are not yet studied thoroughly enough [10], and its use for treatment in pneumonia is not approved in Japan. Therefore, development of an antibiotic regimen independent of colistin or tigecycline is crucial to the treatment of pneumonia caused by CRE. Among the limited antibiotics available, carbapenem is a reasonable option when administered in combination with another active antibiotic. Daikos et al. analyzed data from 138 carbapenem-resistant strains of *Klebsiella pneumoniae* (CR-Kp) infection in patients and found that the lowest mortality rate was observed in patients with carbapenem-based combination therapy when compared to non-carbapenem combination therapy [11]. In those regimens, aminoglycoside was the most common antibiotic accompanied by

carbapenems, suggesting clinical effectiveness of the combination. The combination of carbapenems and aminoglycosides has been studied *in vitro*, displaying synergistic activity against CR-Kp isolates [12], but their effectiveness in the *in vivo* setting has not yet been studied thoroughly.

In this study, we assessed the efficacy of combination therapy of meropenem (MEPM) and amikacin (AMK) in a CR-Kp mouse model of pneumonia.

2. Materials and methods

2.1. Bacterial strains

In preliminary experiments for developing the mouse model, a total of eighteen clinically isolated CR-Kp in Nagasaki University Hospital between January 2009 and June 2015 [13] were screened in four kinds of mice strains (BALB/c, C57/BL6, ddY, and ICR) by instilling saline-based inoculum into their tracheas. Among them, the strain named KP2 showed the strongest pathogenicity against BALB/c, though the infection was inconsistent and lacked reproducibility. Therefore, we decided to inoculate using an agar-based inoculum method, using the KP2 strain. This strain has been confirmed to have IMP-1 gene and produce carbapenemase. [13] The bacteria were stored at -80 °C in a Microbank® bead preservation system (Pro-Lab Diagnostics, Ontario, CA) until use.

2.2. Antimicrobial agents

MEPM, cilastatin (CS), and AMK were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), Hangzhou APIChem Technology Co., Ltd (Hangzhou, China), and Sigma-Aldrich Co. LLC (St. Louis, MO, U.S.A.), respectively.

118

119 *2.3. Animals*

120 We purchased specific pathogen-free male BALB/c mice (6-7 weeks old, 18-22 g) from
121 Japan SLC, Inc. (Shizuoka, Japan). The mice were housed in a pathogen-free environment
122 and received sterile food and water in the Biomedical Research Center at Nagasaki
123 University.

124

125 *2.4. Ethics*

126 All the experimental protocols used in this study were approved by the Ethics Review
127 Committee for Animal Experimentation (approval number 1003310842).

128

129 *2.5. Antimicrobial susceptibility tests*

130 The MICs (minimum inhibitory concentration) for MEPM and AMK were determined
131 using a microdilution method in accordance with the guidelines of the CLSI (M100, 29th
132 ed.).

133

2.6 Murine model of pneumonia using agar-based inoculum

In order to develop a robust pneumonia model, we modified the agar-based inoculum method previously reported by Hoover et al. [14]. The KP2 strain was cultured overnight on Mueller-Hinton II agar (Becton Dickinson, Le Pont-de-Claix, France). They were suspended in sterile saline and the concentration was adjusted to 5×10^6 CFU/mL, as estimated by turbidimetry (DEN-1B Densitometer, WAKENBTECH, Kyoto, Japan). Then, the bacterial suspension was diluted with liquid Mueller-Hinton II agar to 5×10^5 CFU/mL. Subsequently, 20 μL of the agar-based inoculum was inoculated through the outer sheath of the intravenous catheter (1×10^4 CFU/mouse). The procedures were carried out carefully in order not to cause choking from clotted agar. For model validation, survival rate and sequential bacterial load in the lungs were analyzed.

2.7. Treatment protocol

Antimicrobial treatment was initiated 6 h post infection. MEPM was administered intraperitoneally at 100 mg/kg four times a day (every 6 h) combined with 100 mg/kg CS. AMK (100 mg/kg) was administered intraperitoneally twice a day (every 12 h). Treatment

was continued until the evaluation time point. The doses of these antibiotics were determined according to previous studies [15,16] and concentrations that exerted a sufficient antibacterial effect were chosen for this study. For the survival analysis (n=7 for each group), treatment was continued up to 120 h after inoculation and survival was checked every 6 h for 5 days. Simultaneously, bodyweights of the mice were measured twice a day until the end of the treatment. We performed the survival study and the bacteriological study separately.

