The Journal of Veterinary Medical Science

NOTE

Bacteriology

Molecular typing and antifungal drug susceptivity profile of *Rhodotorula mucilaginosa* from canine skin and ear canal

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J. Vet. Med. Sci.
83(10): 1545–1548, 2021
doi: 10.1292/jvms.21-0361

Received: 24 June 2021
Accepted: 21 August 2021
Advanced Epub:
14 September 2021

ABSTRACT. *Rhodotorula mucilaginosa* are saprophytic yeast, and opportunistic infections known as human rhodotorulosis are increasing in immunocompromised patients. In this study, we isolated *R. mucilaginosa* from pet dogs in Japan and determined the minimum inhibitory concentrations (MICs) of antifungal drugs on these isolates to investigate the drug susceptibility pattern. All 10 isolates according to the broth microdilution (BM) assay of the Clinical and Laboratory Standards Institute (CLSI) M27-A2 were resistance to azoles and genetically close to fluconazole (FLZ)-resistant human isolates of *R. mucilaginosa*. Due to resistance, it is expected that treatment will be difficult if they infect humans.

KEY WORDS: antifungal resistance, canine, *Rhodotorula mucilaginosa,* susceptibility testing

Rhodotorula spp. are saprophytic yeast that are usually isolated from moist environmental sources, including bathroom surfaces, dairy products, plant surfaces, and as commensal inhabitants of skin and gastrointestinal and upper respiratory tracts of mammals [8]. Infections with *Rhodotorula* spp. in dogs are very rare, but granulomatous epididymitis, cystitis, and respiratory tract infections have been reported [1, 8]. Opportunistic infection of humans, known as rhodotorulosis, is increasing in immunocompromised patients and is associated with high mortality despite antifungal treatments [6, 11, 13]. Therefore, rhodotorulosis is not a problem for dogs themselves, but might be concern as a zoonosis that can be transmitted directly to immunocompromised patients from healthy dogs, or from medical staff who have pets through medical equipment such as catheters.

In this study, we isolated *R. mucilaginosa* from pet dogs in the Tokyo area of Japan and determined the minimum inhibitory concentrations (MICs) of antifungal drugs on these isolates to investigate their drug susceptibility.

A total of 10 yeast isolates were collected from canine skin and ear canals in 2020; all were isolated from nine pet dogs that were kept in 9 different homes located in the Tokyo region of Japan (Table 1). In this study, seven of nine dogs (all dogs were diagnosed with seborrheic dermatitis or otitis externa) had been treated with oral itraconazole (ITZ) and ketoconazole (KTZ) or a shampoo with miconazole (MIZ) (Table 1). No fungal infections have been found in the owners of the patient dogs.

Culturing (32°C, 4 days) of skin swabs of the canine skins on modified Dixon medium (mDixon; 3.6% malt extract, 0.6% peptone, 2.0% desiccated ox bile, 1.0% Tween 40, 0.2% glycerol, 0.2% oleic acid, 1.2% agar, pH 6.0) yielded orange-colored colonies with straight margins. In fact, they were isolated while attempting to isolate *Malassezia pachydermatis* from the dogs' skin and ear canals.

Yeast colonies were identified as *R. mucilaginosa* based on the sequence homology of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) of ribosomal DNA as described previously [12]. The universal fungal primers ITS-5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) were used to amplify the ITS region of the isolates [12]. Isolates were obtained by culturing cells on Sabouraud's dextrose agar (1% peptone, 2% dextrose, 2% agar) at 32°C for 3 days. The resulting yeast cells (about 1–2 mg) were boiled in lysis buffer (20 mM Tris-hydrochloride (Tris-HCl), pH 8.0, 1 mM EDTA, 1% sodium dodecyl sulfate). High-molecular-weight DNA was purified from the lysate by extraction with phenol followed by ethanol precipitation. The resulting samples were suspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and used as templates for PCR amplification.

DNA samples (100 to 200 ng each) were amplified by PCR in a volume of 30 µl, using a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM each deoxynucleotide triphosphate, 1.0 unit of *Taq* polymerase (Takara, Kyoto, Japan), and 30 µmol of each primer pair. PCR amplification was carried out for 30 cycles consisting

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Table 1. Minimum inhibitory concentrations (mg/l) of antifungal drugs against the tested isolates

