Title

Establishment of a novel model of onychomycosis in the rabbit

for evaluation of antifungal agents

Running title

Establishment of onychomycosis model using rabbits

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ABSTRACT

We developed a novel model of onychomycosis in which we observed the fungi in the deep layer of the nail and used the model to evaluate the efficacy of two topical antifungal drugs. To establish an experimental, *in vivo* model of onychomycosis, we applied *Trichophyton mentagrophytes* TIMM2789 to the nails of the hind limbs of rabbits that underwent steroid treatment. The nails were taken from the rabbits' feet at zero, two, and six weeks after a two-week infection. The localization of the fungi was evaluated histopathologically. Some fungi were seen to penetrate to the nail bed and infection rate in the sample at zero, two and six weeks after infection were 57%, 87% and 93%, respectively. In addition, fungi proliferated and moved proximally into the nail plate in a manner that depended on the duration of infection. Secondly, using this model we evaluated antifungal efficacy, both by the culture recovery method and histopathological examination. Two topical antifungal drugs, 8% ciclopirox nail lacquer and 5% amorolfine nail lacquer, were applied to the nail for four weeks in each group. On histopathological examination, two antifungal treatment groups showed no significant difference...
against non-treated control group. However, there was a significantly low fungus-positive rate and intensity of recovery of fungi on culture between antifungal treatment and non-treated control group. We therefore suggest that we have established an *in vivo* model of onychomycosis that is useful for the evaluation of the efficacy of antifungal agents.
INTRODUCTION

Onychomycosis is an intractable superficial mycosis, and oral administration of antifungal agents is the main modality for clinical treatment (8). However, some of these oral antifungal drugs have well-known drug interactions with other medications, and these interactions limit their usage in older people and those living with diabetes or human immunodeficiency virus (2). These patients eagerly await topical antifungal agents that are both effective and useful.

Tatsumi et al. (20) reported the evaluation of some antifungal agents using an in vivo experimental model of onychomycosis but detailed histological examination was limited, and they do not describe whether fungal infection occurred near the nail bed, as occurs in a clinical setting. Drug efficacy tests that used this model were useful in the evaluation of orally administered drugs, but not for topical agents. Agents that are active following oral administration reach the nail bed via the circulation, and then diffuse towards the dorsal surface of nail. In consequence, if the fungal infection that occurs in this animal model is only superficial, where the lowest concentration of active agent is thought to be

5
following oral administration, drug efficacy can be evaluated adequately. In order to evaluate topical

2 drugs using this model, we need to confirm efficacy in the deeper layers of the nail.

3 Although there are reports that describe histological findings in human onychomycosis and that

4 confirm the value of histopathological examination in making the diagnosis (3, 10, 16, 18), the route of

5 infection and how the fungi behave in the nail plate have not been determined in detail.

6 The rate of morbidity in onychomycosis may be affected by age, smoking, peripheral arterial disease,

7 diabetes, smoking, and immunodeficiency (5, 14, 22). In particular, a multicenter survey (7) reports

8 that the administration of immunosuppressive agents to people with diabetes may be an important

9 factor that predisposes to onychomycosis.

10 In this article, we here attempt to establish an animal model of onychomycosis in immunosuppressed

11 rabbits using *Trichophyton mentagrophytes*, a well-recognized and widely identified pathogen in

12 rabbit (1, 9, 21, 24). We used histopathological examination and the culture recovery method to

13 evaluate the efficacy of 8% ciclopirox nail lacquer (Penlac®, Dermik Laboratories, Sanofi-Aventis,
Bridgewater, NJ, USA) and 5% amorolfine nail lacquer (Loceryl®, Galderma, Lausanne, Switzerland)

known as topical onychomycosis treatment in our model.
MATERIALS AND METHODS

In this article, the terminology we use in describing the histology of the nail tissue of the rabbit is identical to that used in humans.

Animals

Male Japanese white rabbits aged 14 weeks were purchased from KITAYAMA labes and used in this study. The experiment to establish the animal onychomycosis model was performed in three groups with five rabbits in each group, while the experiment that determined therapeutic efficacy was performed in three groups with four rabbits in each group. The nails examined were on the first to third toes of the right and left hind paws, that is, six nails were used per animal. All experimental procedures were evaluated and approved in accordance with the Institutional Animal Care and Use Committee (IACUC) of POLA.
Test organism

T. mentagrophytes TIMM2789, isolated from the guinea pig, was purchased from Teikyo University Institute of Medical Mycology, Tokyo, Japan.

