Comparative analysis of *Mycobacterium abscessus* clinical isolate virulence using an invertebrate infection model

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Abbreviated running headline: *Mycobacterium abscessus* virulence

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Abstract

Aims: *Mycobacterium abscessus* causes chronic skin infections, lung diseases, and systemic or disseminated infections. We investigated the quantitative evaluation of the virulence of *M. abscessus* clinical isolates by calculating the LD_{50} using a silkworm infection model.

Methods and Results: *M. abscessus* subsp. *abscessus* cells were injected into the hemolymph of silkworms. Silkworms died within two days post-infection with *M. abscessus* subsp. *abscessus* when reared at 37˚C. Viable cell numbers of *M. abscessus* subsp. *abscessus* increased in the hemolymph of the silkworms injected with *M. abscessus* subsp. *abscessus*. Silkworms were not killed following injections with heat-killed *M. abscessus* subsp. *abscessus* cells. The administration of clarithromycin, an antibacterial drug used for the treatment of the infection, prolonged the survival time of silkworms injected with *M. abscessus* subsp. *abscessus*. The LD_{50} values of seven clinical isolates were determined using the silkworm infection model and differed by up to nine-fold.

Conclusions: *M. abscessus* proliferation is required to kill the silkworms and that the virulence of *M. abscessus* clinical isolates can be evaluated by the silkworm infection model.

Significance and Impact of the Study: The silkworm infection model with *M. abscessus* is useful for estimating the virulence of the clinical isolates in a short period.

Keywords

Virulence, quantitative evaluation, *Mycobacterium abscessus* subsp. *abscessus*, silkworm, infection

Introduction

The *Mycobacterium abscessus* complex (MABC) is a group of rapid-growing, non-tuberculous mycobacteria (NTM) that includes three subspecies: *M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* (Hoshino and Suzuki, 2015; Oren and Garrity, 2016; Gupta *et al.*, 2018; Tortoli *et al.*, 2018). Because the MABC causes chronic skin infections and lung diseases in immunocompetent patients, and also systemic and/or disseminated infections in immunocompromised patients (Catherinot *et al.*, 2007; Piersimoni and Scarparo, 2008; Esther *et al.*, 2010; Johansen *et al.*, 2020), the patients infected with these subspecies will show heterogenous clinical outcomes, suggesting the different subspecies present various degrees of virulence (Koh *et al.*, 2011; Morimoto *et al.*, 2018). Moreover, the virulence of *M. abscessus* has evolved through stepwise adaptation of host and soil environments (Catherinot *et al.*, 2007; Bryant *et al.*, 2021), which means that it likely varies between clinical isolates. Several mouse infection models with *M. abscessus* were established for evaluating the effects of antibacterial drugs (Obregón-Henao *et al.*, 2015; Maggioncalda *et al.*, 2020). In mouse infection models, since a long time is required to mouse death by the infection of *M. abscessus* (Catherinot *et al.*, 2007), the development of a rapid evaluation system is desired.

Silkworm, an invertebrate, is a beneficial experimental animal for revealing the host-pathogen interactions (Matsumoto, 2020; Kaito *et al.*, 2020; Montali *et al.*, 2020). A large number of silkworms can be reared in a small space compared with mammalian animals (Matsumoto and Sekimizu, 2019). The 3Rs, replacement, refinement, and reduction, are important principles for experiments using mammals in the view of animal welfare (Herrmann *et al.*, 2019). Experiments with invertebrates fit the concept of replacement. Since silkworm is invertebrate, few ethical issues cause by sacrificing a large number of silkworms compared with mammals. Exploiting the benefits

of silkworms to infectious disease research, the LD_{50}, which is the dose of a pathogen required to kill half of the silkworms in a group, can be determined for quantitatively comparing the virulence of strains (Matsumoto *et al.*, 2011; M. Ishii *et al.*, 2017). Silkworm infection models were used as the evaluation systems for first screening to identify virulence-related genes in pathogenic microorganisms (Kaito *et al.*, 2005; Hanaoka *et al.*, 2008; Ueno *et al.*, 2011; Paudel *et al.*, 2020). Therefore, silkworm infection models are useful for the comparing virulence of microorganisms. A silkworm infection model was established for the evaluation of anti-mycobacterial compounds using a type strain (Hosoda *et al.*, 2020). However, comparative analysis of virulence of clinical isolates in *M. abscessus* based on the determination of the LD_{50} values was not demonstrated.

