Quantitative evaluation of *Mycobacterium abscessus* clinical isolate virulence using a silkworm infection model

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Short title: Evaluation of *Mycobacterium abscessus* virulence using silkworms

Abstract

Mycobacterium abscessus causes chronic skin infections, lung diseases, and systemic or disseminated infections. Here we investigated whether the virulence of *M. abscessus* clinical isolates could be evaluated by calculating the median lethal dose (LD_{50}) in a silkworm infection model. *M. abscessus* subsp. *abscessus* cells were injected into the silkworm hemolymph. When reared at 37˚C, the silkworms died within 2 days post-infection with *M. abscessus* subsp. *abscessus*. Viable cell numbers of *M. abscessus* subsp. *abscessus* increased in the hemolymph of silkworms injected with *M. abscessus* subsp. *abscessus*. Silkworms were not killed by injections with heat-killed *M. abscessus* subsp. *abscessus* cells. The administration of clarithromycin, an antibacterial drug used to treat the infection, prolonged the survival time of silkworms injected with *M. abscessus* subsp. *abscessus*. The LD_{50} values of 7 clinical isolates were determined using the silkworm infection model and differed by up to 9-fold. The Mb-17 isolate, which was identified as a pathogenic strain in the silkworm infection model, induced more detachment of human THP-1-derived macrophages during infection than the Mb-10 isolate. These findings suggest that the silkworm *M. abscessus* infection model with can be used to quantitatively evaluate the virulence of *M. abscessus* clinical isolates in a short time period.

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

38 # Introduction

39 The *Mycobacterium abscessus* complex (MABC) is a group of rapidly growing non-tuberculous
40 mycobacteria that includes 3 subspecies: *M. abscessus* subsp. *abscessus*, *M.*
41 *abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletii* [1-4]. The MABC causes chronic
42 skin infections, lung diseases, and systemic or disseminated infections in immunocompromised
43 patients [5-8]. The virulence of MABC has evolved through a stepwise adaptation to the host and
44 soil environments [5,9], and could thus vary between clinical isolates. Several mouse MABC
45 infection models have been used to evaluate the effects of antibacterial drugs [10,11]. Mice
46 infected by MABC, however, die over several months, and thus the development of a more rapid
47 evaluation system is highly desired.

48 Silkworms are invertebrate animals beneficial for use in experiments to reveal host-
49 pathogen interactions [12-14]. A large number of silkworms can be reared in a small space
50 compared with mammalian animals [15]. The 3Rs, replacement, refinement, and reduction, are
51 important animal welfare principles for experiments using mammals [16]. Experiments using
52 invertebrates are consistent with the concept of replacement. Because the silkworm is an
53 invertebrate, fewer ethical issues are associated with the use of a large number of silkworms for
54 experimentation compared with mammals. By exploiting this advantage of silkworms for
55 infectious disease research, the median lethal dose (LD_{50}), which is the dose of a pathogen required
56 to kill half of the animals in a group, can be determined to quantitatively compare the virulence of
57 different strains [17,18]. Silkworm infection models are used as initial screening systems to
58 identify virulence-related genes in pathogenic microorganisms [19-22]. Silkworm infection
59 models are therefore useful for comparing the virulence of microorganisms [15]. A silkworm
60 infection model was established to evaluate anti-mycobacterial compounds using a type strain [23].

61 Virulence among *M. abscessus* clinical isolates based on the LD_{50} values, however, has not been
62 evaluated in a silkworm *M. abscessus* infection model.

63 In the present study, we compared the virulence of *M. abscessus* subsp. *abscessus* clinical
64 isolates by calculating the LD_{50} values in a silkworm infection model. Among the 7 clinical isolates
65 evaluated, the extent of the virulence varied up to 9-fold. Furthermore, using the silkworm
66 infection model, the *M. abscessus* subsp. *abscessus* Mb-17 isolate was identified as a highly
67 pathogenic strain that exhibits higher cytotoxic activity against human THP-1 macrophages
68 compared with the Mb-10 isolate. These findings suggest that the silkworm infection model is a
69 rapid evaluation system for quantitatively estimating the virulence of *M. abscessus*
70 subsp. *abscessus* clinical isolates.

71

72 Materials and Methods

73 Reagents

74 Clarithromycin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) was suspended in 0.9% NaCl
75 solution (saline). Middlebrook 7H9 broth, Middlebrook 7H10 agar, and Middlebrook OADC
76 enrichment were purchased from Becton, Dickinson, and Company (Sparks, MD, USA).
77 Middlebrook 7H9 broth and Middlebrook 7H10 agar were supplemented with 10% Middlebrook
78 OADC Enrichment.

79

80 Bacterial strains and growth

81 The *M. abscessus* subsp. *abscessus* ATCC19977 strain and 7 clinical isolates (Mb-7, Mb-10, Mb-
82 14, Mb-16, Mb-17, Mb-18, and Mb-22) were used in this study. The clinical isolates were obtained

83 from sputum samples of patients infected with *M. abscessus* subsp. *abscessus* at the Keio
84 University School of Medicine. This study was approved by the medical research ethics committee
85 of the National Institute of Infectious Diseases (#1046) and by the Keio University School of
86 Medicine Ethics Committee (#2008-0131-9 sai). Bacterial species were identified with a DDH
87 Mycobacteria Kit (Kyokuto Pharmaceutical Industrial Co., Tokyo, Japan) [24] and multiplex
88 polymerase chain reaction [25]. The *M. abscessus* subsp. *abscessus* strains were grown on a
89 Middlebrook 7H10 agar plate at 37˚C. A single colony was then inoculated into 5 ml of
90 Middlebrook 7H9 broth and incubated at 37˚C for 3 days.
91

