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ORIGINAL ARTICLE

Galleria mellonella as an *in vivo* model for assessing the efficacy of antimicrobial agents against *Enterobacter cloacae* infection

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Background/Purpose: *Enterobacter cloacae* is a well-recognized nosocomial pathogen. Use of a rapid, *in vivo* infection model for *E. cloacae* that can determine the efficacy of antibiotic therapies could help facilitate screening for new treatments. Nonmammalian model systems of infection, such as *Galleria mellonella*, have significant logistical and ethical advantages over mammalian models.
Materials and methods: We utilized *G. mellonella* larvae to determine the utility of this infection model to study antibacterial efficacy. *G. mellonella* killing with heat-killed or live clinical isolates (*E. cloacae* GN1059 and GN0791) was tested. We also investigated the effect of postinoculation incubation temperature on the survival of infected larvae. The protection of administration of antibiotics to infected larvae was investigated. Finally, we determined the *G. mellonella* hemolymph burden of *E. cloacae* after administration of different antibiotics.
Results: With live bacterial inocula, *G. mellonella* killing was significantly dependent on the number of *E. cloacae* cells injected in a dose-dependent manner. Further, we observed that survival was reduced with increasing the postinoculation temperature. Treatment of a lethal *E. cloacae* infection with antibiotics that had *in vitro* activity significantly prolonged the survival of larvae compared with treatment with antibiotics to which the bacteria were resistant.

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The therapeutic benefit arising from administration of antibiotic correlated with a reduced burden of *E. cloacae* cells in the hemolymph.
Conclusion: The *G. mellonella* infection model has the potential to be used to facilitate the *in vivo* study of host—pathogen interactions in *E. cloacae* and the efficacy of antibacterial agents.

Introduction

Enterobacter cloacae is a well-recognized nosocomial pathogen that causes significant infections. It comprises part of the normal flora of the gastrointestinal tract of 40—80% of the population and is widely distributed in the environment.[1,2] Like most members of the Enterobacteriaceae family, these microorganisms are important nosocomial pathogens capable of causing opportunistic infections among hospitalized or debilitated and immunosuppressed patients.[3] *E. cloacae* is responsible for various infections, including bacteremia, lower respiratory tract infections, skin and soft-tissue infections, urinary tract infections, and intra-abdominal infections. Clinical awareness of the potential of *E. cloacae* strains to cause disease has been reflected in the increasing number of epidemiologic studies of these microorganisms showing that they could be a serious cause of nosocomial Gram-negative bacteremia.[4—6] The treatment of *E. cloacae* has become difficult due to resistance to antibiotics. This microorganism is intrinsically resistant to ampicillin and narrow-spectrum cephalosporins, due to a chromosomal cephalosporinase. Additional resistance to broad-spectrum cephalosporins and aztreonam is usually related to the mutational overproduction of the species-specific cephalosporinase, or production of plasmid-mediated extended-spectrum β-lactamases.[7,8] Imipenem resistance has been described in strains with porin alterations combined with hyperproduction of chromosomal cephalosporinase, and in strains producing class A carbapenem hydrolyzing non-metallo-β-lactamases, such as NmcA and IMI-1.[9—11]

Due to the discrepancies between susceptibilities of multidrug resistant *E. cloacae in vitro* and *in vivo*, data determined *in vivo* are essential to reliably inform about the true clinical potential of any novel antibiotic treatment. Thus, use of a rapid, *in vivo* infection model for *E. cloacae* that can determine the efficacy of antibiotic therapies could help facilitate screening for new treatments. Typically, a mammalian system is used, but these experiments are time-consuming, expensive, and require full ethical consideration.[12,13] Hence, there is a need for preliminary and alternative infection models that generate *in vivo* data quickly and inexpensively and that do not require the same ethical considerations. Invertebrate models have gained increased attention as a viable alternative to mammalian models.[14—16] The introduction of a wax moth (*Galleria mellonella*) model of systemic *E. cloacae* infection may prove useful to this end. Larvae of the greater wax moth, colloquially termed "wax worms" due to their natural lifestyle of infesting beehives and consuming beeswax, have already been used to determine the virulence of various human pathogens, and to study therapeutics for infections, including those caused by Gram-positive and Gram-negative bacteria and several pathogenic fungi.[17—22]