In the present study, we focused on evaluating the virulence of *M. abscessus* subsp. *abscessus.* We attempted to compare the virulence of *M. abscessus* subsp. *abscessus* clinical isolates using a systemic infection model using silkworms. We established a silkworm infection model to determine the LD_{50} values of *M. abscessus* subsp. *abscessus* isolates to quantitatively evaluate their virulence. Among the seven clinical isolates, the difference in virulence varied up to nine-fold. The results suggest that the silkworm infection model is a rapid evaluation system for quantitatively estimating the virulence of *M. abscessus* subsp. *abscessus* clinical isolates.

Materials and Methods

Reagents

Clarithromycin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was suspended in 0.9% NaCl solution (saline). Middlebrook 7H9 broth, Middlebrook 7H10 agar, and Middlebrook OADC enrichment were purchased from Becton, Dickinson, and Company (MD, USA). Middlebrook 7H9

broth and Middlebrook 7H10 agar were supplemented with 10% Middlebrook OADC enrichment.

Bacterial strains and growth

The *M. abscessus* subsp. *abscessus* ATCC19977 strain and seven clinical isolates (Mb-7, Mb-10, Mb-14, Mb-16, Mb-17, Mb-18, and Mb-22) were used in this study. The clinical isolates were obtained from the Keio University School of Medicine. This study was approved by the medical research ethics committee of the National Institute of Infectious Diseases (#1046) and by Keio University School of Medicine Ethics Committee (#2008-0131-9 sai). Species were identified with a DDH Mycobacteria Kit (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) (Kusunoki *et al.*, 1991) and multiplex PCR (Yoshida *et al.*, 2021). The *M. abscessus* subsp. *abscessus* strains were grown on Middlebrook 7H10 agar plate at 37˚C. A single colony was then inoculated into 5 ml of Middlebrook 7H9 broth and incubated at 37˚C for three days.

Infection experiments using silkworms

The silkworm infection experiments were performed as previously described (Kaito *et al.*, 2002). Fifth instar larvae were reared with an artificial diet Silkmate 2S (Ehime-Sanshu Co., Ltd., Ehime, Japan) for 24 h. *M. abscessus* subsp. *abscessus* cells grown in Middlebrook 7H9 broth were collected by centrifugation and suspended in sterile saline. Silkworms injected with the *M. abscessus* subsp. *abscessus* cells were incubated at 27˚C or 37˚ C and their survival monitored.

Therapeutic activity tests using the silkworms were performed according to a previous study with slight modifications (Kaito *et al.*, 2002). Either 50 µl of saline or 50 µl of an *M. abscessus* subsp. *abscessus* suspension (6.3 x 10^7 cells) was injected into the silkworm hemolymph. Immediately following inoculation of the silkworm with the *M. abscessus* subsp. *abscessus*

suspension, clarithromycin was injected the silkworms at a concentration of 25 μg g^{-1} larva.

Viable cell counts

Silkworms were injected with an *M. abscessus* subsp. *abscessus* cell suspension (7 x 10^6 cells in 50 μl) and incubated at 37˚C. Hemolymph was harvested from the silkworm larva at either 3 or 30 h post-infection through a cut on the first proleg (Matsumoto *et al.*, 2011). The hemolymph was added to saline and the solution was spread on a Middlebrook 7H10 agar plate. The agar plate was incubation at 37°C for 3 days, the colonies on the agar plate were counted. The total number of viable cells in the sample was calculated.