Strain no.	Patient dog (year old) sex	Diagnosis	Isolation site	Treatment history	FLZ	ITZ	RAZ	VRZ	CTZ	MIZ	LUZ	ITS number
NUBS21001	Toy Poodle (9y 11m) male	Otitis externa	Left ear canal	Ear drop for KTZ	>64	1	<0.03	1	0.5	>32	0.06	LC627504
NUBS21002	Shiba Inu (13y 8m) castrated	Seborrheic dermatitis	Ventral side of neck	Oral administration for KTZ	>64	4	<0.03	16	8	>32	2	LC627505
NUBS21003	Beagle (8y 4m) spayed	Otitis externa	Right ear canal	Oral administration for prednisolone and cefalexin	>64	1	<0.03	1	1	>32	0.5	LC628147
NUBS21004	Westie (14y 9m) female	Otitis externa	Right ear canal	Shampooing with 2% miconazole and oral administration for KTZ	>64	<0.03	<0.03	8	16	>32	2	LC628639
NUBS21005	Same as above	Same as above	Left ear canal	Same as above	>64	1	<0.03	1	0.125	>32	0.125	LC628678
NUBS21006	Dachshund (4y 4m) spayed	Otitis externa	Ear canal	Unknown	>64	2	<0.03	2	0.125	>32	0.25	LC628679
NUBS21007	Pug (11y 10m) spayed	Seborrheic dermatitis	Right elbow	Shampooing with 2% miconazole and oral administration for KTZ	>64	4	<0.03	4	1	>32	2	LC628680
NUBS21008	Shih Tzu (9y 6m) castrated	Seborrheic dermatitis	Inner thigh	Shampooing with 2% miconazole	>64	4	<0.03	16	2	>32	4	LC628681
NUBS21009	Dachshund (4y 10m) castrated	Seborrheic dermatitis	Right forelimbs	Oral administration for KTZ	>64	4	<0.03	8	0.5	>32	4	LC628682
NUBS21010	Shih Tzu (9y 6m) male	Seborrheic dermatitis	Breast	Oral administration for ITZ and KTZ ointment	>64	2	<0.03	16	2	>32	2	LC628683

KTZ: ketoconazole KTZ, NUBS: Nihon University College of Bioresource Sciences, FLZ: fluconazole, ITZ: itraconazole, RAZ: ravuconazole, VRZ: voriconazole, CTZ: clotrimazole, MTZ: miconazole, LUZ: luriconazole, ITS number: GenBank accession number for ITS sequence.

of denaturation for 30 sec at 94°C, primer annealing for 30 sec at 55°C, and extension for 60 sec at 72°C. Final extension was performed at 72°C for 2 min. For each strain/reaction, the amplicon was electrophoresed in agarose, and an approximately 550-bp long DNA band was purified using the ExoSAP-IT® kit (USB Corp., Cleveland, OH, USA), and sequenced on an ABI PRISM 3130 DNA Analyzer (Thermo Fisher Scientific, Inc., Tokyo, Japan) using the PCR primers for sequencing.

Comparative sequence analyses were carried out using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI).

Comparative nucleotide sequence analysis using the BLAST algorithm on the NCBI website showed that the ITS sequences amplified from all 10 isolates were 99 to 100% identical to *R. mucilaginosa* type strain CBS 316^T(GenBank accession No. NR_073296). The region sequences were also 87 to 98% identical to other *Rhodotorula* species. Therefore, all isolates were identified as *R. mucilaginosa.*

The ITS sequences determined in this study have been deposited in the GenBank database: *R. mucilaginosa* strains NUBS21001 to 21010 (Table 1).

The resulting sequences of the ITS region were compared with those of the *Rhodotorula* species type strains, FLZ-resistance strains of *R. mucilaginosa* (CMRP3462 and CMRP3463), and 10 isolates using the ClustalW multiple sequence alignment program [10]. The phylogenetic tree was constructed with the neighbor-joining method using the GENETYX ver.15 program (GENETYX Corp., Tokyo, Japan) [7]. Bootstrap analysis was performed on 1,000 replicates of random samples and analyzed with the ClustalW program [4].

Phylogenetic analysis of the ITS region sequences revealed that the isolates (NUBS21001 to NUBS21010) were grouped in a cluster of *R. mucilaginosa* type strain CBS 316^T and FLZ-resistance human isolates of *R. mucilaginosa* (CMRP3462 and CMRP3463) [6], and were independent of the cluster of the other type strains of *Rhodotorula* species (Fig. 1).

Antifungal susceptibility tests were performed to determine the MICs of clotrimazole (CTZ), MIZ, fluconazole (FLZ), ITZ, luliconazole (LUZ), ravuconazole (RAZ), and voriconazole (VRZ) for the 10 isolates according to the broth microdilution (BM) assay of the Clinical and Laboratory Standards Institute (CLSI) M27-A3 [3]. For quality control, the strain *Candida parapsilosis* ATCC 22019 was used in CLSI M27-A3 to check the accuracy of drug dilution [3]. The experiment was performed in duplicate.

Rhodotorula species were classified as being resistant to FLZ, ITZ, and VRZ according to the clinical breakpoints outlined in the M27-A3 guidelines prepared by the CLSI [2]. The MICs of FLZ, ITZ, and VRZ in the resistant strains were determined to be >64 mg/l, >1 mg/l, and >4 mg/l, respectively [2].

The MICs for all 10 isolates were >32 mg/l for FLZ, <0.03 to 4 mg/l for ITZ, <0.03 mg/l for RAZ, 1 to 16 mg/l for VRZ, 0.125 to 16 mg/l for CTZ, >32 mg/l for MIZ, and 0.06 to 4 mg/l for LUZ (Table 1).