2.8. Bacteriological examinations

For the bacteriological examinations, the infected mice of each group were sacrificed at 18, 30, and 42 h post infection i.e. 12, 24, and 36 h after the initiation of therapy as well as at the end of the treatment of 5 days (n=3 to 4 for each group). Subsequently, they were dissected under aseptic conditions, blood was collected via right ventricular puncture using heparin-coated syringes. The lungs were removed, suspended in 1 mL of normal saline and homogenized with a homogenizer (T10 basic ULTRA-TURRAX®, Yamato, Fukuoka, Japan). Serial dilutions of the lungs and blood were quantitatively cultured in

166 Mueller-Hinton II agar plates. After 12-16 h incubation, we evaluated the number of
167 visible colonies. The lowest level of detectable counts was 1×10^2 CFU/mL.
168
169 *2.9. Histopathological examinations*
170 Whole lungs were removed under aseptic conditions and fixed in a 10% Formalin Neutral
171 Buffer-Methanol Solution (Mildform® 10NM, Wako, Osaka, Japan), and then the lung
172 tissue sections were paraffin embedded and hematoxylin and eosin (HE) stained.
173
174 *2.10. Bronchoalveolar lavage fluid (BALF) cell analysis*
175 BALF analysis was performed on mice different from those used for CFU determination
176 and histopathological analysis, in order to assess inflammatory cell accumulation in the
177 airspace. The chest was opened to expose the lungs and trachea after the mouse was
178 sacrificed, and a disposable sterile tube (Safelet Cath® PU, NIPRO, Osaka, Japan) was
179 inserted into the trachea. BAL was performed three times sequentially using 1.0 mL of
180 0.9% saline each time, and the recovered fluid was pooled on ice. Total cell counts were
181 performed by Turk staining with a hemacytometer (C-chip®, NanoEnTek Inc., Seoul,

Korea), and the percentage of neutrophils was calculated using Diff-Quik staining.

2.11. Statistical analysis

All data were analyzed using Prism ver. 7.0e (GraphPad Software) and were expressed as mean ± standard errors of the mean (SEM). Differences between groups were examined using one-way ANOVA with Tukey's multiple comparison test. A *P* value of <0.05 was considered to indicate a statistically significant difference.

3. Results

3.1. MICs of antimicrobial agents for KP2

The MICs of MEPM and AMK for the bacterial strain KP2 were both 8 mg/L. This strain exhibits resistance to MEPM but susceptibility to AMK.

3.2. Evaluation of the murine pneumonia model using agar-based CR-Kp inoculum

In the evaluation of this pneumonia model, the survival rate and sequential bacterial load were analyzed. Two different bacterial concentrations of 10^4 and 10^3 CFU/mouse were compared in the survival analysis. The mortality of the 10^4 CFU /mouse group was higher than that of the 10^3 CFU /mouse group (Fig. 1A), and the bacterial load in the lungs showed a constant increase (Fig. 1B). Pathological findings were consistent with inflammation caused by a bacterial infection (Fig. 1C).

3.3 Therapeutic effect of combination therapy compared to monotherapy on survival rate and change of bodyweight

The survival rate of the mice is shown in Fig. 2A. Most of the mice in treatment groups