Using a *G. mellonella* model has a number of advantages over traditional mammalian models. First, the insects have sophisticated cellular and humeral defenses, including the production of antimicrobial peptides, which is similar to the innate immune response of mammals. Hemocytes are the major mediator of cellular defenses, which perform similar functions to human macrophages and neutrophils. Second, the larvae can be easily maintained and can be infected by injection without anesthesia. Third, the larvae are not subject to the ethical limitations of mammalian models. Finally, and of great novelty, the *G. mellonella* model is amenable for assessing the efficacy of antimicrobial agents, and can be maintained at temperatures of 37°C, which are well suited to study human pathogens.[20,21]

The aim of this study was to evaluate the potential of *G. mellonella* larvae as an *in vivo* infection model for *E. cloacae* and to determine whether the efficacy of antimicrobial treatment can be assessed.

Materials and methods

Reagents, bacteria and insects

All reagents and culture media were purchased from Sigma-Aldrich Ltd (St. Louis, MO, USA). All solutions were made using sterile deionized water. Water, phosphate buffered saline (PBS), and media were sterilized by autoclaving at 121°C for 15 minutes. The clinical isolates of *E. cloacae* used in this study are summarized in Table 1. Bacteria were cultured overnight in Luria-Bertani broth aerobically at 37°C with shaking, to prepare inocula for antibiotic susceptibility testing *in vitro* and antibiotic efficacy testing *in vivo*. Batches of *G. mellonella* larvae (Kaide ruixin Co., Ltd, Tianjin, China) in their final instar stage were stored in the dark at 4°C and used within 7 days from shipment. Larvae masses varied slightly, but were typically 250 mg, and this value was used to calculate treatment doses.

Determination of minimum inhibitory concentration

The minimum inhibitory concentrations (MICs) of piperacillin (PIP), cefotaxime (CTX), imipenem (IMP), amikacin

Table 1 Minimum inhibitory concentrations (MICs) of antimicrobials against *Enterobacter cloacae* GN1059 and GN0791

Antimicrobials	MIC (mg/L)	
	GN1059	GN0791
PIP	32	256
CTX	0.25	512
IMP	0.5	64
AMK	2	256
CIP	1	16

AMK = amikacin; CIP = ciprofloxacin; CTX = cefotaxime; IMP = imipenem; PIP = piperacillin.

(AMK), and ciprofloxacin (CIP) against *E. cloacae* GN1059 and GN0791 were determined using the microtiter broth dilution method. In brief, an inoculum of 5×10^5 colony-forming units (cfu)/mL was used, and the test was conducted in Luria-Bertani broth. Plates were incubated at 37°C and the MIC was defined as the lowest concentration of antibiotic where no bacterial growth was observed. Strains were classified as resistant to certain antibiotics if they grew in levels equal to or above the MIC breakpoint as defined by the Clinical and Laboratory Standards Institute (CLSI). *Escherichia coli* ATCC 25922 was used for the quality control. Each experiment was performed in triplicate.

G. mellonella killing with heat-killed or live *E. cloacae*

Bacterial infection of *G. mellonella* was performed as described previously,[21] with minor modifications. Briefly, bacterial cells were washed with PBS and then diluted to an appropriate cell density. Bacterial colony counts on Mueller-Hinton (MH) agar were used to confirm all inocula. A 50-μL Hamilton syringe was used to inject 10 μL aliquots of the inoculum into the hemocoel of each larva via the last left proleg. After injection, larvae were incubated in Petri dishes in the dark at 37°C for 5 days. Unless stated, 15 randomly chosen larvae were used for each group of an experiment and each experiment was repeated using larvae from a different batch. For heat-killed inoculum experiments, the bacterial suspension was heated in a water bath at 100°C for 10 minutes. Heat killing was confirmed to contain no viable bacteria by plating them on MH agar and incubating at 37°C for 48 hours. The heat-killed bacterial suspension was diluted with PBS and inocula containing $1 \times 10^6 - 1 \times 10^8$ cfu were used to infect groups of larvae, as above. In all experiments, there were two negative control groups; one group that underwent no injection, while the other group was injected with PBS only, which controlled for the impact of physical trauma.

