Original Article

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Histopathological analysis of filament formation of *Nocardia farcinica* in a silkworm infection model

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SUMMARY The silkworm *Nocardia* infection model has been established as a useful animal model for screening the pathogenicity of *Nocardia* and evaluating the therapeutic effects of antimicrobial agents against *Nocardia* infection. No histopathological analysis of silkworms infected with *Nocardia farcinica* has yet been performed. In this study, we performed histological analyses on organs of silkworms infected with *N. farcinica*. One day after infection with *N. farcinica*, the organism developed a branching filamentous form from coccid cells in the hemolymph. In addition, we evaluated effective doses (ED_{50}) values by treating infected silkworms with amikacin 30 seconds and 24 hours after infection and found that the ED_{50} values treated within 30 seconds and 24 hours after infection were 4.1 μg/larva and 5.6 μg/larva, respectively. Evaluation of treatment with amikacin against the infected silkworms was unaffected by the growth process form of *Nocardia*. These results suggest that the silkworm *Nocardia* infection model is a useful tool for evaluating the antimicrobial therapy in the growth process of the *N. farcinica*.

Keywords *Nocardia farcinica*, silkworm, filament, amikacin, infection

1. Introduction

Nocardia farcinica, a ubiquitous bacterial species in an environment such as soil organic material and water (*1*), is an important opportunistic pathogen, causing infections in the lung, blood, and central nervous system sites in immunocompromised patients (*2,3*). In addition, nocardial brain abscesses by *N. farcinica* rarely occur in healthy individuals (*4*). This organism is more likely to cause disseminated infection and higher mortality than other *Nocardia* species (*5-7*). The whole-genome sequencing identified virulence factors in the organism, including genes responsible for antimicrobial resistance and virulence (*8,9*). However, current research on pathogenesis and virulence using a mouse model on this organism is limited (*10*). In addition, although an antimicrobial susceptibility test is necessary for selecting antimicrobial agents for infection by *N. farcinica*, the correlation between the results of an *in vitro* antimicrobial susceptibility test and the *in vivo* response to antimicrobial therapy in patients with *Nocardia* infection has not been adequately investigated (*11*). These problems are due to very few reports of experimental infections with this organism in a mouse model.

Animal experiments using mice and other mammalian models have contributed to the development of pathological analysis, drug discovery, and medicine related to human diseases (*12*). However, high costs and ethical considerations have been a hindrance to international experimentation with mammalian infections (*13,14*), so it is desirable to develop an experimental animal model that can serve as an alternative to mammals. For this purpose, invertebrate larvae, such as the Waxworm (*Galleria mellonella*), the Hornworm (*Manduca sexta*), and the cotton bollworm (*Helicoverpa armigera*) have attracted attention as alternative models for mice (*13,15,16*), and the silkworm (*Bombyx mori*) is one of non-mammalian animals (*13*).

Silkworms can be reared in a small space at low cost and used for experiments on large numbers of individuals, and they grow to a size that can be used for

experiments after about three weeks of rearing (*13,17,18*). Silkworm infection models have been established to date for various pathogenic microorganisms, including *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus anthracis* (*13,19*), as well as *Aspergillus fumigatus* and *Cryptococcus neoformans* (*20,21*). A silkworm *Nocardia* infection model has been established for the evaluation of pathogenicity of *N. farcinica* (*22*). However, the focal area of *N. farcinica* in the silkworm *Nocardia* infection model is not identified. Histopathological analysis is a direct method of revealing the causal relationship between a lesion and its cause. Recently, those silkworm infection models have yielded to histopathological analyses, to identify and observe the focal site of infection (*20,23,24*), however, not with *N. farcinica*.

The aims of the present study were to observe the accumulation of *N. farcinica* in the hemolymph of infected silkworms using histopathological analysis, then to elucidate if changes in the filament morphology of the organism over time could be observed. We finally determined the utility of the silkworm *Nocardia* infection model, which may represent a potentially useful tool for studying the mechanisms of the growth process of *N. farcinica* in infected lesions.