206 survived through 5 days after infection. No statistical difference was observed in terms
207 of survival between the three treatment groups. The change of bodyweight of the mice is
208 shown in Fig. 2B. In all of the groups, the body weight decreased drastically just after the
209 infection procedure. However, the decrease was suppressed in the combination group
210 after day 2. From day 3 to 4, the body weight of combination group is statistically higher
211 than both of the monotherapy groups ($P < 0.001$ vs. MEPM, $P < 0.05$ vs. AMK).
212
213 *3.4. Bacteriological examinations.*
214 The bacterial load in the lungs was counted and analyzed at each timepoint. The bacterial
215 load started to decrease in the treatment groups after initiation of antibacterial treatment
216 (Fig. 3A). The bacterial counts in the lungs of the control group at 6 h, 18 h, 30 h, and 42
217 h were 4.916 ± 0.11, 8.18 ± 0.14, 9.57 ± 0.20, 10.19 ± 0.11, the MEPM-treated group at
218 18 h, 30 h, and 42 h were 5.06 ± 0.05, 4.55±0.33, and 6.38 ± 0.17, the AMK-treated group
219 at 18 h, 30 h, and 42 h were 3.68 ± 0.10, 3.94 ± 0.05, and 6.17 ± 0.16, and in the
220 MEPM+AMK-treated group at 18 h, 30 h, and 42 h were 3.20 ± 0.11, 2.74 ± 0.07, and
221 2.00 ± 0.00, respectively. The bacterial load of the group receiving combination therapy

222 at 30 h and 42 h (Fig. 3B, C) was statistically lower than that in each monotherapy group.
223 The bacterial load in the blood was also counted and analyzed. The bacterial counts in the
224 blood of the mice of control group, MEPM, AMK, and MEPM+AMK group were 6.12 ±
225 0.46, 0.00 ± 0.00, 0.73 ± 0.73 and 0.00 ± 0.00, respectively (Fig. 3D). The bacterial counts
226 in the lungs of the mice which received thorough course of antibiotic therapy were below
227 the detection limit.
228
229 *3.5. Histopathological examinations*
230 Light microscopy analysis of the HE-stained lungs of the control group at 42 h post
231 infection, revealed infiltration of large numbers of inflammatory cells in the alveolar
232 spaces, alveolar hemorrhage, and destruction of alveolar structures (Fig. 4A). These
233 findings are relatively conservative in the treatment groups. In MEPM+AMK-treated
234 mice, only mild inflammatory changes were observed, supporting a beneficial advantage
235 of using combination therapy.
236
237 *3.6. Analysis of BALF*

KP2 induced an increase in the total number of neutrophils in BALF. The total numbers of neutrophils in BALF were significantly lower in treatment groups, with significant reduction in the MEPM+AMK-treated group compared to MEPM or AMK monotherapy groups (Fig. 4B).

4. Discussion

In this study, we developed a novel CR-Kp mouse model of pneumonia using an agar-based inoculum method. Furthermore, we demonstrated the efficacy of combination MEPM and AMK therapy in this model.

To the best of our knowledge, this is the first study to establish a CR-Kp pneumonia model in immunocompetent mice. *K. pneumoniae* is one of the most common pathogens of community-acquired pneumonia, causing infection in immunocompetent patients [17–19]. Therefore, in this study, the pneumonia mouse model under immunocompetent conditions is more relevant than the neutropenic mouse model commonly used for assumed patient populations. We generated this mouse model using an agar-based inoculum method that was previously reported [14]. Agar-based inoculum enables bacteria to scaffold in the lungs and helps their primary growth. Correspondingly, this method showed robust development of pneumonia with sufficient reproducibility. Furthermore, it has potential adaptability to other pneumonia mouse models, independent of bacterial strains or mouse matching. However, the concern remains that agar itself might induce some inflammatory response, regardless of the existence of an infection.

Therefore, it may be difficult to precisely assess the host-pathogen interaction in this model. However, evidence of infectious inflammation is suggested by the continuous bacterial growth and findings of pathological bacterial infection observed (Fig. 1B, C). Additionally, no deaths were observed in mice exposed to agar-based inoculum without bacterial suspension. For these reasons, we consider this model appropriate in validating the efficacy of antimicrobial agents against pathogens.

The efficacy of MEPM and AMK combination therapy against CR-Kp pneumonia over monotherapy was thoroughly demonstrated by this study. The number of viable bacteria in the lungs decreased and the inflammatory changes were limited in the combination therapy group when compared to those in the monotherapy groups. Several clinical studies have suggested the effectiveness of carbapenem and AMK in combination against CR-Kp pneumonia, [20,21] but little is known about the efficacy of the combination *in vivo*. Hirsch et al. screened combinations of doripenem, AMK, levofloxacin, or rifampin against CR-Kp *in vitro* and found that doripenem and AMK exerted a synergistic effect. [22] They further assessed the combination in immunocompromised mice and demonstrated its efficacy by comparing with a placebo control group. In this study, we