LD_{50} measurement

LD_{50} values were determined according to a previous study, with slight modifications (Matsumoto *et al.*, 2012; Miyazaki *et al.*, 2012). *M. abscessus* subsp. *abscessus* cells grown in Middlebrook 7H9 broth were suspended in saline. Either a two- or four-fold dilution series of the bacterial suspension was prepared, followed by 50 μl injections into silkworm hemolymph (4 x 10^5 – 1 x 10^8 cells), and incubation at 37˚C. The survival number of silkworms was observed at 48 h. The LD_{50} values were determined from the data of three experiments using a simple logistic regression model in Prism 9 (GraphPad Software, LLC, San Diego, CA, USA, https://www.graphpad.com/scientific-software/prism/).

Statistical test

The statistical significance of differences between the viable cell counts of *M. abscessus* subsp. *abscessus* in silkworm groups were determined by the Student *t*-test.

Results

Experimental conditions for the evaluation of *M. abscessus* subsp. *abscessus* virulence in silkworms

We first determined the experimental conditions for evaluating the virulence of *M. abscessus* subsp. *abscessus* in silkworms. The rearing temperature in a silkworm infection experiment is critical because it regulates the body temperature that affects bacterial virulence (Matsumoto and Sekimizu, 2019). Silkworms were died within 48 h by injection of *M. abscessus* subsp. *abscessus* ATCC19977 strain (1.6 x10^9 cells) at 37˚C, but not died at 27°C (Fig. 1). The LD_{50} for *M. abscessus* subsp. *abscessus* ATCC19977 was 1.1 x 10^7 cells under a 37˚C incubating condition (Fig. 2). These results suggest that 37˚C condition is necessary for *M. abscessus* subsp. *abscessus*-induced silkworm death that can be evaluated within two days.

Effect of *M. abscessus* subsp. *abscessus* proliferation in silkworms on virulence

Since the silkworm death caused by *Porphyromonas gingivalis* in a previous report did not require proliferation, we hypothesized that to be a result of a shock, and not a bacterial infection (K. Ishii *et al.*, 2010). To evaluate that possibility for *M. abscessus* subsp. *abscessus* in our model, we evaluated if proliferation was necessary for virulence (see the experimental schematic in Fig. 3A). The *M. abscessus* subsp. *abscessus* viable cell count increased in the silkworm hemolymph at 30 h post-infection (Fig. 3B). The injection of autoclaved *M. abscessus* subsp. *abscessus* cells, however, did not kill silkworms (Fig. 4A). The administration of clarithromycin, an antibiotic clinically used for *M. abscessus* subsp. *abscessus* infections, to silkworms post-infection with *M. abscessus* subsp. *abscessus* prolonged their survival time (Fig. 4B). These results suggest that *M.*

abscessus subsp. *abscessus* virulence in silkworms requires *M. abscessus* subsp. *abscessus* growth.

Comparative pathogenic analysis of *M. abscessus* subsp. *abscessus* clinical isolates

We next determined the LD_{50} values of *M. abscessus* subsp. *abscessus* clinical isolates using the silkworm infection model to compare their virulence. Seven clinical isolates were obtained from sputum samples of patients infected with *M. abscessus* subsp. *abscessus*. Their LD_{50} values ranged from 3.1×10^6 to 2.9×10^7 cells per larva; the LD_{50} value of the Mb-17 isolate was the lowest (Fig. 5). The LD_{50} value of the Mb-17 isolate was nine-fold lower than that of the Mb-10 isolate (Fig. 5). These results suggest that the Mb-17 isolate is highly pathogenic to silkworms compared to the other isolates.

Discussion

In the present study, the virulence of *M. abscessus* subsp. *abscessus* clinical isolates was compared using a silkworm infection model. Among the seven clinical isolates, the virulence, as determined by the LD_{50}, varied by up to nine-fold. The results indicate that the *in vivo* evaluation system is useful for revealing the virulence of *M. abscessus* subsp. *abscessus* clinical isolates in a shorter period of time (within two days).

M. abscessus subsp. *abscessus*-infected silkworms incubated at 37˚C, human body temperature, were more sensitive to infection than those reared at 27˚C, the normal rearing temperature of silkworms. We assumed that the difference was caused by both high-temperature stress in silkworms and the optimal growth temperature for *M. abscessus* subsp. *abscessus*. Hosoda *et. al*. reported to establish a silkworm infection model for evaluating the anti-mycobacterial compounds (Hosoda *et al.*, 2020). Compared to that report, we demonstrated that *M. abscessus*

subsp. *abscessus* grows in silkworm hemolymph and evaluated the virulence of several clinical isolates. Our findings are important for validating the usefulness of silkworm to estimate the *M. abscessus* subsp. *abscessus* clinical isolate virulence.