Preparation of inocula

Freeze-dried T. mentagrophytes was suspended in 1 ml of saline containing 0.05% Tween 80. A volume of 0.05 ml of this suspension was seeded onto Fluid Sabouraud Dextrose agar, and T. mentagrophytes was cultured at 28°C for two weeks in order to prepare microconidia. After subculturing the fungi, microconidia of T. mentagrophytes were taken from the fungi in saline, and the suspension of microconidia was adjusted to give a concentration of $10^8$ conidia/ml by counting with a hemocytometer.

Production of onychomycosis
Four mg/kg of methylprednisolone acetate was injected intramuscularly into the hind limb of each rabbit once a week for four weeks until the end of the infection period. In some cases, the dosage of steroid was decreased depending on the condition of the animal. Two weeks after starting steroid treatment, 0.2 ml of microconidia suspension was dripped onto the nail at a site between the lunula and the proximal nail fold. A gauze patch was then used to wrap together the nail plates of the first to third toes of the hind paw. The treated toe nails were covered with a finger cot (that contained the first to third toes) and 0.5 ml of sterile water was then injected into the finger cot to produce a culture environment around the nail that was suitable for fungal growth. This condition was maintained for a period of infection of two weeks, without any other intervention. The finger cot and the gauze patch were removed after two weeks of exposure, and this condition was maintained for zero, two or six weeks without finger cot and gauze patch, as the “post-infection period”. After each post-infection period was completed, the animals were sacrificed and the nails were taken from the treated paw for histopathological and microbiological examination.
Histopathological examination of nail tissue

The nails sampled for histopathological examination were fixed in 20% (vol/vol) buffered neutral formalin solution for one week, and decalcified in 5% (vol/vol) formalin solution containing 5% (vol/vol) buffered formic acid for one week. They were then neutralized in 5% (vol/weight) sodium sulfate aqueous solution for one week, and embedded in paraffin. The nails were sectioned using a microtome with the paraffin tape-transfer system (Instrumedics, Hackensack, NJ) to support the cohesion of whole nail elements. Thin paraffin sections of the nails were observed by light microscopy after staining with the periodic acid-Schiff stain. The following semiquantitative scoring system was used to describe the intensity of *T. mentagrophytes* infection in the nail plate: Grade 0, no fungus; Grade 1, fungi present but do not form a cluster; Grade 2, a few clusters of fungi present; and Grade 3, numerous clusters of fungi present. The infection rate was calculated as follows. The number of nails in which the grade was above 1 was divided by the total number of nails evaluated in each group. The
histological localization of fungi in the nail plate was evaluated based on the intensity and the infection rate in each of six regions, that is, following division in two in a lengthwise direction from the dorsum of the nail plate to the nail bed, and in three, across from the proximal to distal end of the nail plate (Fig. 1).

Drugs and treatment

In this study, 8% ciclopirox nail lacquer and 5% amorolfine nail lacquer were purchased through an import company and used as test items. Each nail was cleaned with water-soaked cotton just before application of the drug, and then a volume of 3.6 µl of each drug was topically applied to the nails. 8% ciclopirox nail lacquer was applied once a day and 5% amorolfine nail lacquer was applied twice a week according to the drug package insert. Applications of these drugs were carried out from the day following the end of the period of infection, for 28 days. The animals in the untreated control group were underwent the process of infection and removal of the material used for this process, but were not
exposed to the test agents. In cases that underwent the drug efficacy experiment, the nails sampled from
the paw were cut into two lengthwise, and one piece was used for histopathological evaluation while
the second was used for evaluation using culture recovery.

Evaluation of therapeutic efficacy using the culture recovery method

The infected nail intended for evaluation using culture recovery was cut into 10 pieces in cross
section. Each nail piece was implanted onto a plate of Sabouraud dextrose agar (Difco laboratories,
Detroit, Mich., U.S.A.) containing, in 1 liter, 0.5 g of cycloheximide, 100 mg of chloramphenicol, and
50 mg of sisomicin. All plates with implanted nails were incubated for two weeks at 28°C. A nail piece
that had confirmed fungal growth was assessed as culture-positive, and a nail with more than one
culture-positive piece was considered fungus-positive. The extent of fungal burden was assessed with
scores ranging from +10 to 0, based on the number of culture-positive nail pieces out of all 10 pieces
examined.
Statistical analysis

The total infection rate by histopathological examination and the frequency of fungus-positive nails by the culture recovery method in each group was analyzed using Fisher’s exact test. The intensities of the fungal burden of the infected nails in each group were analyzed by Student’s t test. P values of less than 0.05 were regarded as significant.
RESULTS