92 **Infection experiments using silkworms**

93 The silkworm infection experiments were performed as previously described [26]. Fifth instar
94 larvae were reared on an artificial diet (Silkmate 2S, Ehime-Sanshu Co., Ltd., Ehime, Japan) for
95 24 h. *M. abscessus* subsp. *abscessus* cells grown in Middlebrook 7H9 broth were collected by
96 centrifugation and suspended in sterile saline. Silkworms injected with the *M. abscessus*
97 subsp. *abscessus* cells (1.4 x 10^7 cells per larva) were incubated at 27˚C or 37˚C, and their survival
98 was monitored.
99 The therapeutic activity of clarithromycin in silkworms was evaluated according to a previous
100 study with slight modifications [26]. Either 50 µl of saline or 50 µl of an *M. abscessus*
101 subsp. *abscessus* suspension was injected into the silkworm hemolymph. Clarithromycin (25 µg/g
102 larva) was immediately injected into the silkworms. The silkworms were incubated at 37˚C, and
103 their survival was monitored.
104

105 ## Viable cell counts

106 Silkworms were injected with an *M. abscessus* subsp. *abscessus* cell suspension (7 x 10^6 cells in
107 50 μl) and incubated at 37˚C. Hemolymph was harvested from the silkworm larvae through a cut
108 on the first proleg at either 3 or 18 h post-infection [17]. The hemolymph was added to saline, and
109 the solution was spread on a Middlebrook 7H10 agar plate. The agar plate was incubated at 37°C
110 for 3 days, and the colonies on the agar plate were counted.

111

112 ## LD_{50} measurement

113 The LD_{50} values were determined according to a previous study, with slight modifications [27,28].
114 *M. abscessus* subsp. *abscessus* cells grown in Middlebrook 7H9 broth were suspended in saline.
115 Either a 2- or 4-fold dilution series of the bacterial suspension was prepared. The bacterial
116 suspension (4 x 10^5 – 1 x 10^8 cells/50 μl) was injected into the silkworm hemolymph, and the
117 silkworms were incubated at 37˚C. The number of surviving silkworms was counted at 48 h after
118 infection. The LD_{50} values were determined from the data of 3 experiments using a simple logistic
119 regression model in Prism 9 (GraphPad Software, LLC, San Diego, CA, USA, https://www.graph
120 pad.com/scientific-software/prism/).

121

122 ## Cytotoxicity test using human THP-1–derived macrophages

123 To evaluate the cytotoxicities of *M. abscessus* subsp. *abscessus* clinical isolates against human
124 THP-1-derived macrophages, a high-content imaging analysis was performed to count the number
125 of cells attached to the polystyrene surface of a 96-well plate. Cell detachment from the well
126 correlated with cell death [29]. Therefore, the cytotoxicities of the *M. abscessus* subsp. *abscessus*

127 clinical isolates against THP-1–derived macrophages were determined by monitoring the cell
128 detachment. Human THP-1 monocytes were cultured in RPMI1640 medium (Wako Pure Chemical
129 Corporation, Osaka, Japan) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific
130 Inc., Waltham, MA, USA) and ampicillin (50 μg/mL) at 37˚C and 5% CO_2. Cells (2 x 10^4
131 cells/well) were seeded into 96-well plates (Cell Carrier-96well Ultra microplate; PerkinElmer Inc.,
132 Waltham, MA, USA) and differentiated to macrophages with phorbol 12-myristate 13-acetate (10
133 ng/mL) for 72 h. The single monolayer THP-1 macrophages were infected by *M. abscessus*
134 subsp. *abscessus* Mb-10 or Mb-17 isolates at a multiplicity of infection of 50, centrifuged at 1000
135 rpm for 5 min, and incubated for 4 h. To remove the extracellular bacteria, wells were washed
136 twice with phosphate-buffered saline (PBS) and RPMI 1640 with amikacin (200 μg/mL) was
137 added. After 2-h incubation, wells were washed twice with PBS and RPMI 1640 was added. The
138 plates were incubated for 48 h at 37˚C and 5% CO_2. After incubation, infected cells were fixed
139 with 4%(v/v) paraformaldehyde for 10 min at room temperature and washed 3 times with ice-cold
140 PBS before treating them with 0.1% Triton X-100 for 10 min, and then washing 3 times with PBS.
141 The cells were stained with Hoechst 33258 (1:1000; Dojindo Molecular Technologies, Inc.,
142 Kumamoto, Japan) and HCS Cell Mask Deep Red (1:20000; Thermo Fisher Scientific Inc., MA,
143 USA) for 25 min at room temperature and then washed 3 times with PBS. Stained cell images
144 were obtained using a High-Content Imaging System Operetta CLS (PerkinElmer Inc.) with 40x
145 Air/0.6 NA. The number of attached cells (cytoplasm with nuclei) was automatically calculated
146 by Harmony software (PerkinElmer Inc.). Data are expressed as the mean ± the standard deviation
147 (SD). Statistical analysis was performed with GraphPad Prism 9 (GraphPad Software).

