Otitis externa is one of the most frequently occurring canine diseases [2, 3], and although it is not lethal, it is a chronic disease that troubles both dogs and owners for a prolonged period of time [1]. *Malassezia pachydermatis* is the most common microorganism isolated from canine otitis externa cases and even from healthy ear canals [12]. Seven species, *M. furfur*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae*, *M. sympodialis*, and *M. pachydermatis* belong to the genus *Malassezia*, but 6 of the species, all except *M. pachydermatis*, are characterized by a lipid being required for growth [6]. As *M. pachydermatis* is a major component of normal canine microbial flora, infections of the canine ear canal must be opportunistic to multiply when the microenvironment changes [1, 18]. *M. pachydermatis*-related otitis externa is very difficult to control, with repeated recurrences, and requires long-term antifungal agent therapy. Recently, the appearance of multidrug-resistant microbes due to long-term pharmaceutical treatment has come a problem for human and veterinary clinical practices, and drug resistance for the genus *Candida* has been reported [19]. The appearance of resistant strains of *M. pachydermatis* is a concern. Azoles, such as ketoconazole and itraconazole, polyene derivatives, such as nystatin, and allylamines, such as terbinafine HCl, are antifungal agents available in veterinary practice, but they are expensive to use in large quantities such as in ear canal cleaners. The development of more economical antifungal agents is expected.

β-Thujaplicin is a chemical substance with a tropolone base extracted from Aomori Hiba (*Thujopsis dolabrata* SIE-BOLD et ZUCCARINI var. hondai MAKINO, a kind of hiba arborvitae inhabiting north Japan), and the antimicrobial and antifungal effects of this chemical have previously been reported [10, 11]. β-Thujaplicin has anti-inflammatory and deodorant effects, and can be used safely and cheaply outside the medical arena in cosmetics, hair tonics, and deodorant effects. However, it is thought that the potential development of a resistant strain is low, even with continuous infusion for otitis externa therapy. β-Thujaplicin is an inexpensive and safe treatment with anti-inflammatory and deodorant effects that can be recommended as an effective remedy for canine otitis externa.

**MATERIALS AND METHODS**

**Isolation of *Malassezia pachydermatis* from canine cerumens:** Cerumen samples were collected from 61 otitis externa-affected dogs and 30 non-affected dogs that were brought to the Veterinary Teaching Hospital of Osaka Prefecture University and to private animal hospitals within Osaka city. Otitis externa was diagnosed by clinical signs such as waving the head side to side, itching, flare, swelling, and offensive odor, and microscopic findings from cerumen biopsies. Cerumen samples were obtained using a sterilized swab, with approximately 10 mg of gauged cerumen sus-
pended in 1 ml of sterilized phosphate-buffered solution (PBS, pH 7.2). The suspensions were aggregated into 100 µl groups, spread onto Sabouraud’s agar plates (NISSUI, Tokyo, Japan), and incubated at 37°C for 7 days. Growing colonies were confirmed as yeast using Gram staining, and the number of colonies was counted. Each isolate was identified tentatively as *M. pachydermatis* by colony morphology characteristics, bottle-shaped organisms and a no-lipid requirement for growth. The isolated strains were suspended in a 5% glucose solution and stored at –80°C.

**Improvement of the microbial sensitivity test**: Plate methods, plate disk methods, and tube methods are used for microbial sensitivity examinations, but they all require great effort. In particular, fungi require a long period of time because the growth rate is slow. Many methods have previously been devised for sensitivity tests. The Sabouraud’s broth [20] and urea broth [17] liquid culture mediums used for the drug sensitivity test of *M. pachydermatis* have previously been reported. Urea broth (yeast extract 0.1 g/l; potassium dihydrogen phosphate 9.1 g/l; di-sodium hydrogen phosphate 9.5 g/l; urea 20.0 g/l; phenol red 0.01 g/l) has the advantage of easy visual judgment of growth, and was selected and improved for the *M. pachydermatis* sensitivity test. Since *M. pachydermatis* forms an agglutination when incubated with ordinary culture broth, it is difficult to evaluate the fungal growth quantitatively. In addition, it is necessary to use the same inoculum size for sensitivity tests, and this cluster formation is therefore extremely inconvenient. As a result, referring to previous reports [7, 20] and using representative *M. pachydermatis* isolates to achieve uniform suspensions, a culture solution based on Sabouraud’s broth (Oxoid, UK), with 0.2% Brij35 (Wako, Osaka, Japan) and 0.01% oleate, was found to give good results, with no growth influences on the yeasts.

