

Molecular Detection of *Enterocytozoon bieneusi* and Identification of a Potentially Human-Pathogenic Genotype in Milk[∇]

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Milk specimens from 180 dairy cows were examined for the presence of *Enterocytozoon bieneusi*, using molecular assays. Fifteen specimens were found to be positive, of which 3 were identical to the human *E. bieneusi* types, which suggests that some *E. bieneusi* isolates from milk can infect humans. Overall, dairy cows' milk may play a significant role in the transmission of *E. bieneusi* infections to humans.

Microsporidia cause opportunistic infections in a wide range of animals and in humans and have recently been recognized as important emerging pathogens (6). Among the microsporidial species known, *Enterocytozoon bieneusi* is the most common microsporidium found in AIDS patients and in an increasing number of immunocompetent patients (16). AIDS patients infected with *E. bieneusi* suffer from chronic diarrhea, significant wasting, and malabsorption (17). This organism was also reported to be associated with hepatobiliary and pulmonary infections, which cause papillary stenosis, acalculous cholecystitis, bile duct dilatation, and sclerosing cholangitis (15, 19). Despite the large number of studies of this subject, the transmission routes and sources of human infections of this microsporidium are not completely understood. However, collective information from these studies has provided insights into several plausible modes of transmission to humans. Animals are also a likely source of human infections because this organism is released into the environment through the animal's feces and respiratory secretions. *E. bieneusi* has been reported in various animals including pigs, dogs, cats, rabbits, monkeys, and cattle. Many of the genotypes of *E. bieneusi* from these animals have also been found in humans, which supports the likelihood of zoonotic transmission (3, 5, 16).

This study examined the occurrence of *E. bieneusi* in the milk from dairy cattle, using molecular methods and sequence analysis both to assess the genotypic characteristics of *E. bieneusi* and to determine if milk is a potential source of *E. bieneusi* infections in humans.

Milk samples were collected from a total of 180 dairy cows between 3 and 7 years of age at 45 farms located throughout Chonbuk Province in Korea. Two to five samples per farm were collected from each sampling location. After teats were cleaned with sterile saline and initial milk was discarded, approximately 100 ml of milk per head was collected as aseptically as possible. All samples were transported immediately to the laboratory in ice-cooled containers, and 50 ml of each sample was centrifuged in a sterile polypropylene conical centrifuge tube at 1,000 × g for 30 min at 4°C. The fat and

supernatant layers were aspirated, and the pellet from the milk sample was used to extract the DNA for PCR. The pellet was resuspended in a 1/50 volume of phosphate-buffered saline. Ten microliters of the phosphate-buffered saline-resuspended solution was applied to an ISO code Dipstik (Schleicher & Schuell, Dassel, Germany), and the remaining procedure was performed according to the instruction manual. The presence of *E. bieneusi* isolates in the milk samples was examined by nested PCR amplification. The first PCR amplification was performed using the primers EBIEF1 and EBIER1, as described by da Silva et al. (2), and nested amplification was performed using the primers EBIEF5 and EBIER6 (1). The identity of the PCR product was further confirmed by digestion with the MspA1I restriction enzyme (9). The other conditions for PCR have been described previously (10). The secondary PCR products were sequenced, and sequences were compared with those from GenBank. The 15 milk samples testing positive for *E. bieneusi* and the 27 samples testing negative were screened for their somatic cell count (SCC) to determine if the cow(s) producing the milk showed any signs of mastitis. The SCC was determined using a slight modification of the methodology reported previously (13).

Among the 180 cows, 15 (approximately 8.3%) tested positive for *E. bieneusi*. The cows testing positive were from six different cities, which indicated a wide dispersion. Table 1 shows the number of positive samples per collection site. The PCR products of EBIEF5 and EBIER6 and those of primers AL4038 and AL4040 from the *E. bieneusi*-positive samples were purified for sequencing to ensure the specificity of the PCR assay. The homology of the isolate sequences to the published *E. bieneusi* sequences ranged from 99 to 100% for the small-subunit rRNA (SSU-rRNA) portions of each PCR fragment amplified by EBIEF5 and EBIER6 (GenBank accession numbers L16868, L07123, AF024657, AF119100, AF023245, and EF139195 to EF139198). This confirmed that the isolates from the milk samples, detected by PCR, were *E. bieneusi*. The molecular characterization of the 243-bp internal transcribed spacer (ITS) region of the *E. bieneusi* rRNA was examined to determine the genotypes of *E. bieneusi* (1, 16, 18). All of the ITS sequences amplified by AL4038 and AL4040 were identical to at least one or more of the previously published *E. bieneusi* ITS sequences (Table 2). An analysis of the ITS sequences revealed the presence of five distinct genotypes

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TABLE 1. Detection of *E. bieneusi* in the milk of dairy cows