Establishment of an onychomycosis model using rabbits

After a two-week infection period under immunosuppression, the post-infection period was set to zero, two and six weeks in each group, to determine a suitable post-infection period. Some of the infected nails became cloudy on gross appearance, similar to the findings in human onychomycosis (Fig. 2). With a longer post-infection period, these findings were fully confirmed. Correlation between the gross appearance and histopathological changes was, however, not confirmed as clearly. On histopathological examination, hyphae of T. mentagrophytes were seen to penetrate the nail plate and some invading fungi reached the nail bed (Fig. 3). Almost hyphae have septum and some of them were thin as crushed within the nail layer. Chains of spores were also seen near the surface of the nail (Fig. 3). The total infection rate at zero, two and six weeks into the post-infection period was 56.7% (17/30), 86.7% (26/30) and 93.3% (28/30), respectively, and the regions that showed the highest infection rate in the six divided portions at each period of post-infection were the proximal/dorsal side (46.7%), the
distal/ventral side (62.1%) and the distal/ventral side (90.0%), respectively (Table 1). The regions that showed the highest infection intensity were also the proximal/dorsal side (0.73), distal/ventral side (1.45) and distal/ventral side (2.53) in each group. Additionally, the presence of subungual abscess with associated necrosis of the epithelium of the nail bed or matrix was confirmed near the fungi in the nail plate (Fig. 3). For subungual abscesses, changes in the appearance rate with time were similar to those for the infection rate. Therefore, the total appearance rates of subungual abscesses in the groups at zero, two and six weeks post-infection were 33.3% (10/30), 43.3% (13/30) and 93.3% (28/30) respectively (Table 1).

Histopathological evaluation of the drug efficacy in our onychomycosis model

Once-daily topical treatment with 8% ciclopirox nail lacquer or twice-a-week topical treatment with 5% amorolfine nail lacquer was applied to infected nails. Total infection rates in the control, 8% ciclopirox nail lacquer and 5% amorolfine nail lacquer groups were 66.7% (16/24), 47.6% (10/21) and
43.5% (10/23) respectively; and the regions that showed the highest infection rate in the six sections at each groups were the distal/ventral side (65.2%), distal/ventral side (33.3%) and medium/ventral side (30.4%), respectively (Table 1). The region where the intensity of infection was greatest was the distal/ventral side (1.83, 0.90 and 0.67); it was same in all groups. Additionally, the total appearance rates for subungual abscesses in the control, 8% ciclopirox nail lacquer and 5% amorolfine nail lacquer groups were 58.3% (14/24), 38.1% (8/21) and 39.1% (9/23), respectively (Table 2). In statistical analysis, however, there was no significant difference was found in total infection rate between drug treatment groups and non-treat control group.

Microbiological evaluation of drug efficacy in our onychomycosis model

The culture recovery method was used for microbiological evaluation of the antifungal effect of the two reference drugs in the nail. Table 3 shows the recovery rate and fungal burden of infected nails. The recovery rate in the untreated group was 75% (18/24). Conversely, those of the 8% ciclopirox nail...
lacquer and 5% amorolfine nail lacquer groups were 66.7% (16/24) and 20.8% (5/24), respectively.

Furthermore, a statistically significant lower rate was found in 5% amorolfine nail lacquer group on comparing the infection rate with that in both the control and 8% ciclopirox nail lacquer groups, and likewise on comparison the infection intensity with that in the control group.
**DISCUSSION**

The first report of an animal model for onychomycosis was described by Tatsumi et al. in 2002, with the use of guinea pigs. These authors also used this model to evaluate the efficacy of a topical antifungal agent, but the article did not describe the pathophysiological findings in detail, omitting how the gross appearance changed as well as the histopathological findings on examination of the infected nails (20). It was thus not clear whether the drug efficacy found using the animal model reflected the drug’s therapeutic effect against onychomycosis in clinical practice.

In the clinical therapy of onychomycosis, a poor outcome of topical use of antifungals has been mentioned for the management of onychomycosis, most of which attribute to their penetration disability onto the nail plate (19, 23, 26). Some reports suggest that the nail has properties like those of a hydrophilic gel membrane (12, 15) that prevents penetration of the nail plate by chemicals and drugs (4). In addition, histopathological examination of nail biopsies revealed the presence of fungi near the distal and proximal nail bed in distal subungual type (DSO) (10). In animal models in which topical
drug efficacy for onychomycosis is evaluated, it is therefore preferable that the causative organism is able to invade the deeper layers of the nail in order to confirm drug efficacy in this region.