148

149 **Statistical analysis**

150 The statistical significance of differences between viable cell counts of *M. abscessus*
151 subsp. *abscessus* in silkworm groups were determined by the Student *t*-test. Statistically significant
152 differences between survival curves in the silkworm infection experiments were evaluated using a
153 log-rank test in GraphPad Prism 9 (GraphPad Software). The statistical significance between Mb-
154 10 and MB-17 isolates in the infection experiment using human THP-1 macrophages was
155 calculated using the unpaired Student *t*-test. Values of $P < 0.05$ were considered significant.
156

157 # Results

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

160 We first determined the experimental conditions for evaluating the virulence of *M. abscessus*
161 subsp. *abscessus* in silkworms. In silkworm infection experiments, the rearing temperature is
162 critical because it affects bacterial virulence and silkworm health [15]. The standard silkworm
163 rearing temperature is 27˚C, and 37˚C corresponds to human body temperature. We previously
164 reported that administration of *Staphylococcus aureus* (1 x 10^7 cells) to silkworms killed them in
165 3 days under rearing conditions of 27˚C [19]. Within 48 h of injection of *M. abscessus* subsp.
166 *abscessus* ATCC19977 strain (1.4 x10^7 cells), silkworms died at 37˚C, but did not die at 27°C (Fig.
167 1A and B). The viable cell count of *M. abscessus* subsp. *abscessus* in the silkworm hemolymph at
168 18 h post-infection at 37˚C was higher than that at 27˚C (Fig. 1C). The LD_{50} for *M. abscessus*
169 subsp. *abscessus* ATCC19977 was 1.1 x 10^7 cells under a 37˚C rearing condition (Fig. 2). These
170 results suggest that a 37˚C rearing condition is necessary for *M. abscessus* subsp. *abscessus*-
171 induced silkworm death within 2 days.

172

173 **Fig 1. Effects of temperature on the virulence of *M. abscessus* subsp. *abscessus* ATCC19977**

174 **in silkworms.**

175 Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell

176 suspension (1.4 x 10^7 cells per 50 μl) and incubated at (**A**) 27˚C and (**B**) 37˚C. The curves were

177 drawn by the Kaplan-Meier method. n = 7 per group. (**C**) Silkworms were injected with *M.*

178 *abscessus* subsp. *abscessus* ATCC19977 cell suspension (2.9 x 10^8 cells per 50 μl) and incubated

179 at 27˚C and 37˚C. Silkworm hemolymph was harvested at 18 h post-infection. Statistically

180 significant differences between groups were evaluated using the Student *t*-test. n = 3 per group.

181

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

183 Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell

184 suspension (4 x 10^5 – 1 x 10^8 cells per 50 μl) and incubated at 37˚C for 2 days. The numbers of

185 live and dead silkworms are indicated as 1 and 0, respectively. The curve represents data from 6

186 independent experiments combined in a simple logistic regression model. n = 129 per group.

187

188 Virulence effect of *M. abscessus* subsp. *abscessus* proliferation in

189 silkworms

190 Because silkworm death caused by *Porphyromonas gingivalis* in a previous report did not require

191 proliferation, we assumed that the silkworm death was caused by shock not induced by the

192 bacterial infection [30]. We tested whether *M. abscessus* subsp. *abscessus* proliferates in the

193 silkworm body, and if proliferation is necessary for virulence. The *M. abscessus* subsp. *abscessus*

194 viable cell count increased in the silkworm hemolymph at 18 h post-infection (Fig. 3). The

195 injection of autoclaved *M. abscessus* subsp. *abscessus* cells did not kill silkworms (Fig. 4A).
196 Clarithromycin, a bacteriostatic antibiotic, is used to treat clinical *M. abscessus* subsp. *abscessus*
197 infections [8,31]. The administration of clarithromycin to silkworms infected with *M. abscessus*
198 subsp. *abscessus* prolonged their survival time (Fig. 4B). These results suggest that the virulence
199 of *M. abscessus* subsp. *abscessus* against silkworms requires the growth of *M. abscessus*
200 subsp. *abscessus*.
201
202 **Fig 3. Increase of *M. abscessus* subsp. *abscessus* ATCC19977 viable cell counts in silkworms.**
203 (**A**) Experiment schematic. (**B**) Silkworms were injected with *M. abscessus* subsp. *abscessus*
204 ATCC19977 cell suspensions (5 x 10^8 cells per 50 µl) and incubated at 37˚C. Silkworm
205 hemolymph was harvested at 3 or 18 h post-infection. The viable cell number of *M. abscessus*
206 subsp. *abscessus* was measured by counting the colony-forming units (CFU). Statistically
207 significant differences between groups were evaluated using the Student *t*-test. n = 3 per group.
208
209 **Fig 4. Effects of autoclaved cells and antibacterial treatment in silkworms infected with *M.***
210 ***abscessus* subsp. *abscessus***
211 (**A**) Silkworms were injected with either saline (50 µl), *M. abscessus* subsp. *abscessus*
212 ATCC19977 cell suspension (1.1 x 10^7 cells per 50 µl), or autoclaved *M. abscessus*
213 subsp. *abscessus* ATCC19977 cell suspension and incubated at 37˚C. n = 10 per group.
214 (**B**) Silkworms were injected with either saline (50 µl) or *M. abscessus* subsp. *abscessus*
215 ATCC19977 cell suspension (6.3 x 10^7 cells per 50 µl) followed by clarithromycin (25 µg g^{-1} larva).
216 The number of surviving silkworms following incubation at 37˚C was measured for 66 h.
217 Statistically significant differences between groups were evaluated using a log-rank test based on

218 the curves by the Kaplan-Meier method. n = 10 per group.

219

220 Evaluating the pathogenicity of *M. abscessus* subsp. *abscessus* clinical 221 isolates against silkworms

222 We next determined the LD_{50} values of *M. abscessus* subsp. *abscessus* clinical isolates using the
223 silkworm infection model to compare their virulence. Seven clinical isolates were obtained from
224 sputum samples of patients infected with *M. abscessus* subsp. *abscessus*. Their LD_{50} values ranged
225 from 3.1 x 10^6 to 2.9 x 10^7 cells per larva, with the LD_{50} value of the Mb-17 isolate being the
226 lowest (Fig. 5). The LD_{50} value of the Mb-17 isolate was 9-fold lower than that of the Mb-10
227 isolate. The viable cell count of the Mb-17 isolate in the silkworm hemolymph at 18 h post-
228 infection was higher than that of the Mb-10 isolate (Fig. 6). These results suggest that the Mb-17
229 isolate has higher pathogenicity against silkworms than the Mb-10 isolate.

