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 10⁷ 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 10⁶ 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.

**LD₅₀ measurement**

LD₅₀ 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 10⁵ – 1 x 10⁶ cells), and incubation at 37°C. The survival number of silkworms was observed at 48 h. The LD₅₀ 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 x10⁹ cells) at 37°C, but not died at 27°C (Fig. 1). The LD₅₀ for *M. abscessus* subsp. *abscessus* ATCC19977 was 1.1 x 10⁷ 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 \textit{M. abscessus} subsp. abscessus growth.

**Comparative pathogenic analysis of \textit{M. abscessus} subsp. abscessus clinical isolates**

We next determined the LD$_{50}$ values of \textit{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 \textit{M. abscessus} subsp. abscessus. Their LD$_{50}$ values ranged from \(3.1 \times 10^6\) to \(2.9 \times 10^7\) 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 \textit{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 \textit{in vivo} evaluation system is useful for revealing the virulence of \textit{M. abscessus} subsp. abscessus clinical isolates in a shorter period of time (within two days).

\textit{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 \textit{M. abscessus} subsp. abscessus. Hosoda \textit{et. al.} reported to establish a silkworm infection model for evaluating the anti-mycobacterial compounds (Hosoda \textit{et al.}, 2020). Compared to that report, we demonstrated that \textit{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.

**Acknowledgements**

We thank Tae Nagamachi, Asami Yoshikawa, Yu Sugiyama, Eri Sato, and Asuka Toshima (Meiji Pharmaceutical University) for their technical assistance rearing the silkworms. We also thank Maki Okuda, Sayaka Kashiwagi, and Ginko Kaneda for their assistance. This study was supported in part by grants from the Japan Agency for Medical Research and Development/Japan International Cooperation Agency (AMED) to Y.H. (jp20fk0108064, jp20fk0108075, jp21fk0108093, jp21fk0108129, jp21fk0108608, jp21jm0510004, jp21wm0125007,
jp21wm0225004, and jp21wm0325003); by grants-in-aid for Fostering Joint International Research (B) to Y.H. (jp19KK0217) and for Early-Career Scientists to H.F. (jp18K15966); by Grants-in-Aid for Scientific Research (B) to Y.H. (jp20H02282); and for Scientific Research (C) to Y.M. (JP20K07022) from the Japan Society for the Promotion of Science (JSPS). The funders had no role in the study design, data collection, data analysis, the decision to publish, or preparation of the manuscript.

**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 10⁷ 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 LD₅₀ in silkworms.

Silkworms were injected with saline (50 µl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (4 x 10⁵ – 1 x 10⁸ 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 10⁶ 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 10⁷ 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 10⁷ 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 10⁵ – 3.5 x 10⁷ 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 LD₅₀ 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 10⁷ cells/50 μl) and incubated at (A) 27°C and (B) 37°C. n = 7/group.
Figure 2. Determination of the \textit{M. abscessus} subsp. \textit{abscessus} ATCC19977 LD$_{50}$ in silkworms.
Silkworms were injected with saline (50 \textmu l) or \textit{M. abscessus} subsp. \textit{abscessus} ATCC19977 cell suspension (4 x 10$^5$ – 1 x 10$^8$ cells/50 \textmu l) 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 10⁵ – 3.5 x 10⁷ 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 LD₅₀ values determined from A-C.