276 demonstrated the superiority of the combination therapy to monotherapy *in vivo*. We
277 consider these results to be meaningful, based on the findings *in vitro* and the supporting
278 clinical evidence available.
279 On the other hand, a certain degree of reduction in bacterial load and inflammatory cell
280 infiltration were shown by both monotherapy groups. Furthermore, sufficient
281 improvement of survival was observed in both monotherapy and combination treatment
282 groups. These results can be partially explained by the immunocompetent status. In a
283 meta-analysis comparing β-lactam monotherapy with β-lactam-aminoglycoside
284 combination therapy for severe infections, no advantage of the combination therapy on
285 mortality, in patients without neutropenia, was found [23]. Another review of clinical
286 studies suggests that combination therapy is associated with improved outcomes only in
287 severely ill patients. [24] These clinical data suggest that combination therapy contributes
288 to survival rate improvement only in immunocompromised or severely ill patients; this
289 finding is consistent with our results that even monotherapy improved survival rate in an
290 immunocompetent host.
291 There are several limitations to this study. First, we have not performed any

292 pharmacokinetic analysis. Therefore, the drug administration doses are determined
293 according to the previous studies in mice [15,16] and the intervals are set considering the
294 half-life in mice, which is shorter than in humans [25]. In this study, antimicrobial effects
295 were observed in each monotherapy treatment group, and the synergistic effects were
296 confirmed for those situations. Further studies including PK studies are needed in order
297 to confirm concordance between synergies *in vitro* and *in vivo*. Another limitation is that
298 we assessed a single strain of KP2 in this study. We tried other clinical isolates, but the
299 pathogenicity was weak, and it was difficult to create pneumonia stably. This KP2 strain
300 possesses IMP-1 carbapenemase, which is the most dominant type in Japan [13]. It is
301 known that different types of carbapenemase hydrolyze carbapenems to different degrees,
302 thereby exhibiting variations in antimicrobial resistance [26]. For example, most of the
303 strains harboring IMP-1 carbapenemase exhibit susceptibility to aminoglycosides [13],
304 while NDM-1 carbapenemase possessing strains often show high levels of resistance
305 against aminoglycosides [27]. Hence, careful attention is required in applying these
306 results to clinical situations. In order to combat highly carbapenem-resistant pathogens,
307 other antimicrobial combinations against various strains should be further explored and

assessed.

In conclusion, this pneumonia model is robust and consistent enough to evaluate antimicrobial effectiveness. We confirmed that combination therapy of MEPM and AMK was effective in the treatment of CR-Kp-induced pneumonia mouse model. Exploration of additional antimicrobial combinations for selection or to be avoided and further validation against other MIC strains is warranted.

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422 **Figure legends**

423 **Figure 1.** Evaluation of the CR-Kp mouse model of pneumonia. The effects of agar
424 based inoculum of 10^3 and 10^4 CR-Kp/mouse on mouse survival (A, n=5). The number
425 of viable bacteria in lungs over time (B, n=6). Pathological findings show inflammatory
426 cell accumulation, hemorrhaging in the lungs, and alveolar destruction (C).