Effect of postinoculation temperature on larval survival

To investigate the effect of postinoculation incubation temperature on the survival of infected larvae, larvae were inoculated with 5×10^5 cfu/larva of live bacteria and incubated at 25°C, 30°C or 37°C. Negative control groups were set up for each incubation temperature.

Administration of antibiotics

Groups of larvae were infected with a lethal inoculum of *E. cloacae* GN1059 and GN0791 as above, and all larvae were confirmed to be alive at 2 hours postinoculation. Then, antibiotics were administered in PBS by injection into a different proleg. Larvae injected twice with PBS were used as the control group. The antibiotics and doses included PIP (100 mg/kg), CTX (150 mg/kg), IMP (50 mg/kg), AMK (15 mg/kg), and CIP (10 mg/kg). Doses were based on those used for humans.

Determination of *G. mellonella* hemolymph burden of *E. cloacae*

Experiments were performed as described previously,[23] except for minor modifications. In brief, groups of 30 larvae were infected with 5×10^5 cfu/larva of either strain of *E. cloacae*. Two hours after infection, a single dose of antibiotic was administered as above. Larvae were incubated in Petri dishes at 37°C. At 24-hour intervals, five larvae were selected randomly from each treatment group and tested for hemolymph burden. In some cases where all larvae present had died, then five dead larvae were selected. Selected larvae were surface disinfected by vortexing in ethanol. The ethanol was poured off and the larvae were allowed to dry. Once dry, each larva was placed into a separate Eppendorf tube containing 300 μL of sterile PBS. A clean Argos pestle (Argos Technologies Inc., Elgin, IL, USA) was washed in ethanol, flamed, and used to homogenize the larva and release the hemolymph. A further 300 μL of sterile PBS was then added to each tube and vortexed. Larval homogenate (20 μL) was serially diluted in 180 μL of MHB in a 96-well microplate (Thermo Fisher Scientific Co., Ltd, Shanghai, China). This process was repeated for each of the five larvae sampled/treatment group every 24 hours. The dilution series was plated on MH agar plates and incubated at 37°C for 24 hours. Colonies were counted after 24 hours and data were expressed as viable cfu/mL. Using this method, the detection limit was 100 cfu/mL of larval homogenate.

Statistical analyses

For statistical testing, data from duplicate experiments were pooled to give $n = 30$. These pooled survival data were plotted using the Kaplan-Meier method and differences in survival were calculated using the log-rank test, with a p value ≤ 0.05 indicating statistical significance. In all comparisons with the negative control, it was the uninfected control (rather than the unmanipulated control) that was used. All statistical analyses were performed using GraphPad Prism, version 5.04 (GraphPad Software Inc., San Diego, CA, USA).

Results

G. mellonella larvae are highly sensitive to infection by *E. cloacae*

To determine whether *G. mellonella* larvae was a suitable model to study *E. cloacae* pathogenesis, we first defined its infection characteristics. The effect of infection with *E. cloacae* GN1059 and GN0791 on the survival of *G. mellonella* larvae is shown in Fig. 1. With live bacterial inocula, killing was significantly dependent on the number of *E. cloacae* cells injected in a dose-dependent manner during 96 hours of incubation (Fig. 1). One hundred percent of the *G. mellonella* larvae were killed within 48 hours of infection with at least 1×10^6 cfu/larva, whereas very few *G. mellonella* larvae were killed with 1×10^4 cfu/larva or less ($p < 0.05$) (Fig. 1). With both strains, the heat-killed *E. cloacae* inoculum (up to the maximum inoculation dose of 5×10^7 cfu) had no significant effect on larval survival. Further, we observed that postinoculation temperature affected larval survival after injection of live *E. cloacae*. It was shown that survival is reduced with increasing postinoculation temperature. There were no deaths in each of the uninfected control groups (incubated at 25°C, 30°C, and 37°C) (Fig. 2).