2. Materials and Methods

2.1. Bacterial strain, culture condition, and adjustment of bacterial cell suspension

The *N. farcinica* TUTN006 strain was used in this study. This strain was stored at - 80°C in a microbank tube (Iwaki & Co., Ltd., Tokyo, Japan) and cultured on a brain heart infusion agar plate (BHI, Eiken Chemical CO., Ltd., Tokyo, Japan) under CO_2 conditions at 37°C for 72 h. The colonies grown on the BHI agar plate were picked up using a 10 μg loop, suspended in sterile 1 mm glass beads (Fuji Manufacturing CO., Ltd., Tokyo, Japan) and 4 mL sterile saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan), and after 3 minutes of vortex and adjusted to OD_{600}=2.5~2.6 using a UVmini-1240 spectrophotometer (Shimadzu Co., Ltd., Kyoto, Japan). The bacterial cell suspension was diluted 100-fold using sterile saline, and used for silkworm infection experiments.

2.2. Silkworm rearing

Silkworm eggs were purchased from Ehime Sansyu Co. LTD (Ehime, Japan), disinfected, and reared at 27°C. The silkworms were fed Silkmate 2S (Katakura Industries Co., Ltd., Tokyo, Japan) until they developed the fifth molted larva. Fifth-instar larvae were fasting for 24 h and used for the infection experiment.

2.3. Silkworm infection experiment

An experimental protocol on producing silkworm infection models were described elsewhere (*22*). Briefly, either 50 μL bacterial cell suspension of *N. farcinica* TUTN006 cells (3.2×10^6 cells per larva) or saline (the control group) was administered to the silkworm hemolymph by injecting the silkworm dorsally using a 1 ml tuberculin syringe (Terumo Medical CO., Ltd., Tokyo, Japan). The amount of bacteria inoculated into silkworms was measured by plating the used bacterial cell suspension on the BHI agar plate. After inoculation, silkworms were reared in incubators at 25-27°C without feeding and observed over time.

2.4. Histopathological analysis

Silkworms were fixed at 15, 18, 24, 28 and 40 hours after inoculation of bacterial cell suspension. Tissue specimens of silkworms fixed in 10% neutral buffered formalin (Fujifilm Wako Pure Chemicals Co., Ltd., Osaka, Japan) were prepared according to standard pathology physiological techniques. Briefly, for fixation, silkworms were placed on ice in a state of suspended animation, wrapped in gauze as they were to prevent bending, and then immersed in 10% neutral buffered formalin for 72 hours while pinned to an eraser. After fixation was completed, silkworms were cut out by inserting a blade from the dorsal side and slicing them into rings. After paraffin embedding, sections were thinned to 3 μm thickness, pasted onto Super Frost (Matsunami Glass Industries Co., Ltd., Osaka, Japan), stretched at 50°C, and dried at 37°C. Each paraffin section was deparaffinized, de-xyleneated, and rinsed under running water, and then staining of each was performed. Hematoxylin-eosin (HE) staining was performed by staining with Carracci's hematoxylin for 7 minutes, fractionating and coloring with 1% hydrochloric alcohol, rinsing, passing through distilled water, staining with eosin for 3 minutes, dehydrating, permeabilizing, and encapsulation. The specimens were stained with Gram staining (Hucker) methods (Muto Chemical Co., Ltd., Tokyo, Japan), and sealed in xylene. After staining, each specimen was observed and photographed using an OlympusBX43 (Olympus Co., Ltd., Tokyo, Japan) and Olympus DP21 (Olympus Co., Ltd., Tokyo, Japan) optical microscope. For some specimens, the virtual slide scanner NanoZoomer (Hamamatsu Photonics K.K., Shizuoka, Japan) was used to convert the histopathology slides into image data.