**Microorganism sensitivity test**: Fifty-one stored isolates of *M. pachydermatis* were examined for β-thujaplicin sensitivity. For comparison, sensitivity tests for nystatin, ketoconazole, and terbinafine HCl, as antifungal agents working against yeasts and especially *M. pachydermatis* forms an agglutination when incubated with ordinary culture broth, it is difficult to evaluate the fungal growth quantitatively. In addition, it is necessary to use the same inoculum size for sensitivity tests, and this cluster formation is therefore extremely inconvenient. As a result, referring to previous reports [7, 20] and using representative *M. pachydermatis* isolates to achieve uniform suspensions, a culture solution based on Sabouraud’s broth (Oxoid, UK), with 0.2% Brij35 (Wako, Osaka, Japan) and 0.01% oleate, was found to give good results, with no growth influences on the yeasts.

**Microorganism sensitivity test**: Fifty-one stored isolates of *M. pachydermatis* were examined for β-thujaplicin sensitivity. For comparison, sensitivity tests for nystatin, ketoconazole, and terbinafine HCl, as antifungal agents working well in clinical practice, were conducted concurrently. Each *M. pachydermatis* strain was incubated for 7 days in Sabouraud’s broth with 0.2% Brij35 and 0.01% oleate, and then diluted 10 times in urea broth to make the inoculum for the sensitivity tests. The inoculum was incubated on a Sabouraud’s agar plate, and the number of *M. pachydermatis* colonies was counted. Each isolate was identified tentatively as *M. pachydermatis* by colony morphology characteristics, bottle-shaped organisms and a no-lipid requirement for growth. The isolated strains were suspended in a 5% glucose solution and stored at –80°C.

**Resistance acquisition tests of antifungal agents**: *M. pachydermatis* samples were subcultured with β-thujaplicin, nystatin, ketoconazole, and terbinafine HCl at concentrations around the MIC of those drugs, and examined as to whether the organisms acquired resistance to the drugs. Ten strains of stored *M. pachydermatis* were used. Using Sabouraud’s broth, including 0.2% Brij35 and 0.01% oleate, the organisms were incubated for 7 days, and the broths were diluted 10 times in Sabouraud’s broth for the inoculum. This Sabouraud’s broth was added to each well of a 96-well sterilized microtiter plate, 150 µl for each well. The concentration series of β-thujaplicin, nystatin, and terbinafine HCl were adjusted to 0.2–100 µg/ml, and for ketoconazole, it was adjusted to 0.002–1.0 µg/ml. The *M. pachydermatis* inoculum was inoculated as 50 µl, and incubated at 37°C for 3 days. The MIC was assessed using the turbidity of the culture media by visual inspection. After the second subculture, the culture broth at a single step lower concentration than MIC was diluted 10 times as the next subculture inoculum, and subcultures were repeated likewise for 30 generations.

**RESULTS**

**Isolation of *M. pachydermatis* from clinical materials**: Cerumen samples were taken from 61 otitis externa-affected and 35 non-affected dogs. *M. pachydermatis* organisms were isolated from 57.3% of the affected samples and 80% of the non-affected ones, but even if the organism was not isolated microbiologically, *Malassezia*-like yeasts were detected in almost all materials cytologically. The total separation rate was 64.8%. The *M. pachydermatis* count for otitis externa-affected samples was $10^3–10^4$ CFU per 10 mg of cerumen, whereas for non-affected samples, it was $10^2$ CFU per 10 mg.

**Drug sensitivity test**: The MIC distributions of drugs for *M. pachydermatis* are shown in Fig. 1. The MIC range of β-thujaplicin was 1.56–3.1 µg/ml. The lowest drug concentration that inhibited 50% growth of *M. pachydermatis* isolates (MIC50) was 3.13 µg/ml for β-thujaplicin, and the MICs of 47 of the 51 isolates were 3.13 µg/ml. The MIC range of nystatin was 1.56–6.25 µg/ml, the MIC50 of the isolates was 3.13 µg/ml, and the MICs of 47 isolates were 3.13 µg/ml. The MIC range of ketoconazole was as wide as 0.0078–0.2 µg/ml, and the MIC50 of the isolates was 0.016 µg/ml. The MIC range of terbinafine HCl was as wide as 0.002–1.0 µg/ml of ketoconazole. Subsequently, 50 µl of *M. pachydermatis* inoculum suspension was added to the wells. An aliquot of 100 µl of the sterilized liquid paraffin was layered in all wells to prevent any false positive growth by diffusing ammonia from decomposed urea in the surrounding wells. After incubation at 37°C for 3 days, MIC was judged by visual inspection. It is generally accepted to use DMSO to dissolve the drug for an MIC examination of water-insoluble antibiotics for microorganisms [9]. All culture media were adjusted in advance to include 2% DMSO after confirming no influence on *M. pachydermatis* growth.
It was concluded that there were no resistant strains against any drugs among the 51 M. pachydermatis isolates in this investigation because the MIC distribution of the drugs uniformly showed a single peak with no protrusions.

