Original Article

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Evaluation of pathogenicity and therapeutic effectiveness of antibiotics using silkworm *Nocardia* infection model

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SUMMARY *Nocardia* is a ubiquitous environmental microbe that causes nocardiosis against immunosuppressed and immunocompromised hosts. The assay system for the quantitative evaluation of virulence of *Nocardia* sp. or therapeutic effectiveness of antimicrobials for treatment of nocardiosis is not established so far. In this study, we established an infection model of *Nocardia* sp. using silkworm as an alternative animal model. We found that all tested *Nocardia* sp. such as *Nocardia asiatica*, *Nocardia elegans*, *Nocardia exalbida*, *Nocardia farcinica*, and *Nocardia nova* killed silkworm and their killing ability were different by species. *N. farcinica* showed higher pathogenicity among tested strain, similar to the mouse model as previously reported. In addition, we found that antimicrobials such as amikacin and minocycline showed therapeutic effectiveness in silkworms infected with N. farcinica, and we could determine effective doses 50 (ED_{50}) values. These results suggest that silkworm is a useful alternative animal to evaluate the pathogenicity of *Nocardia* pathogen and the therapeutic effects of antimicrobials against *Nocardia* sp. in a quantitative manner.

Keywords *Nocardia*, virulence, silkworm infection model, nocardiosis, *N. farcinica*

1. Introduction

Nocardia species are Gram-positive slow-growing bacteria and an acid-fast aerobic actinomycete, ubiquitous in the environment such as soil organic material and water. *Nocardia* sp. rare, although, sometimes causes localized or systemic disease in humans and animals. Manifestations of the disease range from cutaneous infection caused by traumatic inoculation of the organism in a normal host to severe pulmonary or central nervous system (CNS) disease in an immunosuppressed host (*1*). The severe bacteremia by *Nocardia* shows high mortality. The systemic review suggested that overall all-cause mortality was 40% (*2*). Recently, the genus *Nocardia* expanded and currently contains more than one hundred species (*3*), and *N. asteroids*, *N. brasiliensis*, *N. cyriacigeorgica*, *N. farcinica*, and *N. nova* have been reported as the main bacterial species that cause nocardiosis in the world (*4,5*). However, there is little literature regarding elucidating pathogenic properties of *Nocardia* sp. (*6*). Mice infection models have been established (*7*) and therapeutic efficacy of antimicrobials was reported (*8,9*), however, quantitative evaluation of the effectiveness of those antimicrobials were not reported to the best of our knowledge.

Nowadays, conducting experiments using mammals has been limited due to ethical problems and high breeding costs (*10*). Recently, silkworm, *Bombyx mori*, has paid attention as an alternative animal model since it causes less ethical issues and required small cost and space (*11*). We further established several pathogens infection model to evaluate antimicrobials using silkworm, such as *Staphylococcus aureus* (*12*), *Candida albicans* (*12*), *Cryptococcus neoformans* (*13*), and even for acid-fast aerobic *Mycobacterium* species (*14-16*). In this study, we demonstrated that a silkworm model is a useful model to evaluate the pathogenicity of *Nocardia* sp. and therapeutic efficacy of antimicrobials against *N. farcinica*, the most frequently isolated from the clinical.

2. Materials and Methods

2.1. Bacterial strains, identification and culture

Five pathogenic *Nocardia* strains were isolated from the different patients with the lower respiratory tract infection at Nippon Medical School Hospital, Japan, in 2014. Identification of genus of five strains from clinical specimens was based on positive Gram stain (Gram-positive filamentous bacilli) and positive modified acid-fast stain, colonial morphotypes and conventional biochemical reactions.

The identification of the bacterial species was performed by analyzing the 16S rRNA gene sequence. A portion of the colonies on BHI agar was picked up by toothpick and added to the PCR reaction solution. The primers were E9F (5'-GAGTTTGATCCTGGCTCAG-3'), E1541R (5'-AAGGAGGTGATCCAGCC-3') (*17*) were used. Ten cycles of 98°C for 10 sec, 55°C for 30 sec, and 68°C for 1 min 30 sec were performed and followed by 15 cycles of 98°C for 10 sec, 45°C for 30 sec, and 68°C for 1 min 30 sec were performed. The amplified DNA fragments were separated by agarose gel electrophoresis (1%, 100 V, 30 min). The target DNA fragments were cut from the gel and extracted using the QIAEX II Gel Extraction Kit. 16S rRNA sequences were obtained by cycle sequencing reactions using the Sanger method with the BigDye™ Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Tokyo, Japan) and analyzed by ABI PRISM 3130xl genetic Analyzer (Thermo Fisher Scientific, Tokyo, Japan). The obtained sequences were used to perform a blast search against the Pubmed 16S rRNA database (BLAST: Basic Local Alignment Search Tool (nih.gov)). The bacterial species with the highest nucleotide sequence homology was determined as the species of the sample. These strains were stored at - 80°C using a microbank tube (Iwaki & Co., Ltd., Tokyo, Japan). These stocked strains were spread onto brain heart infusion agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) or 5% sheep blood agar (Eiken Chemical Co., Ltd., Tokyo, Japan), and cultured at 37°C in an atmosphere of 5 % carbon dioxide (CO_2) for three days.