Together, these data indicate that infection with live bacteria is required to cause larval death. Given the above-described killing characteristics, for further *G. mellonella* experiments, we used inocula of 5×10^5 cfu/larva and incubated the infected larvae at 37°C.

In vitro sensitivities of *E. cloacae* to antibiotics correlate with the efficacy of the antibiotics *in vivo*

The MICs of PIP, CTX, IMP, AMK, and CIP against *E. cloacae* GN1059 and GN0791 are shown in Table 1. Antibiotics at the concentrations used as mentioned above had no effect on larval survival compared with the PBS-treated controls ($p < 0.05$) and were therefore deemed to be nontoxic at these doses (data not shown). The effects of single doses of antibiotics 2 hours postinfection with either *E. cloacae* GN1059 or GN0791 (inoculum 5×10^5 cfu/mL) on the survival of *G. mellonella* larvae are shown in Fig. 3. Larvae infected with the susceptible strain GN1059 showed that all five antibiotics resulted in significantly greater percentage survival compared with those larvae treated with PBS only ($p < 0.05$; Fig. 3). In contrast, when *G. mellonella* larvae were infected with an Multidrug-resistant (MDR) *E. cloacae* GN0791, these treatment regimens conferred no therapeutic benefit. In conclusion, the efficacy of the antibiotics *in vivo* closely correlated with the measured *in vitro* sensitivities (Table 1).

Successful antibiotic therapy results in a parallel reduction in the larval burden of *E. cloacae*

For the larvae infected with *E. cloacae* GN1059, significant differences ($p < 0.05$) were observed in the number of log10 cfu/mL recovered from the larvae treated with antibiotics mentioned above and the untreated larvae group (Fig. 4). In contrast, a majority of larvae infected with *E. cloacae* GN0791 and treated with either a single dose mentioned above or PBS were dead after 24 hours. In parallel, under both treatments the larval burden increased significantly for the 96-hour duration of the experiment.

For example, treatment with 150 mg/kg CTX prevented the rapid growth of GN1059 over 24 hours and resulted in a significant reduction in the number of viable bacteria present within the larvae over the entire 96-hour duration of the experiment (Fig. 4). In contrast, a single dose of 150 mg/kg CTX had no inhibitory effect, as there was rapid proliferation of GN0791 within the larvae over a period of 24 hours (Fig. 4) and the numbers of GN0791 recovered were similar to those from infected larvae treated with PBS alone.

In summary, the therapeutic benefit arising from administration of an antibiotic closely correlated with a drastic reduction in the larval burden of bacteria.

Discussion

A number of studies have utilized *G. mellonella* larvae to study the virulence of certain microbial pathogens and the efficacy of antimicrobial therapies against Gram-negative pathogens.[17–23] For example, the efficacy of different antibiotic regimens versus larvae infected with *Acinetobacter baumannii* reflected precisely the known resistance patterns of the strains employed.[21] Desbois and Coote[24] reported that antibiotics are also effective in the larva model for the treatment of infections caused by Gram-

