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NOTE

Bacteriology

Isolation of antifungal-resistant yeasts from bulk milk in Japan

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ABSTRACT. We isolated ascomycetous yeasts including *Candida* species, that originally belonged to the genus *Candida,* from bulk milk in the Aichi area of Japan, and determined the minimum inhibitory concentrations (MICs) of antifungal drugs on these isolates by conducting E-tests. We isolated 7 human pathogenic species (14 isolates) from 14 bulk milk samples: 5 *Candida* species of yeasts, and 2 *Candida*-related species. Two isolates of *C. albicans* and *C. inconspicua* were resistant to fluconazole (MIC >32 mg/l). One isolate of *C. krusei* was resistant to both azoles (fluconazole: >256 mg/ml and itraconazole: 4 mg/l). One isolate of *C. catenulata* might be resistant to amphotericin B (>32 mg/l).

KEY WORDS: antifungal resistance, bulk milk, *Candida*, cow, susceptibility testing

Candidiasis is a common yeast infection that is an opportunistic infection in humans. *Candida albicans* is the most common and significant species that causes candidiasis, although non-albicans *Candida* species, such as *C. glabrata, C. parapsilosis*, *C. guilliermondii,* and *C. krusei* (currently reclassified as *Pichia kudriavzevii*), are also known to be causative agents of human *Candida* infections [1, 2]. Importantly, there has been a recent increase in infections due to non-albicans *Candida* species that exhibit resistance to widely used antifungal agents, such as fluconazole (FLZ) and micafungin, and they are thus associated with a high mortality rate [1, 2]. Moreover, the proportion of non-albicans *Candida* species exhibits geographical differences. *C. glabrata* is generally more common in Western countries, whereas *C. parapsilosis* is frequently isolated in Latin America. *C. tropicalis* is more common in Asia [7]. Therefore, continual surveillance is required to monitor the emergence of these resistant *Candida* species in each country [1, 2]. *Candida* species are also a part of the normal flora of skin, the mouth, vagina, and gastrointestinal tract in humans and animals. As well as being a pathogen of bovine chronic mastitis, it can be extracted from chronic mastitis milk [5].

In this study, we isolated *Candida* species, including pathogenic yeasts that originally belonged to the genus *Candida*, from bulk milk in the Aichi area of Japan, and determined the minimum inhibitory concentrations (MICs) of antifungal drugs on these isolates by conducting E-tests.

A total of 24 bulk milk samples were collected for regular bulk milk tests in 2019; all were Holstein cow milk collected at 24 dairy farms located in the Aichi region of Japan. Each milk sample was inoculated directly onto a CHROMagar™ Candida (Kanto Chemical Co., Inc., Tokyo, Japan) and incubated at 32°C for 2 days. Fungal colonies were then identified as *Candida* species based on the sequence homology of the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) of ribosomal DNA as described previously [6]. 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 [6]. The molecular identification of fungal species was carried out according to a previous report [8].

E-tests were performed to determine the MICs of amphotericin B (AMB), FLZ, itraconazole (ITZ), and voriconazole (VRZ) for the 14 isolates of *Candida* species collected from 14 bulk milk samples according to the E-test Technical Guide 10 (bioMérieux Japan, Tokyo, Japan). *Candida* 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 Clinical Laboratory Standards Institute [4]. 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 [3].

We isolated 7 human pathogenic species among the 14 isolates from the 14 bulk milk samples; the human pathogenic species comprised 5 *Candida* species of yeast (*C. albicans*, *C. catenulate*, *C. inconspicua*, *C. parapsilosis,* and *C. tropicalis*) and 2 *Candida*-related species (*Pichia kudriavzevii* and *Wickerhamiella pararugosa*; Table 1).

The MICs for all 14 isolates were <0.002 to >32 mg/l for AMB, 0.064 to >256 mg/l for FLZ, 0.004 to 4 mg/l for ITZ, and

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

Strain number	Balk milk number	Isolated yeast species	Former name	Amphotericin B	Fluconazole	Itraconazole	Voriconazole
No.4	35P	*Wickerhamiella pararugosa*	*C. pararugos*	0.064	1.000	0.064	0.006
No.5	59P	*Candida albicans*		0.008	1.000	0.023	0.004
No.6	65P	*W. pararugosa*	*C. pararugos*	0.250	2.000	0.094	<0.002
No.7	66P	*C. parapsilosis*		<0.002	0.125	0.004	0.004
No.14	194P	*Pichia kudriavzevii*	*C. krusei*	1.500	>256.0	4.000	0.250
No.15	194W	*C. albicans*		0.380	>256.0	0.250	<0.002
No.16	198W	*C. inconspicua*		0.230	>256.0	0.125	<0.002
No.17	207P	*C. parapsilosis*		0.380	0.064	0.094	0.004
No.18	231P	*W. pararugosa*	*C. pararugos*	0.016	3.000	0.064	0.320
No.19	245P	*C. parapsilosis*		0.064	0.125	0.064	0.006
No.20	247B	*C. catenulata*		>32.00	2.000	0.064	<0.002
No.21	247G	*C. tropicalis*		0.500	0.250	0.064	<0.002
No.22	263P	*C. parapsilosis*		0.016	0.094	0.064	<0.002
No.23	268P	*W. pararugosa*	*C. pararugos*	0.380	1.500	0.250	<0.002

<0.002 to 0.32 mg/l for VRZ (Table 1). Two isolates of *C. albicans* and *C. inconspicua* (Nos. 15 and 16) were resistant to FLZ (MIC >32 mg/l; Table 1). One isolate of *P. kudriavzevii* (No. 14) was resistant to both azoles (FLZ: >256 mg/ml and ITZ: 4 mg/l; Table 1). One isolate of *C. catenulata* (No. 20) might be resistant to AMB (MIC >32 mg/l; Table 1).

To our knowledge, this is the first report of antifungal-resistant human pathogenic yeast isolates from bulk milk. In particular, *C. albicans, C. parapsilosis, C. tropicalis P. kudriavzevii* are intrinsically resistant or less susceptible to antifungals [1, 2], and they have also been isolated from bovine clinical mastitis cases in Japan [5]. Based on our findings, we suspect that the prevalence of antifungal-resistant pathogenic yeasts is detected in bulk milk in Japan. Of course, in Japan, raw milk is pasteurized before it is shipped, so it is unlikely for consumers to become infected by antifungal-resistant yeasts in milk. However, if drug-resistant pathogenic yeast is present in the dairy cow's udder, there is concern that beef contaminated with the yeasts may be shipped to markets.

It has been reported that the increasing use of azole fungicides in agriculture has induced azole resistance in *Aspergillus* species in the environment. The European Centre for Disease Prevention and Control issued a risk assessment report on azole resistance in Aspergillus species and its possible link to the environmental usage of azole fungicides (http://www.life-worldwide.org/media-centre/article/ecdc-issues-risk-assessment-on-azole-resistance-in-aspergillus-from-environ/). An increase in azole antifungal-resistant Aspergillus strains that cause serious human infections has been detected in several European countries. In addition, residual azole fungicides remaining in cattle feed may also have induced resistance. Sidrim *et al*. [9] reported that humans, domesticated animals, wild animals, and aquatic and/or terrestrial environments are likely to carry resistant microorganisms, and can spread them to other animals and natural environments. Therefore, azole-resistant *Candida* species may spread between cattle in the same environment.

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

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