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Occurrence of *Enterocytozoon bieneusi* in Korean Native Cattle Examined by Light Microscopic and Molecular Methods

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Abstract : *Enterocytozoon bieneusi*, a microsporidian species, has emerged as an opportunistic pathogen in AIDS patients. This organism has also been identified in a wide range of animals, and the zoonotic potential of human infections is of particular interest. This study revealed that this organism was found with relatively high prevalence in feces of asymptomatic cattle in Korea. Fecal specimens were obtained from a total of 1,720 cattle in a slaughterhouse located in Chungnam province, Daejeon city and Chonbuk province. After removal of fecal debris by sieving and density gradient centrifugation, samples were examined by microscopic examination and then nested polymerase chain reaction (PCR). Microscopic examination with the modified trichrome staining for the fecal specimens revealed 194 (11.28%) positive calves for microsporidia spore. PCR using the specific primer for *E. bieneusi* revealed 79 (4.59%) positive calves. The infection ratio of microsporidia was higher in March than other season.

Key words **:** *Enterocytozoon bieneusi*, spore, AIDS, cattle.

Introduction

The phylum Microsporidia, intracellular organisms contain nearly 100 genera and more than 1,000 species of microsporidia that infect a wide range of vertebrate and invertebrate hosts (10). The characteristics of these organisms are the presence of a nucleated sporoplasm, a coiled polar tube, and an anchoring disk, and the absence of several eukaryotic characteristics such as a lack of mitochondria, Golgi membranes and eukaryotic ribosomes (13,19,30). In 1857, these parasites were first recognized as pathogens in silkworms (27), and long before they were described as human pathogens, they were recognized as a cause of disease in many nonhuman hosts including insects, mammals and fish. Therefore, they are responsible for considerable infectious disease problems in industries such as fisheries and silk production (4,5).

The first human case of microsporidial infection was reported in 1959 (20), and several species are becoming increasingly recognized as a cause of significant diseases in humans. *Enterocytozoon bieneusi* is the most common microsporidium found in human patients and has been detected in increasing numbers in immunocompetent patients (22,28). The sources of microsporidia infecting humans and its transmission routes are not completely understood. Animal is one of the most likely sources of human infections. The detection of *E. bieneusi* in fecal samples from pigs was described in 1996 (9) and the occurrence of *E. bieneusi* in several other animals such as dogs, cats, rabbits, monkeys and cattle have been reported (8,14,16,18,22). Of these animal studies, epidemiologic research on animals is a critically important parameter for illustrating the sources of human infection as well as for public health. Cattle can be considered a potential source of human infection since the cases of cattle harboring the zoonotic genotypes were reported (25). There are several reports on the characteristics of *E. bieneusi* in cattle, and these reports are in mainly of Europe and North America (9,11,21,23,25). Although many genera of the family Microspora were known to be pathogens of invertebrate and vertebrate hosts, approximately 90% of intestinal infections are caused by *Enterocytozoon bieneusi* (30). However, there is little information of intestinal microsporidiosis in Korea. This study investigated the occurrence of *E. bieneusi* in cattle in Korea using light microscope and PCR analysis.

Materials and Methods

Stool specimens and isolation of spore

Fecal specimens were obtained from a total of 1,720 Cattle in a slaughterhouse located in Chungnam, Daejeon and Chonbuk. About 30 g of feces from 3-5 years old Korean native

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cattle were collected from rectum. After sequential sieving (200, 150 and 100 μm), the suspension was cleaned by centrifugation in 50-ml centrifuge tubes at 1,500 g for 30 min. Supernatant was discarded and the pellet resuspended with sugar solution (600 g sugar in 500 ml dH_2O). The suspension was centrifuged at 1,500 g for 20 min. Next, 5 ml of supernatant was aspirated from the top and washed with dH_2O at 1,500 g for 20 min. The volume of final concentrated specimens was adjusted to 0.3 ml. The isolates were stored at 4°C until microscopical examination, and aliquot of 0.1 ml was stored at -20°C for PCR.

Light-microscopical detection of the spore

Detection of *E. bieneusi* spores in stool specimens was performed by the modified trichrome staining method (29). The concentrated samples were smeared on the slides and fixed with methanol for 5 min. Smears were stained for 10 min at 56°C with the modified staining solution containing 6 g of chromotrope 2R (Harleco, Gibbstown, NJ, USA), 0.15 g of fast green (Allied Chemical and Dye, New York, USA), 0.7 g of phosphotungstic acid, 3 ml of glacial acetic acid and 100 ml of dH_2O. After staining slides were decolorized with acid alcohol for 10 sec and then rinsed briefly in 95% and 100% alcohol. Slides were read under the light microscope at 1,000 times magnification.