427

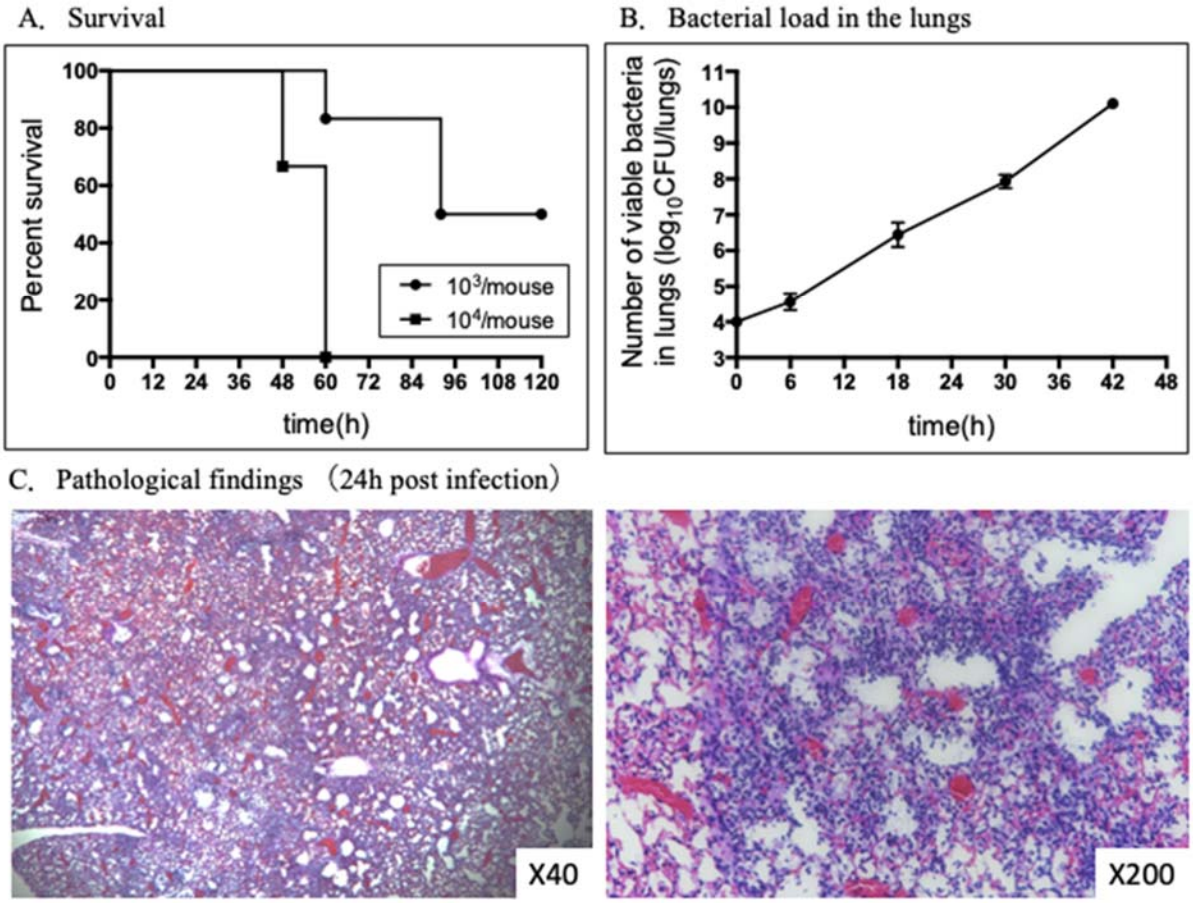
428 **Figure 2.** Treatment effect on survival rate (A, n=7) and bodyweight changes (B, n=7).
429 In bodyweight changes, the data are expressed as relative values, with day 0
430 being 100%. *, $P<0.05$ vs. MEPM group; **, $P<0.01$ vs. MEPM group; ***,
431 $P<0.001$ vs. MEPM group; †, $P<0.05$ vs. AMK group.

432

433 **Figure 3.** Number of viable bacteria in lungs. Agar based inoculum of 10^4 CR-
434 Kp/mouse with antibiotic treatment initiated 6 h post infection. The bacterial load in the
435 lungs (n=3 or 4 per group) was counted and analyzed at 18, 30, and 42 h post infection
436 (A). At 30 h (B), and 42 h (C), combination therapy (MEPM+AMK) significantly
437 reduced bacterial load when compared to each monotherapy. The bacterial load in the

438 blood was counted and analyzed at 42 h post infection (D). All of the control mouse had
439 bacteremia, bacteremia was not seen in the treatment groups, except for a few cases in
440 the monotherapy groups. Data are representative of three independent experiments.
441 *, $P<0.05$; ***, $P<0.001$; ****, $P<0.0001$.
442
443 **Figure 4.** Histopathological examinations of the lungs of mice (A) and the total
444 numbers of neutrophils in the BALF (B). At 42 h after infection, the control group and
445 each treatment group were compared. Inflammatory cells infiltrating the alveolar
446 spaces, alveolar hemorrhage and alveolar destruction were observed in the control
447 group. Those findings were relatively limited in the treatment groups, and most limited
448 in the combination treatment group. BALF analysis demonstrates a decrease in
449 neutrophil counts in the treatment group, with significant reduction in the combination
450 treatment group. *, $P<0.05$; **, $P<0.01$.