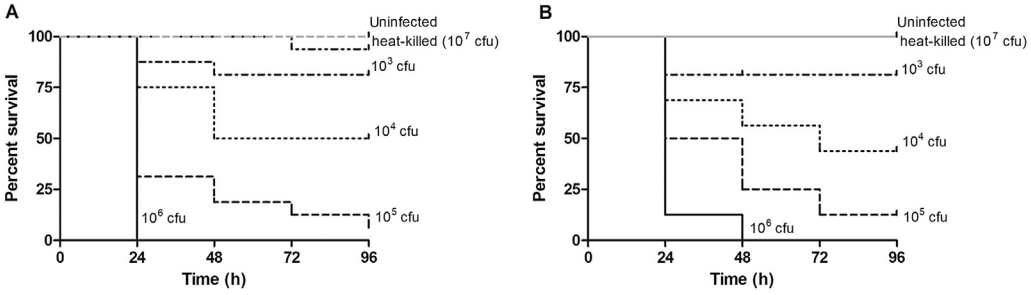

Figure 1. Effect of varying inoculum doses of live *Enterobacter cloacae* (A) GN1059 or (B) GN0791 on the survival of *Galleria mellonella* larvae during incubation at 37°C for 96 hours showing that survival is reduced with increasing doses of bacteria in a dose-dependent manner. Data from the 10^7 cfu of heat-killed bacterial inoculum is also given, showing that heat-killed *E. cloacae* had no significant effect on larval survival.

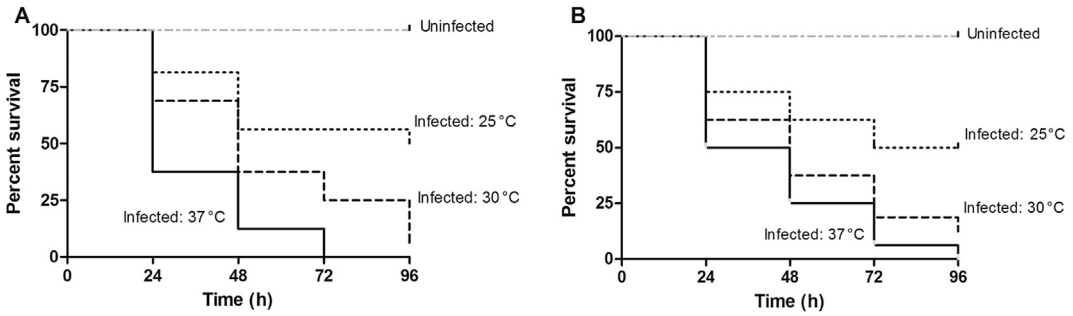

Figure 2. Effect of postinoculation temperature on the survival of *Galleria mellonella* larvae after infection with 5×10^5 cfu/larva of live *Enterobacter cloacae* (A) GN1059 or (B) GN0791 showing that survival is reduced with increasing postinoculation temperature.

positive bacteria. It is a useful preliminary model for assessing the *in vivo* efficacy of candidate anti-staphylococcal agents before proceeding to mammalian studies, which may reduce animal experimentation and expense. However, there are no documented studies that have investigated the suitability of *G. mellonella* larvae for studying the virulence of *E. cloacae* and the efficacy of antimicrobial therapies against *E. cloacae*.

To determine whether *G. mellonella* larvae were a suitable model to study *E. cloacae* pathogenesis, we first defined its infection characteristics. Killing was significantly dependent on the number of *E. cloacae* cells injected. Results revealed a positive correlation between the inoculum number of *E. cloacae* cells and the death rate of larvae. These data suggest that infection with live bacterial cells is required for *E. cloacae* pathogenesis in *G. mellonella* larvae. Similar findings were described previously for other pathogens.[21,23,24]

An important benefit of *G. mellonella* over other invertebrate hosts such as *Caenorhabditis elegans* is its ability to be maintained at temperatures of 37°C, which are well suited to study human pathogens. We found that higher postinoculation temperatures cause greater reductions in larval survival. This is in line with the previous observation.[21,24] These data suggest that either *E. cloacae* have thermoregulated virulence factors or that *G. mellonella* larvae are more susceptible to infection at the higher temperature.