DNA extraction for PCR

Approximately 200 μl of feces was transferred to a 2 ml screw cap conical tube containing 200 μl of 0.5 mm glass beads (Biospec Products, Inc, Bartlesville, OK, USA) and 400 μl of a digestion buffer (100 mM NaCl, 25 mM EDTA, 10 mM Tris-Cl,[pH 8.0], 1% SDS, and 100 μg/ml proteinase K). The sample was then placed in a mini-bead beater at 5000 rpm for 2 minutes and incubated 1 hour at 50°C. The samples were then spun in a micro centrifuge for two minutes at top speed. The supernatant was transferred to a new tube and mixed with an equal volume of phenol/chloroform and 300 μl of the supernatant was added to 50 μl of 5M NaCl. The mixture was incubated for 10 min at 65°C. After incubation, the solution was extracted with an equal volume of chloroform. The DNA was recovered from the resulting supernatant using the Geneclean system (BIO101, OH, USA) according to the manufacture's protocol for liquid samples, and the DNA was resuspended in 20 μl of distilled water. One to two μl of the DNA solution was used as a PCR template.

PCR amplification

The presence of *E. bieneusi* in the feces was examined by nested PCR amplification. The first PCR amplification was performed using the EBIEF1 and EBIER1 primers as described by De Silva *et al.* (7). The cycling parameters consisted of 45 cycles of 94°C for 30s, 55°C for 30s and 72°C for 40s. Nested amplification was performed using the primer EBIEF5: 5'-GCGACACTCTTAGACGTAT-3' and EBIER6: 5'-TGGCCT-TCCGTCAATTTC-3', with 30 cycles of 94°C for 30s, 57°C for 30s and 72°C for 30s. The amplification of the *E. bieneusi* templates with the nested primer pair resulted in a 200-bp DNA fragment. A 350-bp internal control amplified by EBIER1 and EBIEF1 was included in each reaction mixture to test for any false-negative results due to the inhibition of PCR. The amplified products were resolved electrophoretically on a 2% agarose gel and stained with ethidium bromide for visual analysis. The identities of the PCR products were further confirmed by digestion with the *Msp*A1I restriction enzyme (15).

Results

Microscopic examinations of feces by Sheather's sugar floatation method showed the presence of microsporidian organism. A smear stained with the modified trichrome staining technique showed numerous red spores. A rod-shaped central waisted-band is visible in a few spores (Fig 1). Two kinds of spore, small and large size, were detected in microscopical examination. Most of spore was measured within 1.1-1.5 × 0.8-0.9 μm. Some cases were mixed with larger spore than those. The larger spore was measured within 1.9-2.9 × 1.0-1.4 μm.

Prevalence of the microsporidial detection from the feces

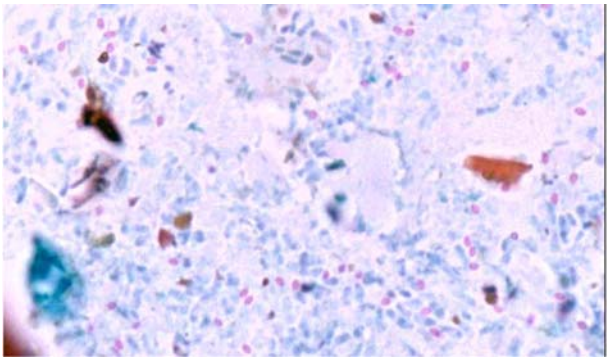

Fig 1. A stool smear stained with the modified trichrome staining technique. Spores are stained with pink color.

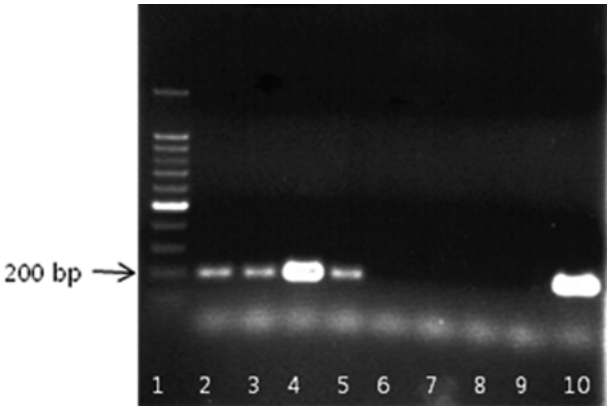

Fig 2. Amplification patterns of PCR products using *E. bieneusi* specific primer set. Lane 9: negative control, D.W. Lane 10: positive control, DNA of cloned *E. bieneusi* ssu-rRNA coding region.

