Molecular-Based Identification of Yeasts Isolated from Bovine Clinical Mastitis in Japan

Tomohito HAYASHI1), Takashi SUGITA2), Eiji HATA1), Ken KATSUDA3), Enshi ZHANG2), Yoshio KIKU3), Kazue SUGAWARA1'), Tomomi OZAWA1'), Tomoko MATSUBARA1'), Takaaki ANDO3'), Tetsu OBAHASHI4), Takaaki ITO5), Takahiro YABUSAKI6), Katsunori KUDO6), Hiroshi YAMAMOTO7), Masateru KOIWA3), Toshio OSHIDA8), Yuichi TAGAWA1) and Kazuhiro KAWAI8)*

1)National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305–0856, Japan
2)Department of Microbiology, Meiji Pharmaceutical University, Kiyose, Tokyo 204–8588, Japan
3)School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069–8501, Japan
4)Tokachi NOSAI, Taiki, Hokkaido 089–0856, Japan
5)Aichi NOSAI, Okazaki, Aichi 444–0816, Japan
6)Ishikari NOSAI, Ebetsu, Hokkaido 069–0055, Japan
7)Okhotsk NOSAI, Kitami, Hokkaido 099–0879, Japan
8)School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 252–5201, Japan

(Received 14 August 2012/Accepted 13 October 2012/Published online in J-STAGE 26 October 2012)

ABSTRACT. This study analyzed molecular-based identification of yeasts that associated with bovine clinical mastitis in Japan. Over 3,200 quarter milk samples from Holstein dairy cows collected in 2011 on Hokkaido and Honshu islands were examined. Yeast isolates were characterized by polymerase chain reaction amplification and sequencing of the D1/D2 region of the 26S rDNA. Molecular characterization confirmed that Candida spp. and Pichia spp. were most frequently isolated species. Our molecular analysis of mastitic milk samples demonstrated the prevalence of Pichia kudriavzevii(22/58) and Candida tropicalis(14/58). In addition, we demonstrated that molecular analysis of the D1/D2 region of the 26S rDNA is a rapid and reliable method for identifying clinically significant yeasts in dairy hygiene, including potentially new or emerging pathogenic species.

KEY WORDS: clinical mastitis, Japan, molecular analysis, yeast.


Bovine mastitis, inflammation of the mammary gland, is among the most important diseases in dairy herds, resulting in reductions of milk yield and milk quality. More than a hundred different microorganisms have been identified as etiological agents of mastitis [2]. In addition to bacteria, several other classes of microorganisms such as yeasts, fungi and algae can cause inflammatory alterations in the bovine udder [2, 5, 8, 20]. However, the incidence of mastitis due to yeast is usually low in dairy herds [5]. Among mastitis-causing yeasts, the most frequent genus is Candida [20]. Other yeast genera such as Cryptococcus, Pichia and Trichosporon also have been isolated from clinical cases with low frequencies [18]. Yeasts are normal flora of the soil and may colonize udder skin in small numbers [14]. Infection by such microorganisms is considered to be opportunistic, producing disease primarily when innate defense mechanisms are lowered.

Identification of this increasing diversity of pathogens by conventional methods is often difficult and sometimes inconclusive [13]. Morphological features and reproductive structures useful for identifying isolated yeast and fungi may take days to weeks to develop in culture, and evaluation of these characteristics requires expertise in mycology [12].

Molecular techniques utilizing amplification of target DNA provide alternative methods for diagnosis and identification [9]. PCR-based detection of fungal DNA sequences can be rapid, sensitive, and specific [11]. Transcribed regions of the 18S, 5.8S and 26S nuclear rRNA genes evolved slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships. Thus, PCR amplification may facilitate the identification of 26S rRNA sequences with sufficient polymorphism to be useful for identifying fungal species [16].