Resistance acquisition tests for antifungal agents: The changes in the MIC$_{50}$ range of antifungal agents after serial subculture for 30 generations are shown in Table 1. There were no changes in the MIC$_{50}$ of β-thujaplicin after 30 repeated subcultures. For nystatin, a 2-fold shift of MIC$_{50}$ from 6.25 µg/ml to 12.5 µg/ml, was recorded, and 4 out of 10 strains showed an MIC shift after 32 subcultures. For ketoconazole, a 16-fold shift of MIC$_{50}$ from 0.031 µg/ml to 0.5 µg/ml, was recorded, and 7 out of 10 strains changed after subculture. For terbinafine HCl, an 8-fold shift of MIC$_{50}$ from 0.2 µg/ml to 1.56 µg/ml, was found, and the MIC of 1 strain was up-regulated after 32 subcultures.

DISCUSSION

A wide range of isolation rates for M. pachydermatis from otitis externa-affected dogs have been reported [15], with some reports as high as 82.8% [14]. These rates depend on the existence and type of treatment. In non-
affected dogs, many reports have shown an isolation rate of approximately 20%, although one was as high as 35.9% [5]. In this investigation, the *M. pachydermatis* isolation rate for otitis externa-affected dogs was 57.3%, similar to previous reports. For non-affected dogs, however, the isolation rate for *M. pachydermatis* was as high as 80%. It was thought that there was a seasonal influence because most of the samples were collected during the summer [8]. The possible effects of humidity in Japan on *M. pachydermatis* growth in the ear canal have previously been considered, even without clinical signs. Therefore, a modified antifungal sensitivity test using urea broth and a 96-well microplate was therefore reviewed. In this method, the growth of *M. pachydermatis* can be evaluated easily by visual inspection for MIC. Furthermore, this method is advantageous because only a small amount of drugs and culture media are needed. The modified method in this study is reliable because of its good reproducibility. Sensitivity tests for *M. pachydermatis* to thujaplicin, nystatin, ketoconazole and terbinafine-HCl were conducted. The MICs previously reported for *M. pachydermatis* were under 0.03 μg/ml for ketoconazole and 0.2–12.5 μg/ml for terbinafine-HCl [4, 16, 17, 20]. The MIC 50 results for ketoconazole (0.016 μg/ml) and terbinafine HCl (1.56 μg/ml) were almost identical to previous reports. The MIC of nystatin in this experiment was evaluated as being identical to those for reports in which the disk diffusion method was used [13]. Thujaplicin suppressed the growth of *M. pachydermatis* in clinically applicable concentrations similar to those of other antifungal agents. As thujaplicin has a wide antibacterial spectrum [11], this drug is estimated to be effective in the treatment of canine otitis externa with a variety of microorganisms [14]. In regard to anti-inflammatory and deodorant action, thujaplicin possibly controls inflammation and offensive odors resulting from otitis externa. As thujaplicin is approximately 100 to 1,000 times less expensive than other antifungal agents, it is used in large quantities with ear canal cleaners, in comparison with other expensive antifungal agents.

*M. pachydermatis* resistance acquisition tests were conducted for β-thujaplicin, nystatin, ketoconazole, and terbinafine HCl. As a result of 30-generation subcultures, MIC 50 shifts were found at 2-fold, 16-fold and 8-fold larger concentrations for nystatin, ketoconazole, and terbinafine HCl, respectively. For β-thujaplicin, no change in MIC 50 change was identified. It is probably difficult for *M. pachydermatis* to develop resistance to β-thujaplicin, compared with other antifungal agents. Long-term treatment using β-thujaplicin for otitis externa therapy is recommended, because the potential is low for a strain to appear that is resistant to β-thujaplicin. There were no resistant strains among the 51 *M. pachydermatis* isolates in this investigation.

In conclusion, since β-thujaplicin has stable anti-*Malassezia* effects, a wide antibacterial spectrum, anti-inflammatory effects, and deodorant actions, in addition to being safe and inexpensive, it is expected to be a remedy for canine otitis externa.

**REFERENCES**


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