Table 1. Detection of microsporidia in stool samples from Korean native cattle

Month	Stool Samples	Stain Positive (%)	PCR Positive (%)
1	139	8 (05.76)	3 (2.16)
2	108	16 (14.81)	6 (5.56)
3	93	34 (36.56)	12 (12.90)
4	107	26 (11.56)	6 (3.53)
5	225	26 (11.56)	10 (4.44)
6	100	10 (10.00)	7 (7.00)
7	149	16 (10.74)	10 (6.71)
8	200	11 (05.50)	6 (3.00)
9	189	14 (07.41)	5 (2.64)
10	138	14 (10.14)	6 (4.35)
11	168	11 (06.55)	4 (2.38)
12	104	8 (07.69)	4 (3.85)
Total	1,720	194 (11.28)	79 (4.59)

was summarized in Table 1. Microscopic examination revealed 194 (11.28%) positive calves for microsporidia spore from 1,720 samples. The spore detected all the year round and the detection ratio showed some seasonal variation. The infection ratio of microsporidia was higher in March than other months. In the second round of nested PCR, the samples were positive with PCR, producing an amplification of the same size (200 bp). Nested PCR using specific primer for *E. bieneusi* revealed 79 (4.59%) positive calves. In comparison with microscopical and PCR examination, both results were not matched and the positive ratio of PCR was lower than that of microscopical examination.

Discussion

In recent years the prevalence study on *Enterocytozoon bieneusi* has been extensively performed. However, the sources of infection and its transmission routes are not clearly illustrated. This organism has been isolated from many animals, and the isolates from the animals have revealed high genetic similarity to those of the human isolates. This indicates that the animals are the possible sources of human infections in *E. bieneusi* (3,8,9,16,18,19,22,24,30). This study revealed that *E. bieneusi* is a common parasite in Korean native cattle. Since the beginning of the AIDS outbreak, many human cases of *E. bieneusi* infection have been documented in the literature (2). This parasite is the microsporidian species most frequently found in human infections. It is recognized as a true pathogen, especially in immunocompromized patients (6). However, the papers of reservoir hosts about this pathogen are a few. The first detection of *E. bieneusi* in fecal sample of cattle (22) raised the question of the significance of this parasite. And this cattle-derived *E. bieneusi* might have a zoonotic significance. In the recent report (21), broiler chicken was identified as the host of *E. bieneusi*. This is the first detection in non-mammalian hosts.

In this study, we conducted a survey in a slaughterhouse to determine the prevalence of *E. bieneusi* and the seasonal variation in Korean native cattle. We examined 1,720 stool samples and found 4.59% positive for *E. bieneusi* using PCR techniques and 11.28% for microsporidial spore using the modified trichrome staining technique. Because of the multiple developmental stages, intracellular location, small size, and variable staining characteristics, the diagnosis of microsporidia due to *E. bieneusi* can be difficulties. Detection of microsporidia by PCR analysis has the potential to overcome many of these difficulties (26). The difference in the detection rate between both techniques may reflect that the staining technique is not species specific but the PCR technique is specific for *E. bieneusi*.

Buckholt *et al.* (3) surveyed swine feces at a slaughterhouse in Massachusetts during 18 months. They indicated that there is a trend toward higher prevalence in the summer, especially warmer months. But, our data was higher prevalence in spring than summer months. We think that the difference due to the weather and the summer is a rainy season in Korea.

Fournier *et al.* (12) estimated the rate and seasonal variation of *E. bieneusi* contamination of surface water. Sequential samples of water from the River Seine in France were collected during a 1-year period. He reported that 16 of 25 specimens were positive (64%) for microsporidia and was identified in only one sample by specific hybridization. The presence of various microsporidia DNA in water is not surprising in regard to the ubiquity of these parasites (1). In public health, these different species of microsporidia and their presence in the environment may represent a potential risk for human (17).

We report *E. bieneusi* prevalence of apparently healthy Korean native cattle in Korea. The prevalence indicates that cattle may be a significant source of environmental contamination.

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광학 현미경 및 분자생물학적 방법을 적용한 한우의 *Enterocytozoon bieneusi* 역학조사

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요 약 : 미포자충인 *Enterocytozoon bieneusi*는 최근에 AIDS 환자에 기회감염 되는 병원체로 부각되었다. 이 병원체는 여러 동물에서 발견되고 있으며 인수공통 기생충으로서 특별한 관심의 대상이 되고 있다. 충남, 대전, 전북 지역의 도축장으로부터 특별한 증상을 보이지 않은 건강한 한우 총 1,729 마리의 직장변을 취하여 *E. bieneusi*의 감염율을 조사하였다. 조사방법은 Trichrome 염색 변법과 중합효소연쇄반응(PCR)을 이용한 분자생물학적 기법을 적용하였다. Trichrome 염색변법을 적용한 광학현미경 관찰에서 194 (11.28%) 개체에서 미포자충이 관찰되었으며, 특히 3월에 가장 높은 감염율을 보였다. 한편 분자생물학적 기법을 적용한 시험에서는 79 (4.59%) 개체에서 양성 반응을 보였으며, 이 역시 3월에 가장 높은 감염율을 나타내었다.

주요어 : *Enterocytozoon bieneusi,* 포자, AIDS, 한우.