Few descriptions of yeasts obtained from bovine mastitis milk in Japan have been published. In this study, we amplified and identified the D1/D2 sequences of the 26S rDNA from 58 yeast strains recovered from over 3,200 bovine clinical mastitis milk samples. Our data indicate that the D1/D2 regions of the 26S rRNA gene are useful for identifying medically important yeasts and may facilitate taxonomic and phylogenetic classification of potentially new pathogenic species.

A total of 3,244 quarter milk samples from Holstein dairy cows with clinical mastitis were collected during 2011 from the dairy belt located in three regions of Hokkaido island (Ishikari, Okhotsk and Tokachi) and two prefectures.
of Honshu island (Ibaraki and Aichi) in Japan. The yeast isolates were originally classified at the genus level based on morphological characteristics. For this initial classification, yeasts were isolated on trypticase soy agar (TSA) II with 5% sheep blood (Becton, Dickinson and Company, Paramus, NJ, U.S.A.) with aerobic incubation at 37°C for 24 hr. A total of 58 yeasts were isolated from 3,244 quarter milk samples (Table 1). These 58 yeast isolates corresponded to 13 (out of 1,220 quarters) from Ishikari, 6 (out of 220 quarters) from Okhotsk, 20 (out of 975 quarters) from Tokachi, 4 (out of 127 quarters) from Ibaraki and 15 (out of 702 quarters) from Aichi. Each yeast isolate was further characterized using rDNA sequence analysis. For each isolate, genomic DNA was extracted by the method of Makimura et al. [11]. The D1/D2 region of the 26S rRNA gene was amplified by PCR with the primer pair NL-1 (5'-GGTCCGTGTTTCAAGACGG-3') and NL-4 (5'-GCATATCAATAAGCGGAGGAAAAG-3') [9]; the resulting PCR product was sequenced directly using the same primers [16]. The sequence data were analyzed with the National Center for Biotechnology Information (NCBI; Bethesda, MD, U.S.A.) BLAST system (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome). Strains with a ≥99% D1/D2 26S rDNA sequence similarity were defined as conspecific. DNA sequences were aligned using CLUSTAL W [19] for the neighbor-joining analysis [15]; distances between the sequences were calculated using Kimura’s two-parameter model [17]. A bootstrap analysis was performed using 1,000 replications.

From a total of 3,244 samples, we isolated 58 yeast colonies using TSA-blood plates. This corresponded to an udder yeast infection rate of 1.8% of the clinical mastitis samples (Table 1). Subsequent speciation was performed using molecular analysis of rDNA sequences. Twenty-three out of the 58 yeast colonies could be assigned to the genus Candida. C. tropicalis was the most frequently isolated species (14/58), followed by C. rugosa (3/58), C. albicans (1/58), C. ethanolic (1/58), C. parapsilosis (1/58), C. maltosa (1/58), C. pararugosa (1/58) and an unknown species (1/58). Thus, based on molecular phylogenetic analysis, our 23 Candida isolates could be assigned to a total of 8 distinct Candida species, of which 7 were previously described species. The remaining isolate showed greatest sequence similarity (541/581 nucleotide identity, or 93%) with Candida freyschussii. As the level of dissimilarity of the D1/D2 26S rDNA sequences was >1%, we concluded that this isolate represents a new species of Candida (Fig.1). In addition to the Candida strains, 6 other yeast genera were identified among our isolates, including those of Pichia kudriavzevii (22/58; anamorphic name, Candida kruzei), Clavispora lusitaniae (5/58; anamorphic name, Candida lusitaniae), Kluiveromyces marxianus (4/58; anamorphic name, Candida kefyr), Trichomonas ciferri (2/58; anamorphic name, Stephaonasascus ciferri), Debaryomyces hansenii (1/58) and Yamadazyma mexicana (1/58; anamorphic name, Pichia mexicana). Yeasts are found in a wide variety of environments such as soil, plants and water [14]. Most of these organisms represent opportunistic infectious agents, with possible sources of infection including the skin of udder, floor, straw, feed, water and milking machine [8, 14]. Environmental contamination resulting from poor hygiene during the milking process and poor equipment cleaning may lead to the development of mastitis. In different countries, the frequency of yeast isolation varies considerably, with reported rates of 1.3% in Denmark [1], 9.6% in Poland [7] and 12.1% in Brazil [3]. In the present study, yeasts were isolated from 1.8% of total clinical samples.