M. abscessus subsp. *abscessus* virulence may correlate with severe infections (Catherinot *et al.*, 2007; Bryant *et al.*, 2021). Therefore, understanding the *M. abscessus* subsp. *abscessus* clinical isolate virulence is information for infection control. *M. abscessus* subsp. *abscessus* showed different virulence among clinical isolates in the silkworm model. We demonstrated that the silkworm infection model with *M. abscessus* subsp. *abscessus* is a beneficial evaluation system that enables a quantitative determination of clinical isolate virulence by calculating the LD_{50} values within just two days. Moreover, the LD_{50} values among the clinical isolates were differed by up to nine-fold. Mb-17 was the most pathogenic isolate in silkworms among the *M. abscessus* subsp. *abscessus* clinical isolates used in this study; it may harbor virulence-related genes that enhance the infection process. The virulence genes of several pathogens were identified by avirulent mutant screening using silkworm infection models from a mutant library (Kaito *et al.*, 2005; Hanaoka *et al.*, 2008; Ueno *et al.*, 2011; Paudel *et al.*, 2020). The method for constructing *M. abscessus* subsp. *abscessus* gene-deletion mutants is well established (Küssau *et al.*, 2020; Foreman *et al.*, 2020). Further studies are needed to determine the virulence factors that the *M. abscessus* subsp. *abscessus* Mb-17 isolate harbours that are responsible for its virulence in silkworms.

M. abscessus vertebrate infection models using a zebrafish, *Danio rerio*, and a tadpole, *Xenopus laevis*, were reported (Küssau *et al.*, 2020; Kim *et al.*, 2021; Lopez *et al.*, 2021). These infection models are needed for the evaluation of anti-mycobacterial drugs and the virulence within 15 days. Since these model animals are vertebrates, ethical problems and protocols must be

managed to ensure animal welfare. The 3Rs, replacement, refinement, and reduction, are important principles for experiments using mammals in the view of animal welfare (Herrmann *et al.*, 2019). Silkworms are invertebrates, which have merits as an alternative animal for infection experiments requiring large groups. Moreover, *M. abscessus* subsp. *abscessus* virulence factors, which are expressed in humans, might be identified by the silkworm infection model with 37˚C conditions.

We propose that the silkworm infection model with *M. abscessus* subsp. *abscessus* is a beneficial assay system for determining the virulence of *M. abscessus* subsp. *abscessus* clinical strains. The silkworm infection model may contribute to revealing the molecular mechanisms *M. abscessus* subsp. *abscessus* infections.

Author's contribution

YM designed the study, performed experiments, collected data, and wrote the initial draft of the manuscript. FH, NH, and YH prepared resources such as the *M. abscessus* subsp. *abscessus* clinical isolates. YH, FH, and TS contributed to data interpretation and critically reviewed the manuscript. All authors read and approved the final manuscript.

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Conflict of interest

The authors declare no conflict of interest.

Data availability statement

All data identified in the present study are provided in the paper and its supplementary information (S1 Dataset).

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macrolide susceptibility. *EBioMedicine* **64**: 103187.

Figure legends

Figure 1 Effects of temperature on the virulence of *M. abscessus* subsp. *abscessus* ATCC19977 in silkworms.

Silkworms were injected with saline (50 µl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (1.4 x 10^7 cells per 50 µl) and incubated at (**A**) 27˚C and (**B**) 37˚C. n = 7 per group.

Figure 2 Determination of the *M. abscessus* subsp. *abscessus* ATCC19977 LD_{50} in silkworms.

Silkworms were injected with saline (50 µl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (4 x 10^5 – 1 x 10^8 cells per 50 µl) and incubated at 37˚C. The number of live and dead silkworms are indicated as 1 and 0, respectively. The curve is data from six independent experiments combined in a simple logistic regression model.