Figure 1


A. Survival
Percent survival
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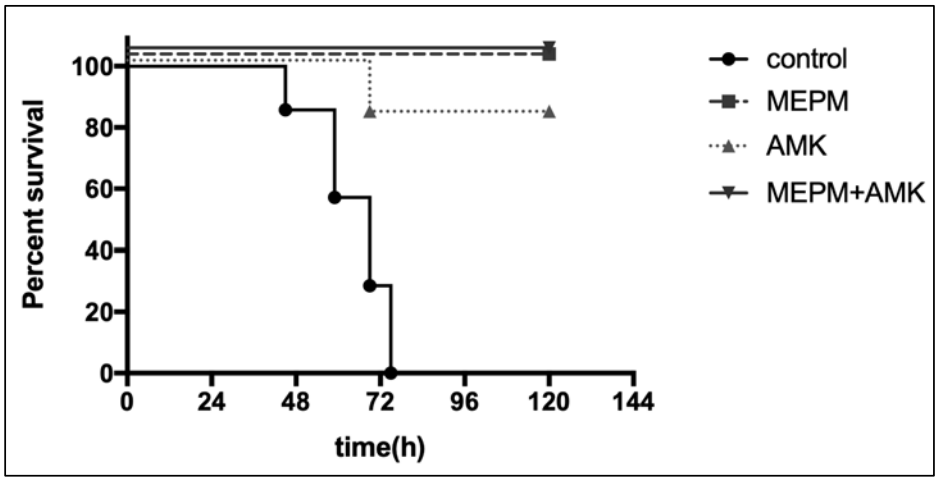
0
0 12 24 36 48 60 72 84 96 108 120
time(h)
10³/mouse
10⁴/mouse
B. Bacterial load in the lungs
Number of viable bacteria in lungs (log10CFU/lungs)
11
10
9
8
7
6
5
4
3
0 6 12 18 24 30 36 42 48
time(h)
C. Pathological findings （24h post infection）
X40
X200

Figure 2

A. Survival



B. Bodyweight

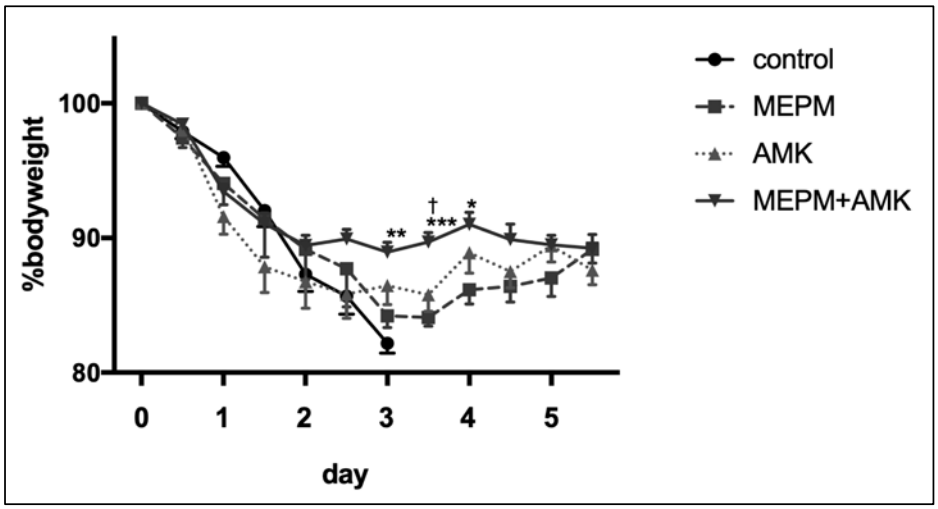
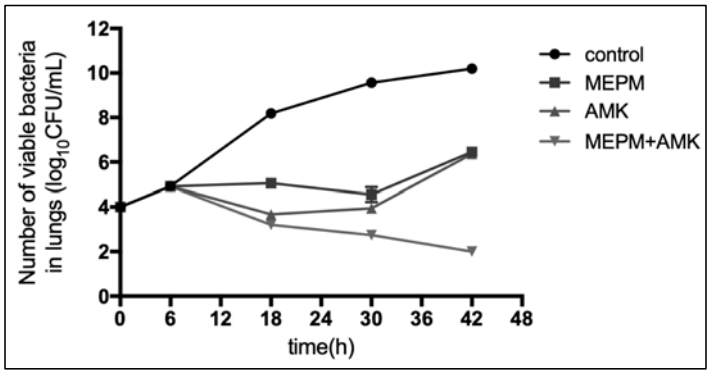
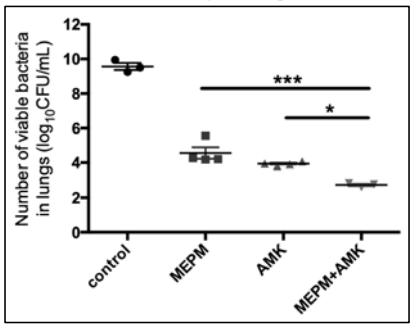