230

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

233 (**A**-**C**) Silkworms were injected with either saline (50 µl), *M. abscessus* subsp. *abscessus*, Mb-7,
234 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
235 50 µl) and incubated at 37˚C. Live and dead silkworms are indicated as 1 and 0, respectively.
236 Curves represent data from 3 independent experiments combined in a simple logistic regression
237 model. n = 39-54 per group.

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

239

240 **Fig 6. Viable cell counts of *M. abscessus* subsp. *abscessus* Mb-10 and Mb-17 isolates in**
241 **silkworms.**

242 Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* Mb-10 cell
243 suspension (3.6 x 10^8 cells per 50 μl) or Mb-17 cell suspension (3.8 x 10^8 cells per 50 μl) and
244 incubated at 37˚C. Silkworm hemolymph was harvested at 18 h post-infection. n = 5 per group.
245 Statistically significant differences between groups were evaluated using the Student *t*-test.

246

247 Evaluating the cytotoxicities of *M. abscessus* subsp. *abscessus* clinical
248 isolates against human THP-1–derived macrophages

249 Human THP-1-derived macrophages can adhere to the polystyrene surface of a 96-well plate.
250 Chemical induced cell detachment from the well correlates with cell death [29]. We next
251 determined the cytotoxicities of the Mb-10 and Mb-17 isolates against THP-1–derived
252 macrophages by monitoring cell detachment. The number of macrophages attached to the well was
253 decreased by infection with these strains at a multiplicity of infection of 50 (Fig. 7). The Mb-17
254 isolate led to a decrease in the number of attached macrophages compared with the Mb-10 isolate
255 (Fig. 7). The result suggests that the Mb-17 isolate, which was identified as a highly pathogenic
256 strain using the silkworm infection model, induces a greater detachment of THP-1–derived
257 macrophages during infection than the Mb-10 isolate.

258

259 **Fig 7. Attached-cell counts of human THP-1 macrophages after infection with *M. abscessus***
260 ***subsp. abscessus* Mb-10 and Mb-17 isolates.**

261 Attached-cell counts of human THP-1 macrophages at 48 h after infection with *M. abscessus* subsp.

262 *abscessus* Mb-10 or Mb-17 cells at a multiplicity of infection of 50. The number of nuclei of
263 macrophages attached to the well was calculated using High-Content Imaging System Operetta
264 CLS with Harmony software. n = 3 per group. Statistically significant differences between groups
265 were evaluated using the Student *t*-test.

266

267 Discussion

268 In the present study, the virulence of *M. abscessus* subsp. *abscessus* clinical isolates was compared
269 using a silkworm infection model. Among the 7 clinical isolates, the virulence, as determined by
270 the LD_{50}, varied up to 9-fold. These results indicate that the *in vivo* silkworm evaluation system is
271 useful for revealing the virulence of *M. abscessus* subsp. *abscessus* clinical isolates with a short
272 time period (2 days).

273 *M. abscessus* subsp. *abscessus*-infected silkworms incubated at 37˚C were more sensitive
274 to infection than those reared at 27˚C. We assumed that this difference was due to both high-
275 temperature stress in silkworms and the optimal growth temperature for *M. abscessus*
276 subsp. *abscessus*. Hosoda *et al*. reported establishing a silkworm infection model for evaluating
277 anti-mycobacterial compounds [23]. Here, we demonstrated that *M. abscessus* subsp. *abscessus*
278 grows in the silkworm hemolymph and compared the virulence of several clinical isolates. Our
279 findings are important toward validating the usefulness of the silkworm infection model for
280 estimating the virulence of *M. abscessus* subsp. *abscessus* clinical isolates. The LT_{50} value, which
281 is the incubation time required to kill half of the silkworms in a group, differed slightly among
282 time-course experiments. Therefore, a secondary evaluation to determine the LD_{50} values based
283 on multiple dose-dependent experiments is needed.

284 *M. abscessus* subsp. *abscessus* virulence may correlate with severe infection [5,9].

285 Therefore, understanding *M. abscessus* subsp. *abscessus* clinical isolate virulence is useful
286 information for infection control. *M. abscessus* subsp. *abscessus* exhibited a different extent of
287 virulence among clinical isolates in the silkworm model. We demonstrated that the silkworm
288 infection model with *M. abscessus* subsp. *abscessus* is advantageous for quantitative
289 determination of clinical isolate virulence by calculating the LD_{50} values within 2 days. Moreover,
290 the LD_{50} values among the clinical isolates differed up to 9-fold. The Mb-17 isolate was the most
291 pathogenic against silkworms among the *M. abscessus* subsp. *abscessus* clinical isolates used in
292 this study. We hypothesized that the Mb-17 isolates harbor virulence-related genes that enhance
293 the infection process. Moreover, the cytotoxicity of the Mb-17 isolate against human THP-1–
294 derived macrophages was higher than that of the Mb-10 isolate. These results suggest that the Mb-
295 17 isolate, which was identified as a highly pathogenic strain using the silkworm infection model,
296 is also highly cytotoxic to human macrophages. Future studies will include experiments aimed at
297 revealing the relationship between the information on disease parameters in patients and the
298 virulence against silkworms. The virulence genes of several pathogens have been identified by
299 avirulent mutant screening using silkworm infection models from a mutant library [19-22]. The
300 method for constructing *M. abscessus* subsp. *abscessus* gene-deletion mutants is well established
301 [32,33]. Further studies are needed to determine the virulence factors harbored by the *M. abscessus*
302 subsp. *abscessus* Mb-17 isolate that are responsible for its virulence in silkworms.