In this study, our experimental animal model succeeded in encouraging *T. mentagrophytes* to invade the deeper layers of the nail plate that was clearly confirmed our histopathological examination. Furthermore, our model allowed assessment of growth into the nail plate and changes in localization of the fungi. This is the first report of fungal behavior in the nail plate in an experimental animal model of onychomycosis.

Haneke (10) has investigated the localization of infected fungi on the longitudinal section of the plate and adjacent soft tissue from patients with onychomycosis of subungual onychomycosis of both proximal and distal subtypes, and reported that the former showed high density of fungal cell from the eponychium to the proximal nail bed and the latter showed that from the nail matrix to the distal nail bed. In our study, the regions seriously involved by fungal infection were the proximal nail plate in the group immediately post-infection (zero weeks), and the distal nail plate at two and six weeks. The fact
suggests that the findings in our model are similar to the clinical diagnoses of PSO and DSO, and that a
transformation from PSO to DSO may depend on the interval that follows infection. However, a few of
chains of spores were also identified near the nail surface. This finding has also been reported as
“superficial onychomycosis” in humans (10). Our model could therefore also partially re-create this
type of superficial onychomycosis.
Of relevance is that the proximal nail fold was thought to be the point of invasion of dermatophytes
into the nail in PSO (27). Another report of a human experiment (25) showed a similar result, in that
the lunula was the most susceptible area. In this study, the proximal/dorsal side was the commonest
area of involvement of fungal infection, which revealed the highest infection rate immediately after the
end of the infection period, a finding that supports the above suggestion.
And on the one hand, in our model, fungi within the nail plate did not move in the opposite direction
to nail growth. The period of drug treatment may therefore be limited, because in this model there is a
possibility of complete cure over a longer experimental period. In this model it was however confirmed
that the causative organism was present for at least six weeks after the infection period. We therefore confirmed that our model could be used to conduct a drug efficacy test for onychomycosis, within this interval.

As noted above, in this drug efficacy experiment, we arranged a drug treatment period of four weeks. Some of the infected nails became cloudy on gross appearance, however, the gross appearance of the infected nails and shape of fungal cell did not clearly differentiate between groups with and without the treatment. And total infection rate clarified by histopathological examination showed no statistical significant difference in drug treated groups compared with non-treated control group. However, a statistical significant reduction was observed in recovery culture method. The possible reason for the difference in the results of histopathological examination and the culture recovery method is that the limits of classical histological stain and a semiquantitative histopathological scoring. Especially, carbohydrates originated from fungal wall were intensely stained by the periodic acid-Schiff stain even with reduced viability of the fungi (11). Therefore, it may be thought that outcome of histopathological
examination could not be reflected the real drug efficacy.

On the other hand, the culture-positive rate in controls, and with 8% ciclopirox nail lacquer and 5% amorolfine nail lacquer was 75.0%, 66.7% and 20.8%, respectively. It has been reported that the clinical cure rate for 5% amorolfine nail lacquer was 38 to 52% (13, 17) and that of 8% ciclopirox nail lacquer was 8% (6). When each rate of culture-positivity in the drug-treated groups was subtracted from that in the non-treated group, the differences were 54.2% for 5% amorolfine nail lacquer and 8.3% for 8% ciclopirox nail lacquer. This figure was similar in the above clinical reports. Moreover, the infection rate and frequency of subungual abscesses that were calculated based on the histological findings in drug-treated groups were low compared to those in the control group, and these frequencies were similar in the 8% ciclopirox nail lacquer or 5% amorolfine nail lacquer treatment groups. Efficacy of these drugs was thus detected using this animal model. In addition, a difference in the efficacy of 8% ciclopirox nail lacquer and 5% amorolfine nail lacquer was confirmed by the culture recovery methods.

In general, subungual abscesses are thought to be rare in humans with onychomycosis. But our
procedure induced subungual abscess which was mostly resulted by induced immunocompromised condition. Whereas the model usually complicated with subungual abscess formation, pathophysiology of the onychomycosis of subungual subtype in human, infected in deep layer of the nail, is faithfully reproduced which comprises the site of initial proliferation (cuticle) and the manner of supply, movement, positioning, and proliferation of fungi in the nail plate. So, the fungal dynamics which was confirmed in our model was thought to be suitable for evaluation of antifungal agent, especially topical ones.

Moreover, evaluation of topical drug efficacy by the culture recovery method confirmed achieved results similar to those encountered in a clinical setting. We have therefore established an animal model of onychomycosis and confirmed that our model may be useful in evaluating the efficacy of antifungals.
Acknowledgements

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Competing interests

Dr. Shibuya reports receiving research grants from Pfizer Inc., Janssen Pharmaceutical K.K., and Dainippon Sumitomo Pharma Co. All authors declare that they have no competing interests.
REFERENCES


through the three layers of the human nail plate. J. Pharm. Pharmacol. 51:271-278.