2.5. Assessment of survival rates and ED_{50} values in the infected silkworms to injection of amikacin at two points in time

The *N. farcinica* TUTN006 strain suspension (3.5×10^7 CFU/larva•g in 50 μL saline) was injected into the hemolymph of silkworms, followed by an injection of various amounts (1.0, 2.0, 4.1, 5.4, 8.1, 16.25, 43, 65

and 130 μg/larva) of amikacin (FUJIFILM Wako Pure Chemical CO., Ltd., Osaka, Japan) within 30 seconds and 24 hours (the reference group). Silkworms were kept without feeding in an incubator at 27°C. Each group had 20 silkworms. The survival rate of silkworms after infection was determined at 48 hours. Next, the ED_{50} values were determined as the amount of amikacin of silkworms required for a 50% survival rate at 72 hours post-infection. To confirm the dilution concentrations of amikacin, the quality control strains used in this study include *Staphylococcus aureus* ATCC 29213 and *Escherichia coli* ATCC 25922.

3. Results

3.1. Histopathological analysis of the silkworm *Nocardia* infection model

No tissue damage was observed in the infected silkworms compared with non-infected controls (Figure 1). In the HE stained tissue specimen of the silkworms with infection, bacteria were found in the hemolymph, and showed filamentous branching bacilli (Figure 1). The formation of filament morphology of the bacterium indicated that the bacterium had established and grown in the hemolymph. The location of the filament morphology of the bacterium was mainly around the fat body in the hemolymph, not in the muscular and capsular cell layer of the mid-gut, and no invasion into the fat body tissue was observed.

Next, we analyzed the histopathological finding of the silkworms infected with *N. farcinica* TUTN006 at various time points. Gram staining of thin sections showed the Gram-positive, filamentous branching bacilli and beaded structure at 15-40 hours after inoculation with the bacterial cell suspension, whereas none of the control silkworms. Compared to druse at 15 hours (Figure 1d), the size of druse was observed to grow over time more than twice with growing filamentation (Figures 1e-1h). In addition, bacterial cells surrounded a granule-like mass, which disappeared over time.

3.2. Effect of different time amikacin inoculation on silkworm survival

Silkworms in the reference group, which was inoculated with bacterial cell suspension alone, started to die at 48 hours after inoculation, and all the silkworms died after 72 hours, whereas all the silkworms in the sterile saline (control) and amikacin inoculation groups survived (Figure 2). These results indicate that the effect of administration of bacterial cell suspension alone on the survival of silkworms tended to increase with time after 48 hours, while that of amikacin alone did not.

Next, we evaluated silkworm survival rates by inoculating infected silkworms with amikacin within 30 seconds and 24 hours (Figure 2). In the group of silkworms inoculated with amikacin 24 hours later, mortality increased predominantly at high amikacin concentrations (more over 16.5 μ/mL).