303 *M. abscessus* vertebrate infection models using a zebrafish, *Danio rerio*, and a tadpole,
304 *Xenopus laevis*, have been reported [32,34,35]. These infection models are used to evaluate anti-
305 mycobacterial drugs and virulence within 15 days. Because these model animals are vertebrates,
306 however, more ethical problems are associated with their use with respect to animal welfare. The
307 3Rs, replacement, refinement, and reduction, are important principles for experiments using

308 mammals [16]. Silkworms are invertebrate animals with several advantages as an alternative
309 model animal for infection experiments requiring a high number of animals. Moreover, *M.*
310 *abscessus* subsp. *abscessus* virulence factors relevant to human pathogenicity could be identified
311 using this silkworm infection model.
312

313 Conclusion

314 We propose that the silkworm infection model with *M. abscessus* subsp. *abscessus* is an
315 advantageous assay system for determining the virulence of *M. abscessus* subsp. *abscessus* clinical
316 strains in a short time period. The silkworm infection model may contribute to revealing the
317 molecular mechanisms of *M. abscessus* subsp. *abscessus* infections.
318

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Supporting information

S1 File. Datasets included in this study.

Fig 1.

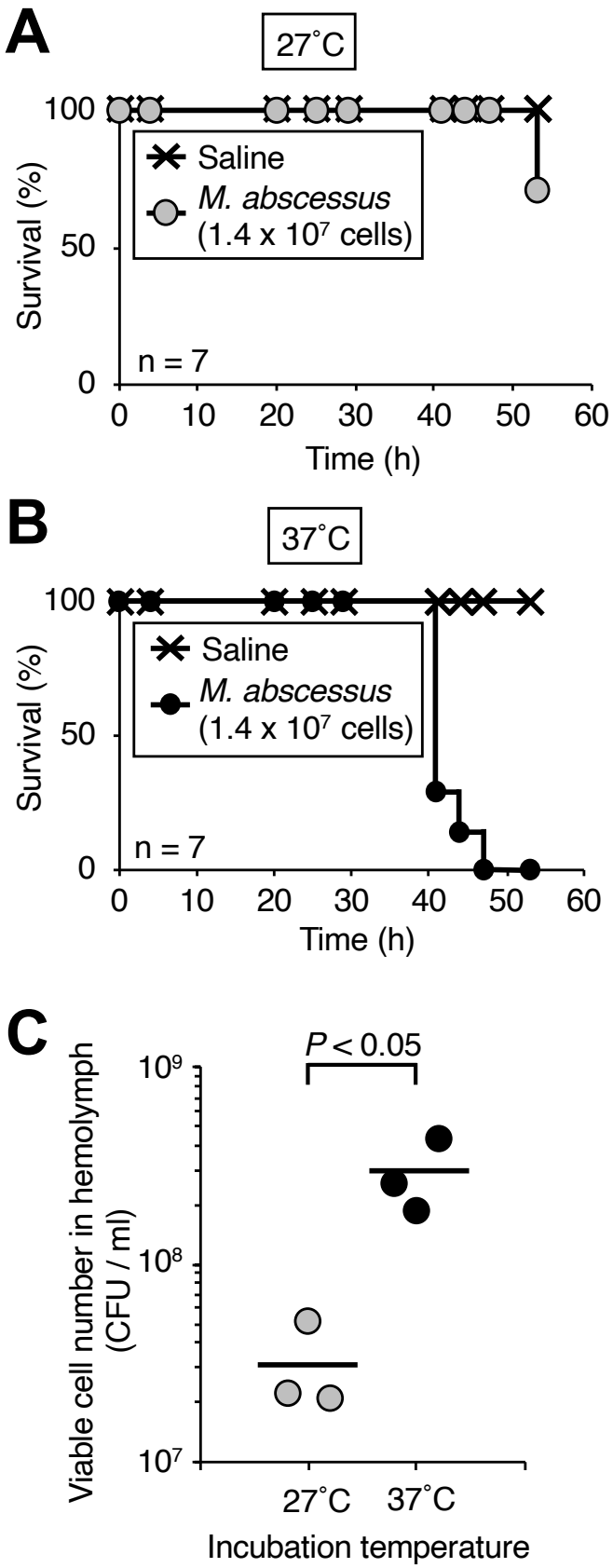

Fig 1. Effects of temperature on the virulence of *M. abscessus* subsp. *abscessus* ATCC19977 in silkworms.
Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (1.4 x 10^7 cells per 50 μl) and incubated at (**A**) 27˚C and (**B**) 37˚C. The curves were drawn by the Kaplan-Meier method. n = 7 per group. (**C**) Silkworms were injected with *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (2.9 x 10^8 cells per 50 μl) and incubated at 27˚C and 37˚C. Silkworm hemolymph was harvested at 18 h post-infection. Statistically significant differences between groups were evaluated using the Student *t*-test. n = 3 per group.

Fig 2.