The majority of yeasts are considered saprobic, although in some cases these fungi are considered potential pathogens. In our study, Candida species represented 38% of the total

### Table 1. Molecular based identification of yeasts isolated from bovine clinical mastitis in Japan

<table>
<thead>
<tr>
<th>Species</th>
<th>Ishikari (N=1,220)</th>
<th>Okhotsk (N=220)</th>
<th>Tokachi (N=975)</th>
<th>Ibaraki (N=127)</th>
<th>Aichi (N=702)</th>
<th>Total (N=3,244)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida albicans</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Candida ethanolic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida maltosa</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Candida parapsilosis</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Candida pararugosa</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Candida rugosa</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Candida tropicalis</td>
<td>2</td>
<td>6</td>
<td>6</td>
<td>10</td>
<td>14</td>
<td>30</td>
</tr>
<tr>
<td>Candida novel species</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Clavispora lusitaniae</td>
<td>1</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>Debaryomyces hansenii</td>
<td>1</td>
<td></td>
<td>1</td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Kluyveromyces marxianus</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>Pichia kudriavzevii</td>
<td>8</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>22</td>
</tr>
<tr>
<td>Yamadazyma mexicana</td>
<td>1</td>
<td></td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Trichomonas ciferri</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>Number of yeasts</td>
<td>13</td>
<td>6</td>
<td>20</td>
<td>4</td>
<td>15</td>
<td>58</td>
</tr>
<tr>
<td>Frequency in area (%)</td>
<td>(1.1)</td>
<td>(2.7)</td>
<td>(2.1)</td>
<td>(3.1)</td>
<td>(2.1)</td>
<td>(1.8)</td>
</tr>
</tbody>
</table>
yeast isolates; however, C. albicans, which is considered a normal member of the gastrointestinal microbiota of mammals, was rarely detected (1 of 58 isolates). Non-albicans species have previously been isolated from bulk milk and cows with mastitis, and these species have also been reported as triggers of several kinds of infections [4, 8]. In relation to outbreaks of bovine mastitis, some reports point to a non-albicans source, represented by species such as C. tropicalis, K. marxianus (anamorphic name; C. kefyr) and C. rugosa [4, 5, 10, 18]. In our study, C. maltosa which has been reported to cause chronic mastitis [6] was only detected in 1.7% (1/58) of isolates. We also detected multiple Candida species, including C. tropicalis, C. kefyr, C. krusei and C. rugosa, and other yeast genera including Pichia (as well as Y. mexicana, anamorphic name; P. mexicana), Debaromyces and Trichomonas (anamorphic name; Stephanascus). The presence of yeasts may trigger alterations in the milk and dairy products due to the release of extracellular enzymes such as lipases and proteases. Antibiotic therapy is not only ineffective against yeasts, but may even serve as a nitrogen source for fungal growth. It is therefore critical to identify yeasts in mastitis samples in a timely manner, and it may be necessary to begin administering antifungal agents or perform continual stripping.

In conclusion, we performed the first analysis of the D1/D2 region of the 26S rDNA from 58 yeast isolates derived from over 3,200 bovine clinical mastitis samples in Japan. We anticipate that this approach will facilitate the rapid diagnosis of fungal infections directly from milk samples.

ACKNOWLEDGMENTS. This work was supported by Grants-in-Aid from the Mastitis Treatment Project (223) of the National Agriculture and Food Research Organization of Japan, and the Project for Developing Practical Technologies to Promote New Policies in Agriculture (22019) from the Forestry and Fisheries of the Ministry of Agriculture, Forestry and Fisheries of Japan. The authors wish to thank Dr. Petr Slama for insightful discussions.

REFERENCES