2.2. Silkworm rearing

Silkworm eggs (Hu·Yo × Tukuba·Ne) were purchased from Ehime Sansyu (Ehime, Japan) and fed Silkmate 2S (Katakura Industries Co., Ltd. Tokyo, Japan) until they developed to the fourth molted larva. On the first day of fifth-instar larvae, silkworms were fed for one day with an antibiotic-free artificial food, Silkmate (Katakura Industries Co., Ltd., Tokyo, Japan).

2.3. Comparison of silkworm killing ability of *Nocardia* sp. strain

The colonies cultured on BHI agar medium (Eiken Chemical, Tokyo) were picked up using a loop, suspended in sterile saline and adjusted to OD_{600} = 0.175. A two-step dilution series of this bacterial solution was prepared using sterile saline and injected 50 µL into the hemolymph of silkworms. Silkworms were kept in an incubator at 27°C without feeding, and the number of surviving silkworms on 4 days after injection was counted (n = 3). To calculate the number of bacterial cells injected into the silkworm, the solution used for injection was diluted with sterile saline solution, and 100 µL was spread on BHI agar medium. After incubated under aerobic conditions at 37°C for three days and appeared colonies were counted. The LD_{50} value was defined as the activity that killed half of the silkworms (50% lethality).

2.4. Antimicrobial susceptibility test

Antimicrobial activity was determined by the microdilution method according to CLSI (*18*). After 72 hours of incubation at 37°C under aerobic conditions, bacterial growth was visually confirmed. The minimum concentration of antimicrobial agent that completely inhibited bacterial growth was determined as the minimum inhibitory concentration (MIC). The antimicrobial agents used were amikacin, minocycline, imipenem, sulfamethoxazole, trimethoprim, linezolid, erythromycin, oxacillin (FUJIFILM Wako Pure Chemical Corporation Tokyo, Japan), levofloxacin (Tokyo Pure Chemical Corporation Tokyo, Japan).

2.5. Therapeutic trial for *Nocardia* infected silkworms

Determination of the 50% effective dose (ED_{50}, µg/larva) of antimicrobial agents against *N. farcinica* TUTN006 strain was performed as described previously by Hamamoto H *et al.* (*12*).

Fifty microliters of *N. farcinica* TUTN006 (3.3×10^7 CFU/mL) suspended in 0.9% sterile saline were injected into hemolymph of silkworm on the day 2 of 5^{th} molted. Then 50 µL of antimicrobial agent diluted in saline was injected into the hemolymph (n = 3) by 27G needle with a syringe (Terumo, Tokyo Japan). After injection, the silkworms were kept without feeding in an incubator at 27°C. The survival number of silkworms were counted on day 3. The ED_{50} values were determined as the amount of drug of silkworm required for 50% survival.

3. Results

3.1. Establishment of a *Nocardia*-infected silkworm model

First, we performed the identification of bacterial species, the clinical isolates of *Nocardia* strain used in this study named TUTN001 to 007, by 16S rRNA sequencing. These strains were identified as *N. asiatica*, *N. elegans*, *N. exalbida*, *N. farcinica*, and *N.*

Table 1. Identification of bacterial species

Strain Name	Identification	Identities (%)	Top hit of Accession no.	Origin
TUTN001	*Nocardia asiatica*	1425/1432 (99)	NR_117244.1	Clinical
TUTN002	*Nocardia elegans*	1443/1443 (100)	NR_042353.1	Clinical
TUTN003	*Nocardia elegans*	1443/1443 (100)	NR_042353.1	Clinical
TUTN004	*Nocardia elegans*	1443/1443 (100)	NR_042353.1	Clinical
TUTN005	*Nocardia exalbida*	1432/1432 (100)	NR_117321.1	Clinical
TUTN006	*Nocardia farcinica*	1446/1446 (100)	MN100049.1	Clinical
TUTN007	*Nocardia nova*	1442/1444 (99)	AB630968.1	Clinical

Table 2. LD_{50} of *Nocardia* in silkworm

Strain Name	Species	LD_{50} at 4days (CFU/larva)
TUTN001	*N. asiatica*	2.0×10^6
TUTN002	*N. elegans*	7.0×10^5
TUTN003	*N. elegans*	6.3×10^5
TUTN004	*N. elegans*	1.4×10^5
TUTN005	*N. exalbida*	1.4×10^4
TUTN006	*N. farcinica*	1.4×10^4
TUTN007	*N. nova*	4.6×10^5

nova, respectively (Table 1). We injected suspension of these cells into silkworm hemolymph, and these strains killed silkworms. Next, we compared the killing ability of these species was examined by determining the LD_{50} value. The results showed that the killing ability of silkworms differed among the species, with *N. farcinica* and *N. exalbida* having the lowest LD_{50} values compared to the other *Nocardia* species (Table 2). These results suggested that *N. farcinica* and *N. exalbida* showed high virulence in the silkworm model. This result was consistent with the previous report (*7*) that *N. farcinica* showed higher virulence than *N. nova* in a mouse model. Therefore, we considered that the *Nocardia*-infected silkworm model was established.