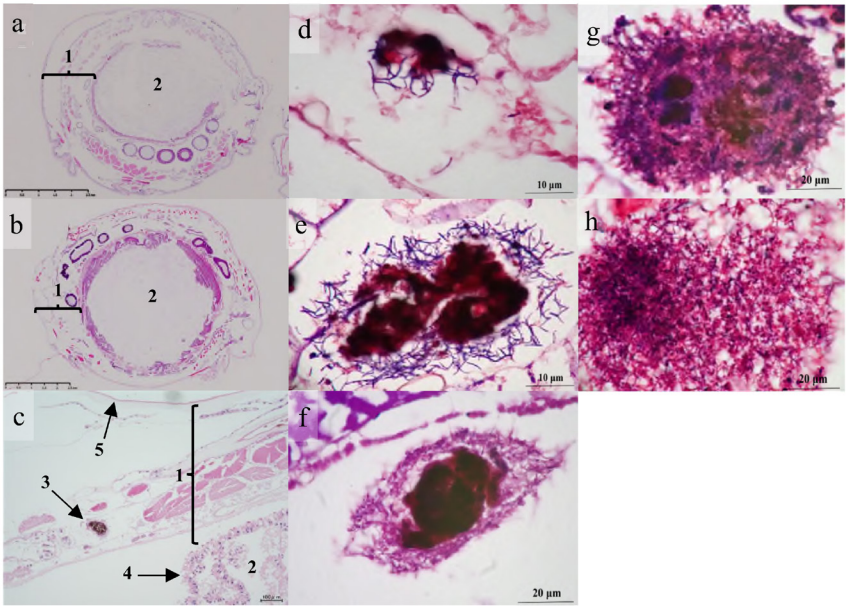

Figure 1. Histopathological analysis. Hematoxylin-eosin staining (100× magnification) was shown non-infected with *N. farcinica* TUTN006 strain (control, **a**) and infected with *N. farcinica* TUTN006 strain (**b**). (1) Hemolymph. (2) Gut lumen. No tissue damage was observed in the infected silkworms. Hematoxylin-eosin staining and observed under a microscope (200 × magnification) was showed infected with *N. farcinica* TUTN006 strain (**c**). (1) Hemolymph. (2) Gut lumen. (3) Branching bacilli. (4) Intestinal membrane cell layer. (5) Skin. Bacteria were found in the hemolymph, and showed filamentous branching bacilli. Gram staining (1000 × magnification) was showed at 15 (**d**), 18 (**e**), 24 (**f**), 28 (**g**), and 40 (**h**) hours after infection showing the Gram-positive, filamentous branching bacilli and beaded structure. Bacterial cells surrounded a granule-like mass.

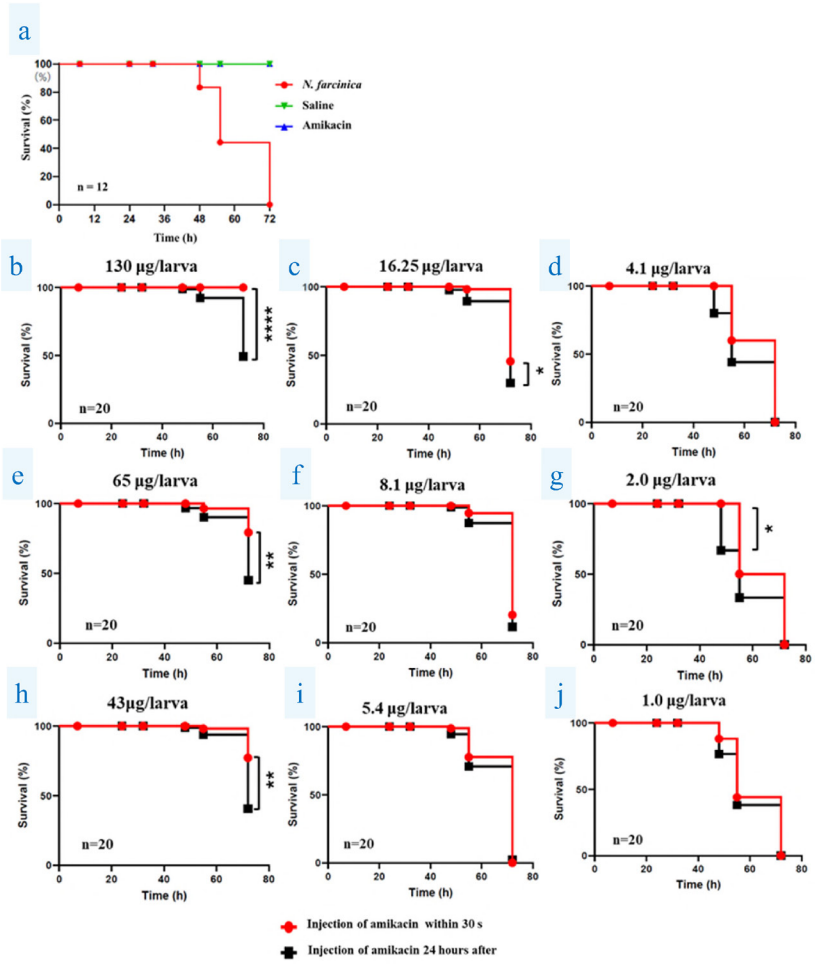

Figure 2. Evaluation of survival rates. Silkworms were injected with saline (50 μL) (control), *N. farcinica* TUTN006 strain cell suspension (1.75×10^7 cells per 50 μL) or amikacin (130 μg/larva) at 27°C, (**a**) The survival curves were drawn using the Kaplan-Meier method. Evaluation of survival rates. Silkworms were injected with *N. farcinica* TUTN006 cell suspension (1.75×10^7 cells per 50 μL). Amikacin at 1.0-130 μg/larva was then administered within 30 seconds or 24 hours after (**b-j**). The survival curves were drawn using the Kaplan-Meier method. Each symbol indicates the following: $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$.