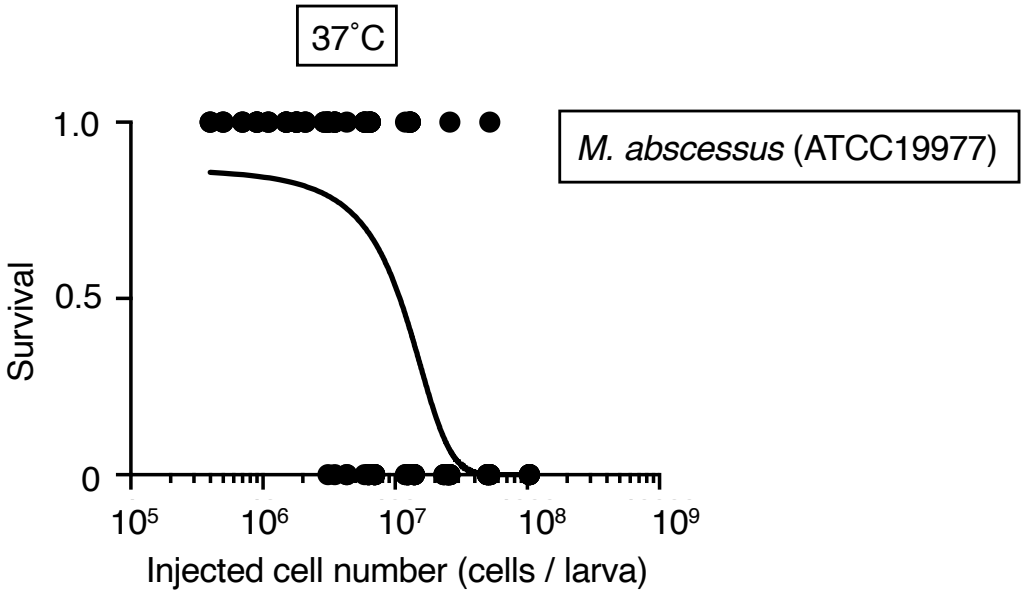

Fig 2. Determination of the *M. abscessus* subsp. *abscessus* ATCC19977 LD_{50} in silkworms.
Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (4×10^5 – 1×10^8 cells per 50 μl) and incubated at 37˚C for 2 days. The numbers of live and dead silkworms are indicated as 1 and 0, respectively. The curve represents data from 6 independent experiments combined in a simple logistic regression model. n = 129 per group.

Fig 3.

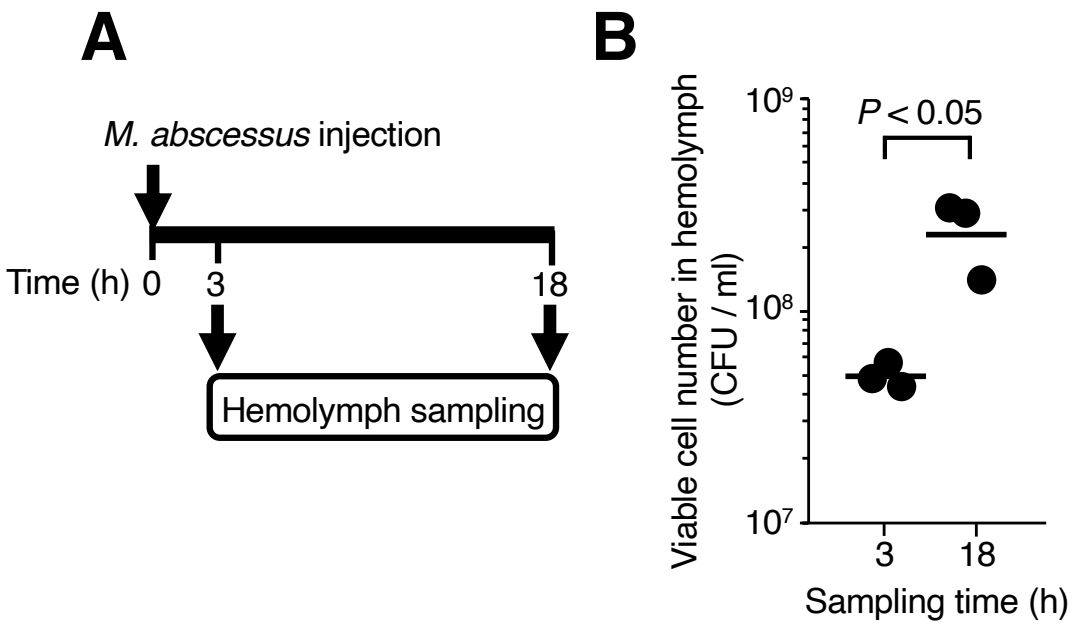

Fig 3. Increase of *M. abscessus* subsp. *abscessus* ATCC19977 viable cell counts in silkworms.
(**A**) Experiment schematic. (**B**) Silkworms were injected with *M. abscessus* subsp. *abscessus* ATCC19977 cell suspensions (5 x 10^8 cells per 50 μl) and incubated at 37˚C. Silkworm hemolymph was harvested at 3 or 18 h post-infection. The viable cell number of *M. abscessus* subsp. *abscessus* was measured by counting the colony-forming units (CFU). Statistically significant differences between groups were evaluated using the Student *t*-test. n = 3 per group.

Fig 4.