Figure 3 Increase of *M. abscessus* subsp. *abscessus* ATCC19977 viable cell counts in silkworms.

(**A**) Experiment schematic. (**B**) Silkworms were injected with *M. abscessus* subsp. *abscessus* ATCC19977 cell suspensions (7 x 10^6 cells per 50 µl) and incubated at 37˚C. Silkworm hemolymph was harvested at 3 or 30 h post-infection. The viable number of *M. abscessus* subsp. *abscessus* cells in the samples was measured by counting the colony-forming units (CFU). Statistically significant differences between groups were evaluated using Student *t*-test. n = 3 per group.

Figure 4 Effects of autoclaved cells and antibacterial treatment in silkworms infected with *M. abscessus* subsp. *abscessus*

(**A**) Silkworms were injected with either saline (50 µl), an *M. abscessus* subsp. *abscessus* cell suspension (1.1 x 10^7 cells per 50 µl), or an autoclaved *M. abscessus* subsp. *abscessus* cell suspension and incubated at 37˚C. n = 10 per group.

(**B**) Silkworms were injected with either saline (50 µl) or an *M. abscessus* subsp. *abscessus* cell suspension (6.3 x 10^7 cells per 50 µl) followed by clarithromycin (25 µg g^{-1} larva). The number of surviving silkworms following incubation at 37˚C was measured during 66 h. Statistically significant differences between groups were evaluated using a log-rank test. n = 10 per group.

Figure 5 Comparison of virulence among *M. abscessus* subsp. *abscessus* clinical isolates in a silkworm infection model

(**A**-**C**) Silkworms were injected with either saline, *M. abscessus* subsp. *abscessus*, Mb-7, Mb-10, Mb-14, Mb-16, Mb-17, Mb-18, or Mb-22 cell suspensions (2 x 10^5 – 3.5 x 10^7 cells per 50 µl) and incubated at 37˚C. Live and dead silkworms are indicated as 1 and 0, respectively. The curves are data from three independent experiments combined in a simple logistic regression model.

(**D**) A plot of LD_{50} values determined from A-C.

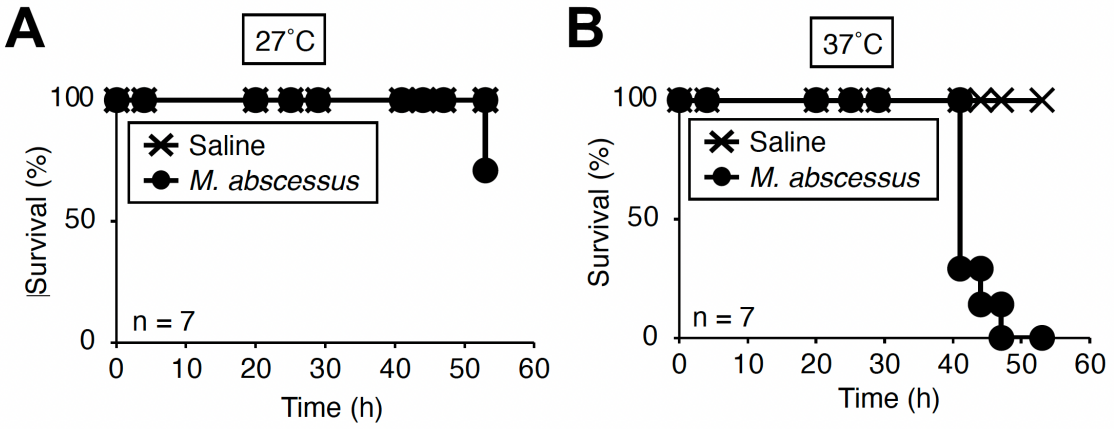

Figure 1. Effects of temperature on the pathogenicity of *M. abscessus* subsp. *abscessus* ATCC19977 in silkworms.
Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (1.4 x 10^7 cells/50 μl) and incubated at (**A**) 27˚C and (**B**) 37˚C. n = 7/group.