To our knowledge, this is the first report of antifungal-resistant profiles for *R. mucilaginosa* isolates from canine skin and ear canals. All isolates were resistant to azoles and genetically close to FLZ-resistance human isolates of *R. mucilaginosa* (Table 1 and Fig. 1) [6]. Due to resistance, it is expected that treatment will be difficult if it infects humans. However, the prevalence of resistant strains of *R. mucilaginosa* in dogs was unclear in this study, but we found more isolates than expected (the original purpose of this study was the isolation of *M. pachydermatis*). Unfortunately, there are few reports of drug susceptibility testing of *R. mucilaginosa*

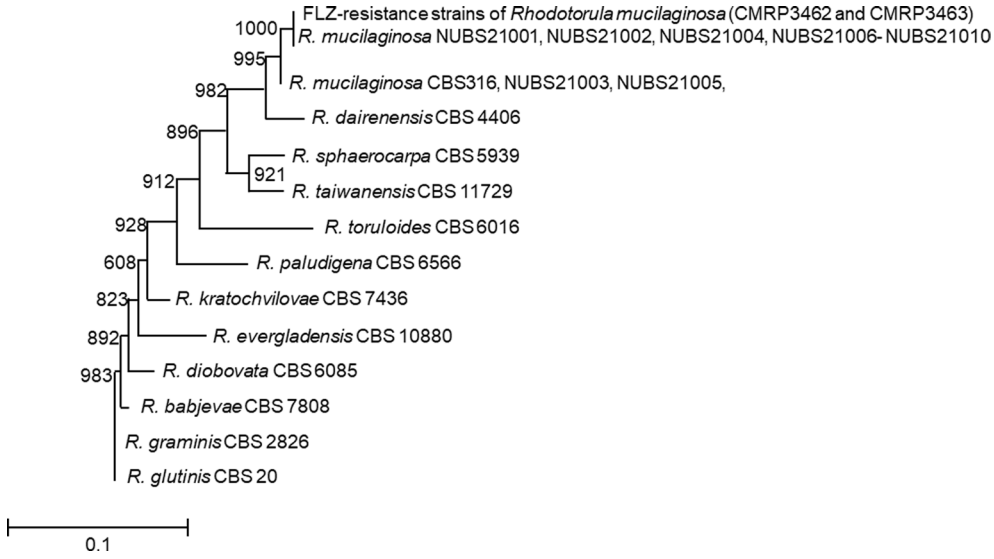

Fig. 1. Phylogenetic tree created with the neighbor-joining method showing the molecular taxonomy of the internal transcribed spacer (ITS) region. The numbers at the branches are bootstrap values and indicate 1,000 repeat sub-samples in monophyletic groups. FLZ-resistant strains of *Rhodotorula mucilaginosa* include CMRP3462 (GenBank accession no. MK453051) and CMRP3463 (MK453052). Isolates from canine skin and ear canals include NUBS21001 (LC627504), NUBS21002 (LC627505), NUBS21003 (LC628147), NUBS21004 (LC628639), NUBS21005 (LC628678), NUBS21006 (LC628679), NUBS21007 (LC628680), NUBS21008 (LC628681), NUBS21009 (LC628682), and NUBS21010 (LC628683). Also included are *R. mucilaginosa* CBS 316 (NR_073296), *R. dairenensis* CBS 4406 (KY104735), *R. sphaerocarpa* CBS 5939 (NR_073269), *R. taiwanensis culture* CBS 11729 (KY104910), *R. toruloides* CBS 6016 (KY104925), *R. paludigena* CBS 6566 (NR_073265), *R. kratochvilovae* CBS 7436 (AF444520), *R. evergladensis* 10880 (NR_137709), *R. diobovata* CBS 6085 (AF444502), *R. babjevae* CBS 7808 (NR_077096), *R. graminis* CBS 2826 (NR_073273), and *R. glutinis* CBS 20 (NR_073294).

isolated in the environment or from the skin of healthy humans and dogs, so it is unknown whether it was resistant from the beginning or acquired resistance by antifungal treatments.

Tang *et al.* investigated canine skin and ear microbiomes using next-generation sequencing assays and did not detect *Rhodotorula* spp. genes from healthy and clinically affected dogs [9]. From their report, *R. mucilaginosa* is not expected to be a major microbe of the skin and ear canal [9]. However, we isolated *R. mucilaginosa* easily.

The history of antifungal treatment may have led to microbial replacement by *R. mucilaginosa*, that is resistant to antifungal agents. The desired isolation of *M. pachydermatis* was not possible from these cases. Only *R. mucilaginosa* was isolated from all the growing colonies on mDixon, which is the isolation medium for *Malassezia* species from canine skin [5]. We speculate that treatment with antifungal agents may be due to fungal alternation, in which *M. pachydermatis* on the skin surface, which was originally desired to be isolated, was reduced and *R. mucilaginosa* is increased.

If artificial changes in the microbial flora to drug-resistant yeasts occur in the skin and ear canals of Japanese pet dogs, there is concern that the risk of zoonotic diseases will increase. In future research, it is necessary to investigate the microbial flora of the skin and ear canals of Japanese pet dogs by microbiome analysis using next-generation sequence analysis.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

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