In our study, two *E. cloacae* strains with contrasting antimicrobial resistance profiles were used to discriminate the effect of treatment with a range of antimicrobials on larvae infected with these two strains. Our study showed that the efficacies of the antimicrobials on infected larvae closely correlate with the known drug susceptibilities of the two strains *in vitro*. Treatment of a lethal *E. cloacae* infection with antibiotics that had *in vitro* activity against the infecting *E. cloacae* strain significantly prolonged the rate of survival of *G. mellonella*. Determination of larval burden postinfection showed that effective antimicrobial therapy drastically reduced the number of bacteria detected inside the larvae. However, it was not possible from this to ascertain whether this was due to a bactericidal effect reducing the inoculum size, or to a bacteriostatic effect on the inoculum preventing subsequent outgrowth in the hemocoel. Taken together, these findings confirm that the *G. mellonella* model may prove useful for evaluating the *in vivo* efficacy of new antimicrobial agents.

In conclusion, very little is known about the pathogenic mechanisms of *E. cloacae*, and thus, the *G. mellonella* infection model described herein serves as a useful tool for

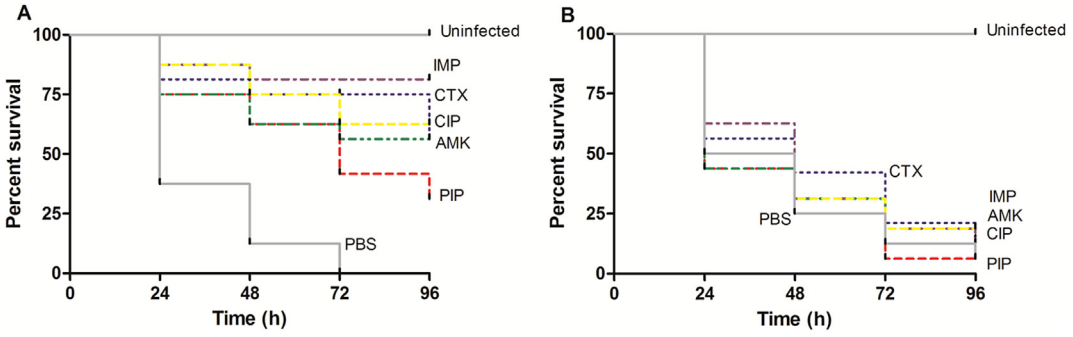

Figure 3. Effect of antibacterial treatment on survival of *Galleria mellonella* larvae infected with *Enterobacter cloacae* GN1059 or GN0791. Antibacterials that are active against *E. cloacae* can prolong the survival of *G. mellonella* larvae. (A) After infection with a lethal dose of *E. cloacae* GN1059, piperacillin (PIP) (100 mg/kg), cefotaxime (CTX; 150 mg/kg), imipenem (IMP; 50 mg/kg), amikacin (AMK; 15 mg/kg), and ciprofloxacin (CIP; 10 mg/kg), to which the strain was susceptible, significantly prolonged the survival of *G. mellonella* larvae [$p < 0.05$ for comparison with phosphate buffered saline (PBS)]. (B) However, for GN0791 infected *G. mellonella* larvae, the antibacterials mentioned above, to which the strain was resistant, caused no difference in killing compared with PBS treatment.