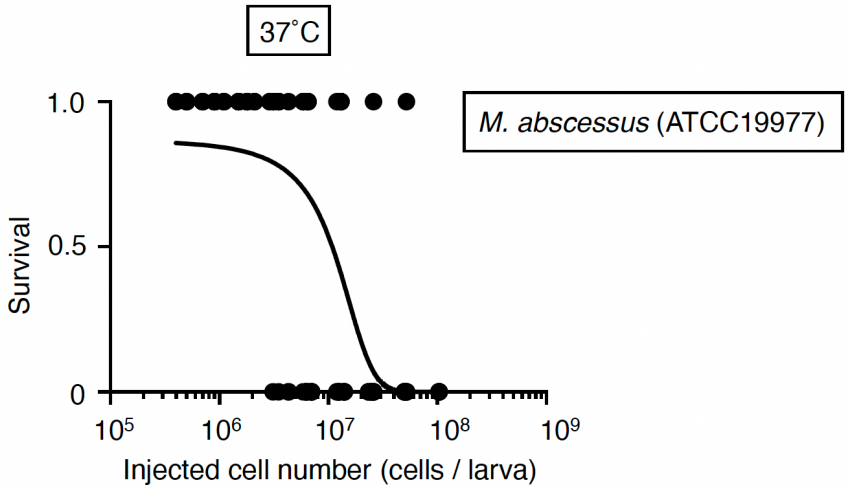

Figure 2. Determination of the *M. abscessus* subsp. *abscessus* ATCC19977 LD_{50} in silkworms.

Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (4 x 10^5 – 1 x 10^8 cells/50 μl) and incubated at 37˚C. The number of live and dead silkworms are indicated as 1 and 0, respectively. The curve is data from six independent experiments combined in a simple logistic regression model.

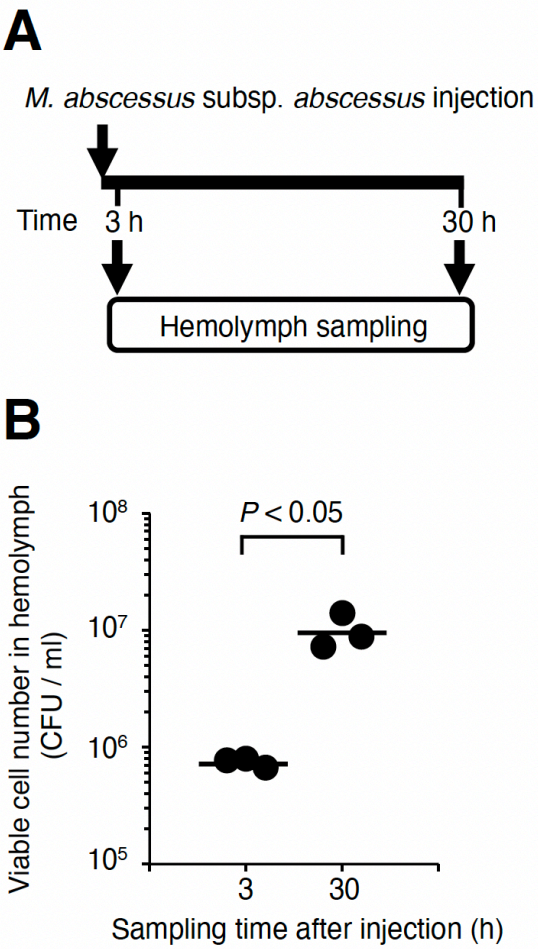

Figure 3. Increase of *M. abscessus* subsp. *abscessus* ATCC19977 viable cell counts in silkworms.

(**A**) Experiment schematic. (**B**) Silkworms were injected with *M. abscessus* subsp. *abscessus* ATCC19977 cell suspensions (7 x 10^6 cells/50 μl) and incubated at 37˚C. Silkworm hemolymph was harvested at 3 or 30 h post-infection. The viable number of *M. abscessus* subsp. *abscessus* cells in the samples was measured by counting the colony-forming units (CFU). Statistically significant differences between groups were evaluated using Student *t*-test. n = 3/group.