Collection site city	No. of farms	No. of samples examined	No. of positive samples	Genotype(s) (no. of positive samples) ^a
Wanju	7	29	1	CEbB (1)
KimJe	9	37	4	CEbB (1), CEbA (2), CEbD (1)
Namwon	5	21	3	CEbA (1), CEbC (1),
Jangsu	4	15	0	CMITS1 (1)
Imsil	8	32	3	CEbB (1), CEbA (1), CMITS1 (1)
Jeongup	5	17	2	CEbA (1), CEbD (1)
Buan	3	11	0	
Iksan	4	18	2	CEbA (2)
Total	45	180	15	

^a Genotypes refer to those shown in Table 2.

of *E. bieneusi* in the 15 isolates characterized. Three isolates of the genotype were homologous to type J, CEbB, or BEB1, which were originally isolated from cattle and chickens. Seven isolates were homologous to type I, CEbA, or BEB2, and two isolates were homologous to type CEbD, all of which were originally isolated from cattle. *E. bieneusi* has host-specific genotypes and broad host-adapted genotypes. These two genotypes might be cattle-specific genotypes because they have been found only in cattle, and phylogenetic analysis showed that they form a cluster consisting of the isolates from cattle only (10, 16, 18). On the other hand, one isolate of the genotype was identical to the *E. bieneusi* ITS type D, PigEBITS9, CEbC, or WL8, which were originally isolated from humans, pigs, cattle, and wild animals such as foxes, beavers, and raccoons. Two isolates of the newly named genotype originating from milk, CMITS1, were identical to type IV, K, or BEB5, which were originally isolated from humans, cats, and cattle. These two genotypes have been detected in many different hosts and in many different countries, such as Germany, Switzerland, Korea, and the United States (1, 4, 10, 12, 16, 18). Therefore, these genotypes have broad host adaptation and can be transmitted from animals to humans. The detection of isolates from these genotypes suggests that milk from dairy cows is a potential source of human *E. bieneusi* infection. The electron-dense proteinaceous exospores and the chitinous endospore layers of this microsporidium appear to provide protection from a variety of environmental conditions, such as chlorination, low and high temperatures, dehydration, high humidity, and low and high pH (11, 14). This suggests that this organism might be resistant to the milk pasteurization processes and may pose a risk of transmission to humans through drinking milk.

TABLE 3. SCC of the milk samples testing PCR positive and PCR negative for *E. bieneusi*

SCC/ml	No. (%) of samples	
	Positive	Negative
<10 ⁵	3 (20)	20 (64)
>10 ⁵	12 (80)	7 (26)
Total	15 (100)	27 (100)

The SCC in milk is an indicator of the level of inflammation in the mammary gland. An inflammatory response is initiated when microbial pathogens enter the mammary gland. The infection status is a major factor influencing the SCC because an elevation of the SCC occurs in response to an insult to the mammary gland and is modulated by inflammatory mediators (8). The SCC was obtained for each of the 15 PCR-positive and 27 PCR-negative milk samples. A milk sample containing $\geq 10^5$ SCC/ml was considered to indicate a case of mastitis (7, 8). Of the 15 PCR-positive milk samples, the SCC was more than 10^5 cells/ml in 12 samples (80%), whereas the SCC in 3 of the PCR-positive samples (20%) was less than 10^5 cells/ml (Table 3). Of the 27 PCR-negative milk samples, the SCC was more than 10^5 cells/ml in only 7 samples (26%). The significance of *E. bieneusi* as a cause of the disease is unclear because other possible pathogens in these milk specimens, which also cause mastitis, were not examined. Nevertheless, there was a significant association between the presence of *E. bieneusi* in the milk and the occurrence of mastitis, because 12 (80%) of the 15 PCR-positive milk samples that tested positive for *E. bieneusi* contained more than 10^5 cells/ml, while only 7 (26%) of the 27 PCR-negative milk samples tested positive for mastitis. This suggests that the presence of *E. bieneusi* in milk might be an indication of an infection by this organism in the mammary gland rather than an accidental contamination (i.e., through feces). However, more study will be needed to determine if this pathogen can cause mastitis.

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TABLE 2. Summary of 15 ITSs of *E. bieneusi* found in cow milk in this study

No. of samples found	Identical genotype(s) ^a	GenBank accession no.	Reference(s)
3	J, BEB1, CEbB	AF135837, AY331005, EF139196	10, 16, and 18
7	I, BEB2, CEbA	AF135836, AY331006, EF139195	10, 16, and 18
2	CEbD	EF139198	10
1	D, PigEBITS9, WL8, CEbC	AF101200, AY237216, AF023245, EF139197	1, 10, 16, and 18
2	Type IV, KBEB5	AF242478, AF267141, AY331009	4, 12, and 18

^a Previously known genotypes that were identical to the genotypes found in this study.

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