Table 1. Localization of fungi, total infection rate and frequency of subungual abscess in an experiment to establish a model of onychomycosis

<table>
<thead>
<tr>
<th>Infection rate by location* (%)</th>
<th>Post-infection period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 week</td>
</tr>
<tr>
<td></td>
<td>D</td>
</tr>
<tr>
<td>Dorsal side</td>
<td>(0.00)</td>
</tr>
<tr>
<td>Ventral side</td>
<td>(0.23)</td>
</tr>
<tr>
<td>Total infection rate** (%)</td>
<td>56.7</td>
</tr>
</tbody>
</table>

Appearance rate of subungual abscesses, by locationc (%)

<table>
<thead>
<tr>
<th>Post-infection period</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 week</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>Dorsal side</td>
</tr>
<tr>
<td>Ventral side</td>
</tr>
<tr>
<td>Total appearance rate of subungual abscessesd (%)</td>
</tr>
</tbody>
</table>

D, Distal; M, Middle; P, Proximal

*Localization of infection rate = (number of histologically fungus-positive nails in each region / number of nails tested in each region) ×100

**Total infection rate = (number of histologically fungus-positive nails / number of nails tested) ×100
1  c localization of appearance rate = \frac{\text{number of nails with subungual abscesses in each region}}{\text{number of nails tested in each region}} \times 100

2  d \text{Total appearance rate} = \frac{\text{number of nails with subungual abscesses}}{\text{number of nails tested}} \times 100

3  \text{()}: \text{Average of infection intensity grades in each region}
Table 2. Localization of fungi, total infection rate and frequency of subungual abscesses in an experimental evaluation of drug efficacy.

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>8% ciclopirox nail lacquer</th>
<th>5% amorolfine nail lacquer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>M</td>
<td>P</td>
</tr>
<tr>
<td>Dorsal side</td>
<td>0.0</td>
<td>26.1</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>(0.00)</td>
<td>(0.54)</td>
<td>(0.13)</td>
</tr>
<tr>
<td>Ventral side</td>
<td>65.2</td>
<td>34.8</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>(1.83)</td>
<td>(0.86)</td>
<td>(0.13)</td>
</tr>
<tr>
<td>Total infection rate</td>
<td>66.7</td>
<td>47.6</td>
<td>43.5</td>
</tr>
<tr>
<td>Appearance rate of subungual abscesses, by location</td>
<td>58.3</td>
<td>38.1</td>
<td>39.1</td>
</tr>
</tbody>
</table>

D, Distal; M, Medium; P, Proximal

- Localization of infection rate = (number of histologically fungus-positive nails in each region / number of nails tested in each region) × 100
- Total infection rate = (number of histologically fungus-positive nails / number of nails tested) × 100
- Localization of appearance rate = (number of nails with subungual abscesses in each region / number of nails tested in each region) × 100
Total appearance rate = (number of nails with subungual abscesses / number of nails tested) × 100

(): Average of infection grades in each region
Table 3. Culture recovery: evaluation of efficacy of two topical antifungal drugs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Duration of treatment (day)</th>
<th>Number of fungus-positive nails / total no. of nails (%)</th>
<th>Average fungal burden of the infected nails</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28</td>
<td>18 / 24 (75.0)</td>
<td>+4.4</td>
</tr>
<tr>
<td>8% ciclopirox nail lacquer</td>
<td>28</td>
<td>16 / 24 (66.7)</td>
<td>+2.8</td>
</tr>
<tr>
<td>5% amorolfine nail lacquer</td>
<td>28</td>
<td>5 / 24 (20.8)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+0.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.01 versus the untreated control and 8% ciclopirox nail lacquer-treated groups.

<sup>b</sup> P < 0.01 versus the untreated control group.
FIGURE LEGEND

Fig. 1. The diagram of histopathology of rabbit nail. The histological examination was carried out according to the six regions separated: a, dorsal and distal; b, dorsal and medium; c, dorsal and proximal; d, ventral and distal; e, ventral and medium; and f, ventral and proximal side of nail plate.

Fig. 2. Gross appearance of infected nails at six weeks post-infection in an experiment to establish a model of onychomycosis. A cloudy appearance like that of human onychomycosis was observed in some infected nails.

Fig. 3. Histological findings in infected nails at six weeks post-infection (A) and two weeks post-infection (B). Many of fungi and a cluster of necrotic tissue in the nail deep layers were observed (A) and some of infected nails showed the chains of spores near the surface of the nail (B). Bar = 100µm.