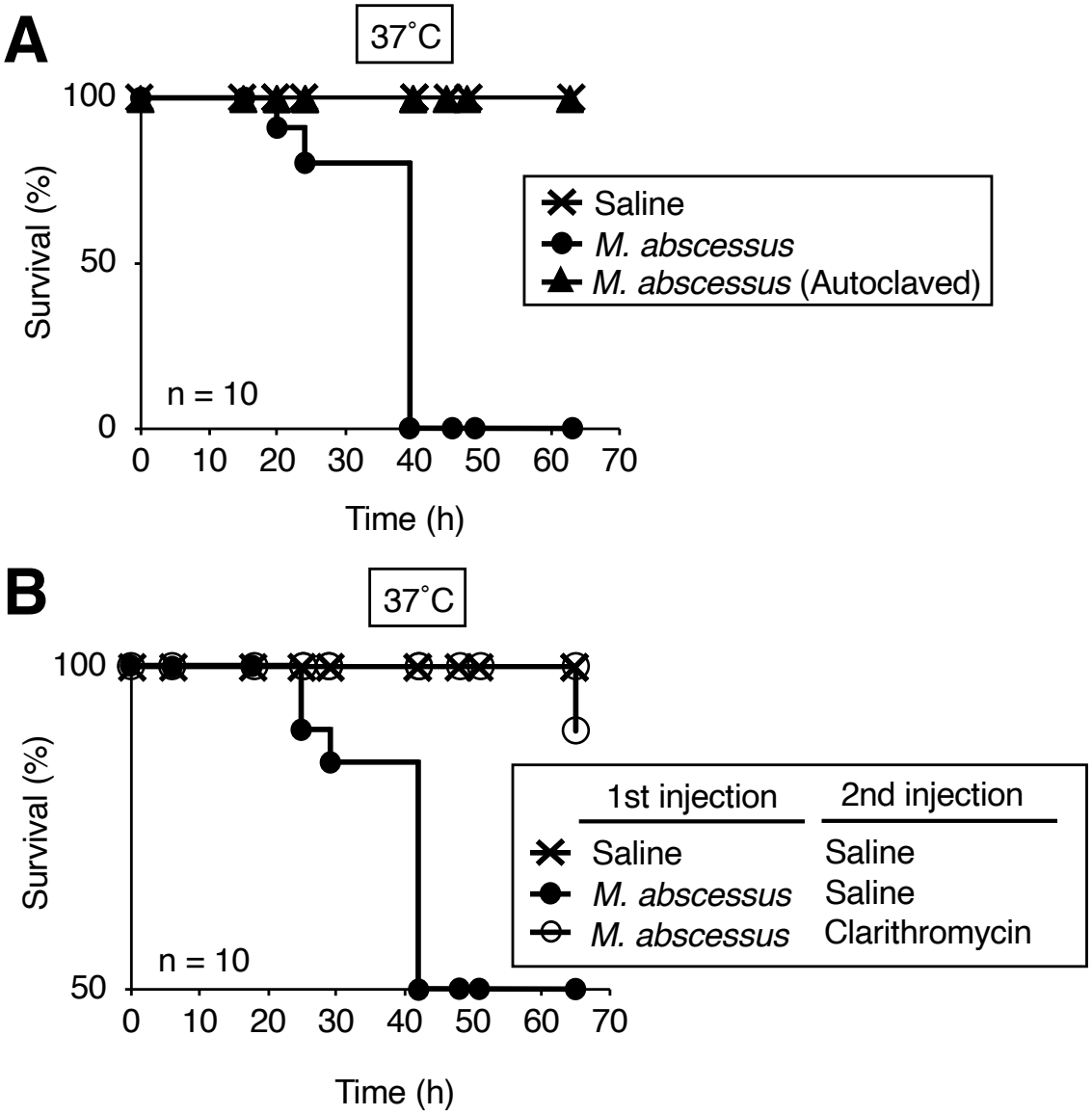

Fig 4. Effects of autoclaved cells and antibacterial treatment in silkworms infected with *M. abscessus* subsp. *abscessus*

(**A**) Silkworms were injected with either saline (50 μl), *M. abscessus* subsp. *abscessus* ATCC19977 cell suspension (1.1 x 10^7 cells per 50 μl), or autoclaved *M. abscessus* subsp. abscessus ATCC19977 cell suspension and incubated at 37˚C. n = 10 per group.

(**B**) Silkworms were injected with either saline (50 μl) or *M. abscessus* subsp. *abscessus* ATCC19977 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 for 66 h. Statistically significant differences between groups were evaluated using a log-rank test based on the curves by the Kaplan-Meier method. n = 10 per group.

Fig 5.