3.3. ED_{50} values in the infected silkworms to amikacin inoculation at two points time

We evaluated ED_{50} values by inoculating infected silkworms with amikacin 30 seconds and 24 hours after infection. The ED_{50} values of inoculation within 30 seconds and 24 hours of amikacin were 4.1 μg/larva and 5.6 μg/larva, respectively. These results are consistent with our previously reported results (*22*) and are reproducible.

4. Discussion

In this study, our histopathological analyses demonstrated that *N. farcinica* develops filamentous forms *in vivo* in the silkworm *Nocardia* infection model. To our knowledge, this study is the first report to demonstrate the filamentous forms of *Nocardia* species *in vivo* in the silkworm. It was found in the present study that the coccoid form of *Nocardia* is transformed into a filamentous form during the growth process. This is one of the characteristics of *Nocardia* transformation (*25,26*). *N. farcinica* is often observed as branched bacterial filaments by Gram staining in clinical specimens from patients with nocardiosis (*27,28*). The study using a mouse model similarly developed a filamentous form (*29*). Therefore, the growth process of *Nocardia* in the silkworm infection model was consistent with that reported in the mouse model. The form of coccid and filament in *Nocardia* has been shown to undergo both complex structural and chemical modification (*30*). These changes in structure have significant effects on nocardial virulence and host-parasite interactions. Virulence and toxicity in the filamentous form were consistently stronger than the coccoid cells in the study using the infected mouse model (*31-33*).

In this study, silkworms started to die after 48 hours when the filament morphology of the organism becomes larger. This finding suggests that the growth of filamentation may contribute to survival in the silkworm

infection model. The silkworm *Nocardia* infection model may be a useful alternative to the mouse model for studying the pathogenicity *in vivo* form during the growth process of *Nocardia*. However, the tissue damage in silkworms, unlike a murine model, was quite limited in the present study, suggesting that silkworms may secrete protective agents or maintain their immune system against *Nocardia* infection, which may be elucidated by further molecular analysis.

The morphology of the organism at the lesion site in patients with *N. farcinica* infection is a filamentous form (*27,34-36*) . Therefore, antimicrobial therapy in many cases is directed against filamentous forms of the organism. However, no quantitative evaluation of the effectiveness of antimicrobials against filamented organisms has been reported to date. Also, no studies were identified that evaluated the therapeutic effects of antimicrobial agents against filamentous forms of *Nocardia* in mice. Thus, this study demonstrated that the silkworm *Nocardia* infection model is able to quantitatively evaluate the therapeutic effect of amikacin against filamentous forms. In addition, the evaluation of the therapeutic effect of amikacin by differences in morphology of the coccoid forms and filamentous form of *Nocardia* was consistent. This trend in humans was consistent with that reported in Nocardiosis in Japan (*37*). Therefore, the silkworm model is useful for evaluating the therapeutic effectiveness of antimicrobial agents adapted to various morphological changes in *Nocardia* infections. Furthermore, the survival rate of silkworms in the high-concentration amikacin-only inoculation group was the same as that in the sterile saline-only group, and it maintained a high survival rate of silkworms. Therefore, high concentrations of amikacin are not toxic to silkworms. However, we found that groups of silkworms inoculated with high concentrations of amikacin after 24 hours in the infected silkworms had significantly higher mortality rates. These findings raised the following possibilities: the administration of high concentrations of amikacin is effective in the early stages of *Nocardia* infection, but it may weaken the host's resistance to infection once the infection has progressed. The establishment of a silkworm model for the purpose of screening for the side effects of antibiotics will be an important future research topic.

The present study was able to reproduce the bacterial morphology of *N. farcinica* in the silkworm *Nocardia* infection model over time. We further speculated that this model has the potential for a more clinical assessment of antimicrobial efficacy against morphological changes of *Nocardia* in antimicrobial susceptibility testing.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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