3.2. ED_{50} of antibiotics against silkworm *N. farcinica* infection model

Next, the drug susceptibility test of the bacteria used in this study was conducted. As a result, all strains used in the experiment showed high susceptibility to amikacin, minocycline, linezolid and erythromycin. On the other hand, several strains showed low susceptibility to oxacillin, levofloxacin and ST fixed-dose combination (Table 3). These results are consistent with reports that a large proportion of *Nocardia* spp. are highly susceptible to amikacin, linezolid and minocycline (*19-21*). Next, we investigated whether the therapeutic efficacy of antimicrobial agents could be assessed using a *Nocardia*-infected silkworm model. We examined amikacin and minocycline, and found that these antimicrobials showed therapeutic effects on *Nocardia*-infected silkworms in a dose-dependent manner (Table 4). The ED_{50} values were determined as 5.2 μg/larva for amikacin, and 60 μg/larva for minocycline. The ED_{50} values for minocycline was higher than expected from its MIC, although, these results were consistent with the previous report in the mice model (*22*). In that mice model, amikacin treatment reduced the number of bacterial cells in the brain of *Nocardia*-infected mice. In contrast, minocycline treatment did not reduce cells in the brain, although amikacin and minocycline showed high antibacterial activity against the strain used for infection assay *in vitro*. Therefore, we concluded that a quantitative evaluation system for the therapeutic effects of antimicrobial agents using the *Nocardia*-infected silkworm model was established.

4. Discussion

In this study, we aimed to establish a model of *Nocardia* infection using the silkworm. The silkworm is an alternative animal, with the advantages of low cost, fewer ethical issues, and the ability to use large numbers of individuals for experiments. We found that silkworms were killed by all tested strains. Furthermore, killing ability of *Nocardia* sp. against silkworm is different among tested strain. The results were consistent with reports from mouse models (*7*). Therefore, we conclude that we have established a *Nocardia* infection model using the silkworm. Recently, novel virulence factors of *S. aureus*, *Serratia marcescens*, *C. neoformans* and enterohemorrhagic *Escherichia coli* have been identified using the silkworm model (*23-28*). These virulence factors also required for exerting virulence of pathogens against the mouse. So far, there has been little evaluation of virulence factors in *Nocardia* using animal models (*7,29*). Thus, this silkworm model would facilitate the research regarding the virulence of *Nocardia* sp.

Furthermore, we found that clinically used antimicrobial agents showed therapeutic effectiveness on *Nocardia*-infected silkworms. There are very few studies that have evaluated the therapeutic effects of antimicrobials in mice (*9,30,31*), and these studies were not quantitative. Thus, this study is the first report to quantitative evaluation of the therapeutic effect of antimicrobial agents in the *Nocardia* infection model. In addition, we found a discrepancy in the therapeutic efficacy of amikacin and minocycline in the silkworm model, compared with the susceptibility of these antimicrobials against *N. farcinica in vitro*.

Table 3. Antimicrobial susceptibility to *Nocardia*

Strain Name	*Species*	MIC(μg/mL)							
		AMK	MINO	LZD	MPIPC	LVFX	TMP/SMX	IMP	EM
TUTN001	*N. asiatica*	< 0.8	< 0.8	< 0.8	13	50	< 0.8	3.1	< 0.8
TUTN002	*N. elegans*	< 0.8	< 0.8	< 0.8	6.3	3.1	13	< 0.8	< 0.8
TUTN003	*N. elegans*	< 0.8	< 0.8	< 0.8	25	6.3	50	< 0.8	< 0.8
TUTN004	*N. elegans*	< 0.8	< 0.8	< 0.8	6.3	3.1	< 0.8	< 0.8	< 0.8
TUTN005	*N. exalbida*	< 0.8	< 0.8	< 0.8	6.3	3.1	50	< 0.8	< 0.8
TUTN006	*N. farcinica*	0.8	< 0.8	< 0.8	> 100	6.3	< 0.8	< 0.8	< 0.8
TUTN007	*N. nova*	< 0.8	< 0.8	< 0.8	50	13	25	< 0.8	< 0.8

Table 4. ED_{50} of antifungal agents in a silkworm model with *N. farcinica*

Antimicrobial	ED_{50} (μg/larva)
amikacin	5.2
minocycline	60

This trend was consistent with that reported in the mouse model (*9*). Therefore, the silkworm model is useful that to evaluate the therapeutic effectiveness of antimicrobial agents against *Nocardia* infections. We further speculated that this model is applicable to the development of novel antimicrobials that are effective against *Nocardia* infection.

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

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