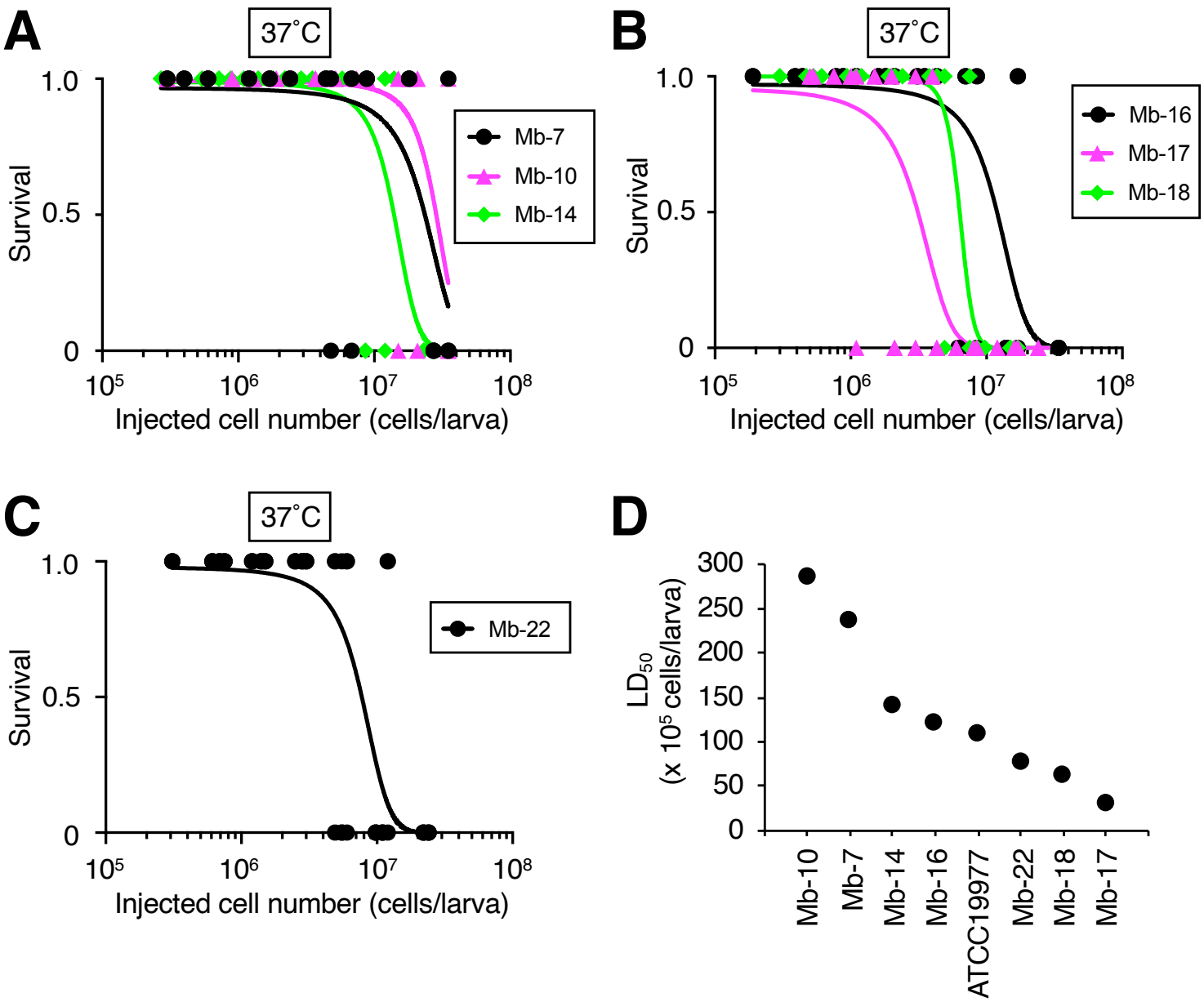

Fig 5. Comparison of virulence among *M. abscessus* subsp. *abscessus* clinical isolates in a silkworm infection model

(**A**-**C**) Silkworms were injected with either saline (50 μl), *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. Curves represent data from 3 independent experiments combined in a simple logistic regression model. n = 39-54 per group.

(**D**) Plot of LD50 values determined from **A**-**C**.

Fig 6.

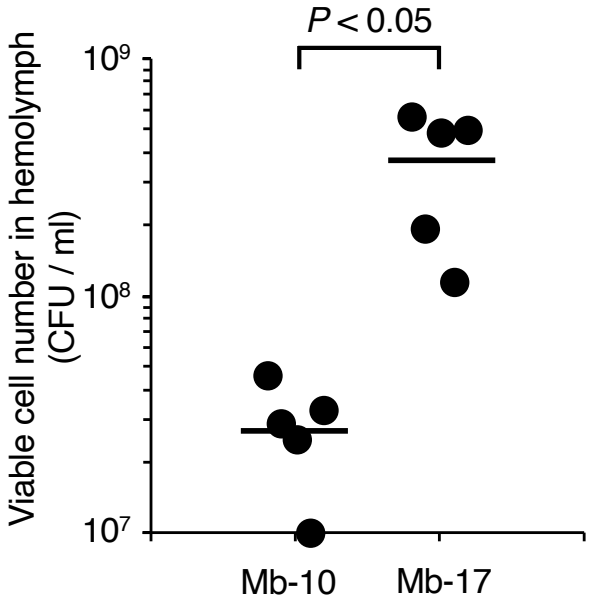

Fig 6. Viable cell counts of *M. abscessus* subsp. *abscessus* Mb-10 and Mb-17 isolates in silkworms.

Silkworms were injected with saline (50 μl) or *M. abscessus* subsp. *abscessus* Mb-10 cell suspension (3.6 x 10^8 cells per 50 μl) or Mb-17 cell suspension (3.8 x 10^8 cells per 50 μl) and incubated at 37˚C. Silkworm hemolymph was harvested at 18 h post-infection. n = 5 per group. Statistically significant differences between groups were evaluated using the Student *t*-test.

Fig 7.

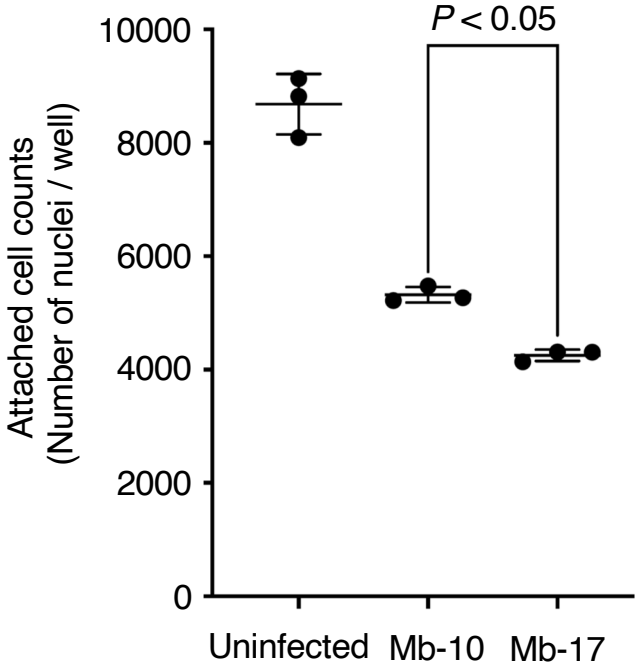

Fig 7. Attached-cell counts of human THP-1 macrophages after infection with *M. abscessus* subsp*. abscessus* Mb-10 and Mb-17 isolates.
Attached-cell counts of human THP-1 macrophages at 48 h after infection with *M. abscessus* subsp. *abscessus* Mb-10 or Mb-17 cells at a multiplicity of infection of 50. The number of nuclei of macrophages attached to the well was calculated using High-Content Imaging System Operetta CLS with Harmony software. n = 3 per group. Statistically significant differences between groups were evaluated using the Student *t*-test.