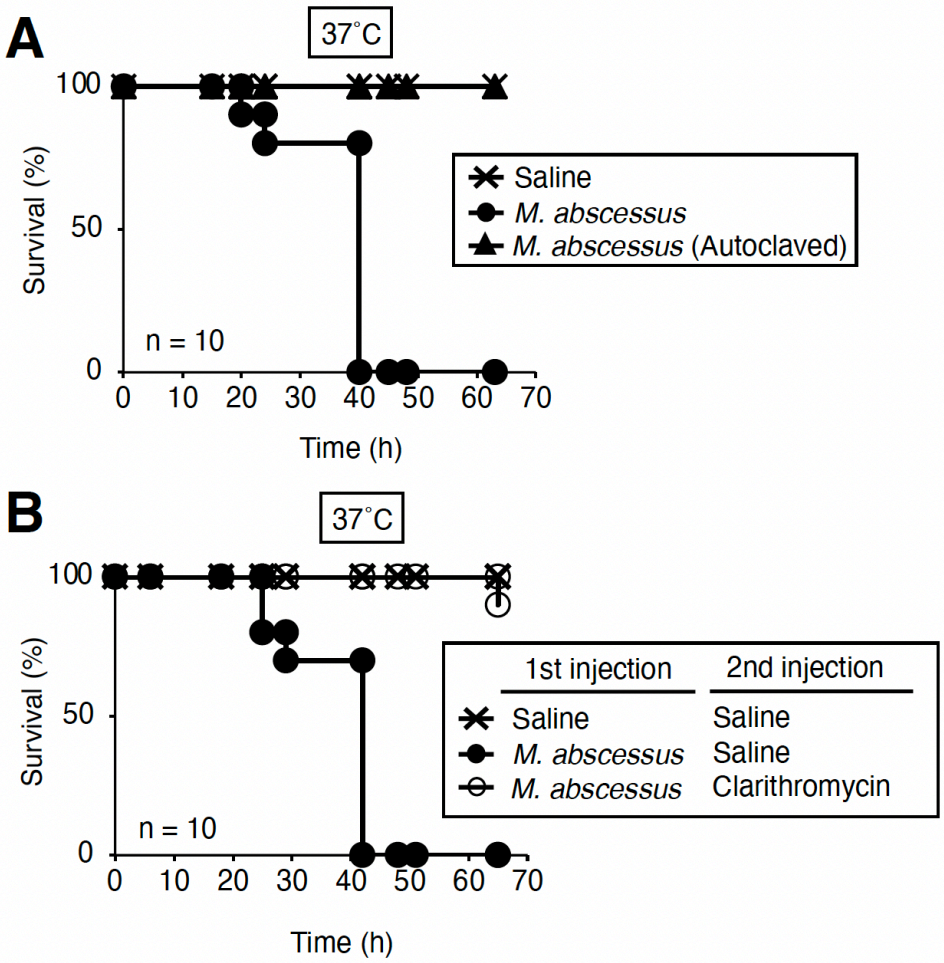

Figure 4. Effects of autoclaved cells and antibacterial treatment in silkworms infected with *M. abscessus* subsp. *abscessus*

(**A**) Silkworms were injected with either saline (50 μl), an *M. abscessus* subsp. *abscessus* cell suspension (1.1 x 10^7 cells/50 μl), or an autoclaved *M. abscessus* subsp. *abscessus* cell suspension and incubated at 37˚C. n = 10/group.

(**B**) Silkworms were injected with either saline (50 μl) or an *M. abscessus* subsp. *abscessus* cell suspension (6.3 x 10^7 cells/50 μl) followed by clarithromycin (25 μg/g larva). The number of surviving silkworms following incubation at 37˚C was measured during 66 h. Statistically significant differences between groups were evaluated using a log-rank test. n = 10/group.