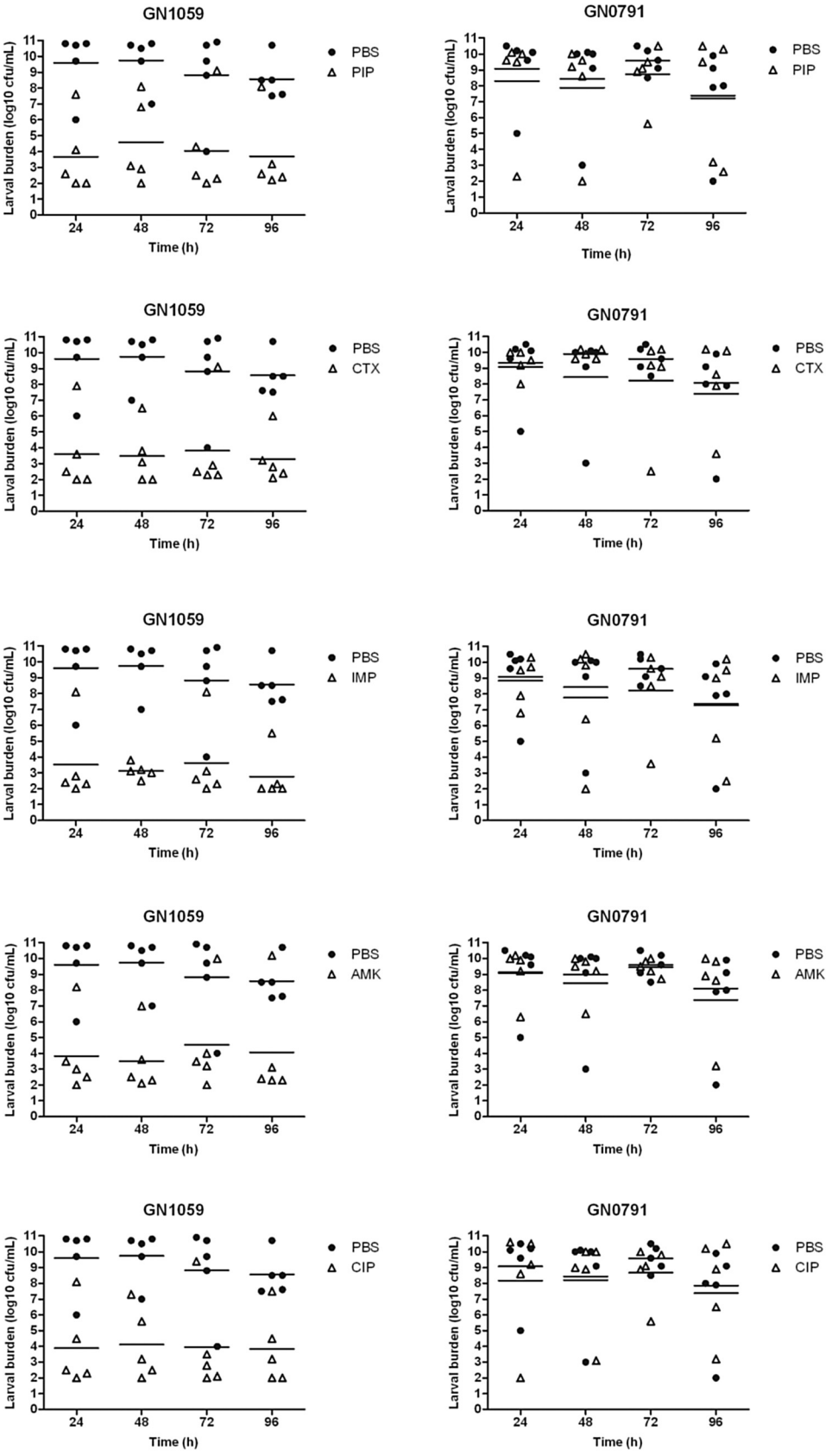

Figure 4. Effect of a single dose of piperacillin (PIP; 100 mg/kg), cefotaxime (CTX; 150 mg/kg), imipenem (IMP; 50 mg/kg), amikacin (AMK; 15 mg/kg), and ciprofloxacin (CIP; 10 mg/kg), on larval burden of *Enterobacter cloacae* GN1059 or GN0791. Larvae were infected with 5×10^5 cfu/mL of either strain. For the susceptible strain *E. cloacae* GN1059, a significant difference in larval burden between groups treated with phosphate buffered saline (PBS) or each antibacterial ($p < 0.05$); for the Multidrug-resistant (MDR) strain *E. cloacae* GN0791, no difference in larval burden between groups treated with PBS or each antibacterial.

future research. The model also has the potential to be used to study novel compounds for *in vivo* activity against *E. cloacae* without the logistical, ethical, and financial barriers that exist with mammalian models.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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