Figure 3

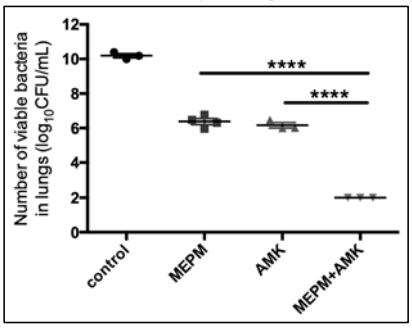
A. Sequential bacterial load in lungs



B. Bacterial load in lungs at 30h post infection



C. Bacterial load in lungs at 42h post infection



D. Bacterial load in blood at 42h post infection

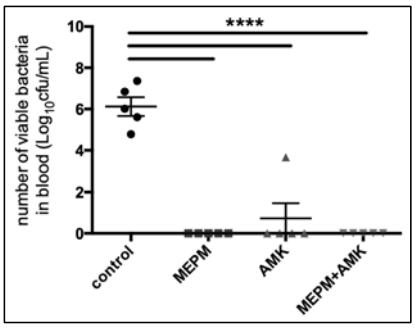
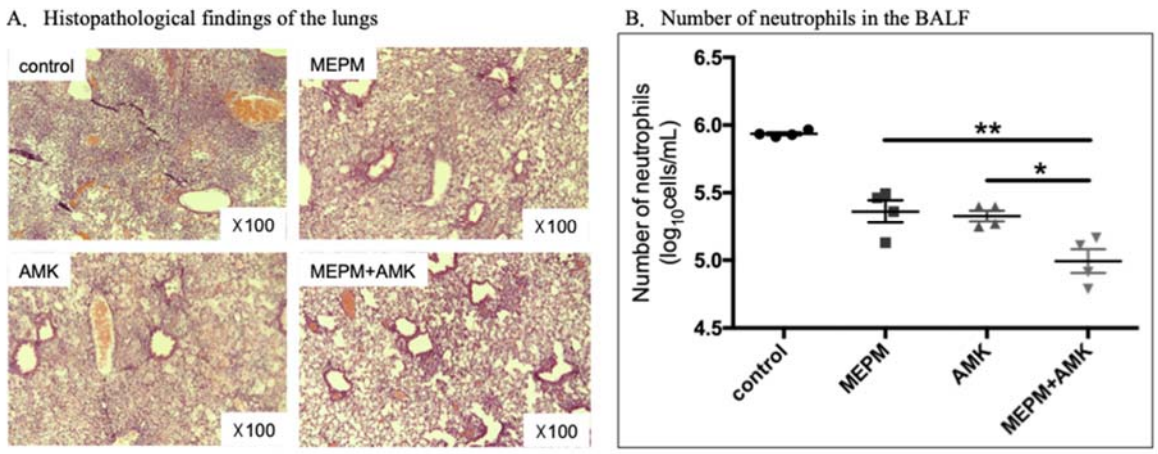


Figure 4


A. Histopathological findings of the lungs
control
MEPM
AMK
MEPM+AMK
X100
X100
X100
X100
B. Number of neutrophils in the BALF
Number of neutrophils
(log10cells/mL)
6.5
6.0
5.5
5.0
4.5
**
*
control
MEPM
AMK
MEPM+AMK