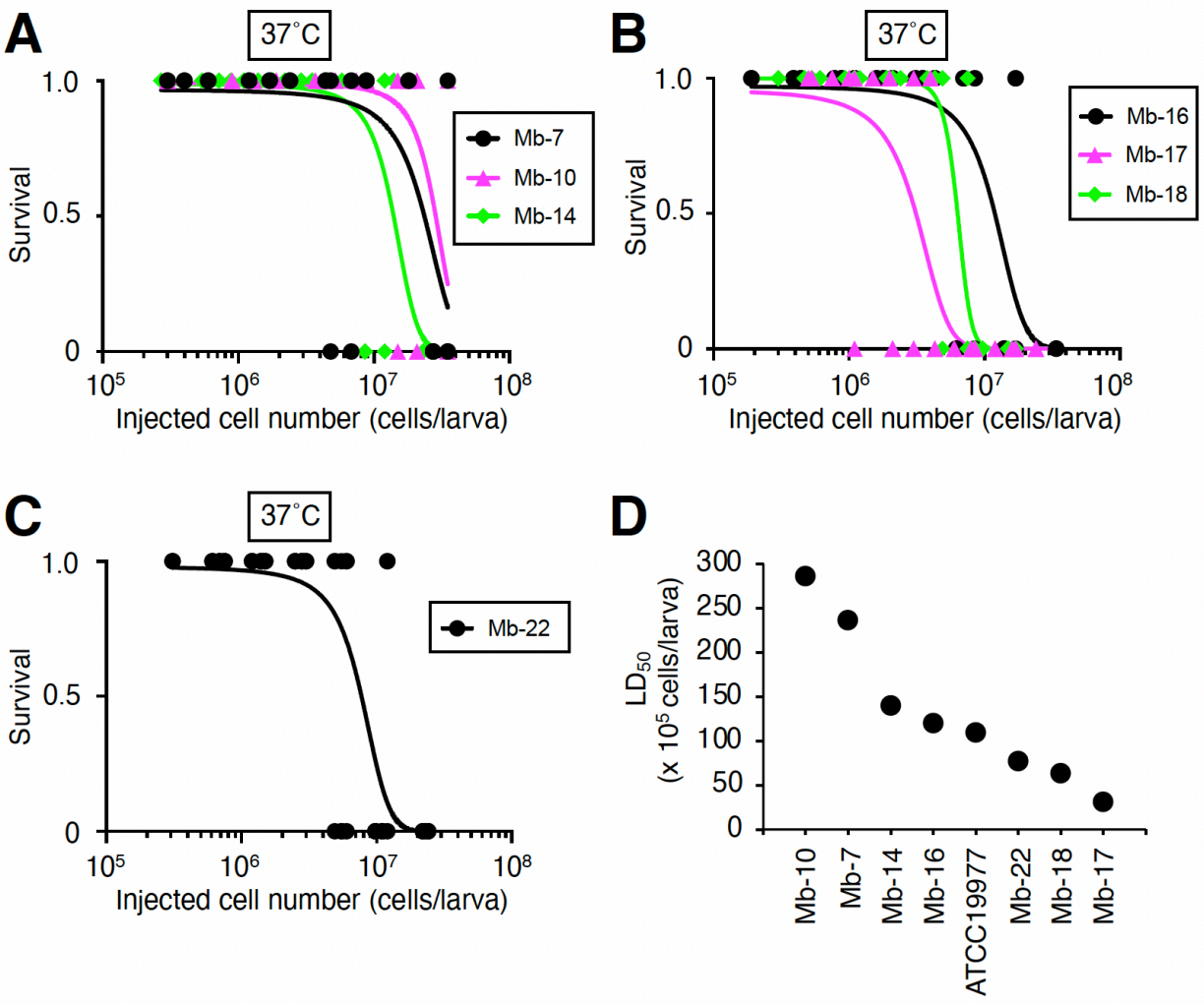

Figure 5. Comparison of pathogenicity among *M. abscessus* subsp. *abscessus* clinical isolates in a silkworm infection model

(**A**-**C**) Silkworms were injected with either saline, *M. abscessus* subsp. *abscessus*, Mb-7, Mb-10, Mb-14, Mb-16, Mb-17, Mb-18, or Mb-22 cell suspensions (2 x 10^5 – 3.5 x 10^7 cells/50 μl) and incubated at 37˚C. Live and dead silkworms are indicated as 1 and 0, respectively. The curves are data from three independent experiments combined in a simple logistic regression model.

(**D**) A plot of LD_{50} values determined from A-C.