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Evaluation for detection of *Cryptosporidium* oocysts in diarrheal feces of calves

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Abstract: For the detection of *Cryptosporidium* oocysts, fecal samples were collected from 201 calves which showed diarrhea. Among the 201 samples, 29 samples (14.4%) were positive for *Cryptosporidium* spp. by the DMSO-modified acid-fast stain (MAFS), 23 samples (11.4%) were positive by commercial kit (Meridian Diagnostics, Cincinnati, Ohio) and 23 by the indirect immunofluorescence antibody (IFA) assay employing the monoclonal antibody (mAb C6). When tested by both IFA and MAFS, 20 fecal samples were positive for *Cryptosporidium* oocysts whereas 169 fecal samples were negative. If the MAFS is considered a standard method for oocyst detection, the IFA showed 69% of sensitivity and 98% of specificity. When tested by both IFA and commercial kit, 22 fecal samples were positive for *Cryptosporidium* oocysts while 177 samples were negative. One sample tested by IFA was found to be false negative, when compared with the results by commercial kit. The sensitivity of IFA was calculated as high as 96%; the specificity as 99% and the predictive value was also 99%. In the present study, IFA employing the mAb C6 revealed that 23 samples (11.4%) were positive among the 201 calves showing diarrhea. Of 23 IFA positive samples, 4 samples (5%) showed cryptosporidial oocysts more than 10^5 OPG. Therefore, it is concluded that the calves showing cryptosporidial oocysts more than 10^5 OPG in the feces were highly associated with clinical cryptosporidiosis.
Key words: *Cryptosporidium*, calf, diarrhea, immunofluorescence antibody assay

INTRODUCTION

Cryptosporidium species have a wide range of hosts including man and many other mammalian species, birds, reptile and fish. Infections can be transmitted directly from man to man or animal to man (Moon and Woodmansee, 1986). The parasites mainly cause diarrhea in several mammalian species especially in neonates. Although the infected host discharges small number of oocysts in feces, it may also be a reservoir of infections. Thus any animal, once infected, can act as a source of infections for another (Angus, 1987). Clinical diagnosis of cryptosporidiosis is primarily based on the detection of oocysts from feces. Also, serological diagnostic methods using monoclonal antibodies (mAbs) are often used (Arrowood and Sterling, 1989; Rusnak *et al.*, 1989).

Recently, it was possible to find *Cryptosporidium* oocysts from feces of the human (Cho *et al.*, 1993) as well as from feces of the many animals (Rhee *et al.*, 1991) in Korea. To diagnose cryptosporidiosis quickly and easily, we produced the monoclonal antibody C6 (mAb C6) against *C. parvum* oocysts isolated from a mouse as previously described (Wee *et al.*, 1995). In the present study, we carried out the prevalence of *Cryptosporidium* caused diarrhea in calves and

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evaluation for detection of *Cryptosporidium* in diarrheal feces of calves.

MATERIALS AND METHODS

Fecal specimens Two hundred and one fecal specimens were collected to test the presence of *Cryptosporidium* oocysts by indirect immunofluorescence antibody (IFA) assay with mAb C6, DMSO-modified acid-fast stain and commercial kit by Meridian Diagnostics (Cincinnati, Ohio) as the Merifluor *Cryptosporidium* kit. The fecal specimens were collected from diarrheal calves under 4 months old. And fecal sample were preserved in 10% formalin solution.

DMSO-modified acid-fast stain (MAFS) Twenty μl of nonconcentrated fecal samples in 10% formalin solution were smeared on the slides. All steps were performed as described by Bronsdon (1984).

Procedure of IFA with mAb C6 To diagnose cryptosporidiosis, we produced the mAb C6 against *C. parvum* oocysts isolated from a mouse as previously described (Wee *et al.*, 1995). For IFA testing, 20 μl of non-concentrated fecal samples in 10% formalin solution were smeared on the slide. The slides were allowed to dry completely at room temperature. Forty μl of mAb C6 were added to each well, and the slides were incubated in a humidity chamber for 30 min at room temperature. Slides were rinsed with phosphate-buffered saline (PBS, pH 7.4). Twenty μl of fluorescein-conjugated goat anti-mouse IgM (KPL, 1:100) dropped and added equal volume of counterstain solution (Evans blue 1:250) to each well. After incubation for 30 min at room temperature, and given a final rinse with PBS. Wells of IFA slides were scanned by fluorescence microscopy (Carl Zeiss, SH-250) at × 250 or × 500 magnification.

Procedure of commercial Merifluor *Cryptosporidium* kit Twenty μl of nonconcentrated fecal samples were applied to slides provided in the kit. The slides were then processed and examined according to the manufacturer's instructions.

Oocysts per gram of fecal specimens (OPG) Ether extraction method (Riggs and Perryman, 1987) was applied to remove the fat or lipid from feces samples. Sediments were washed by centrifugation (1,500 × g for 10 min, 3 times) in PBS, then the washed sediments were sieved sequentially through stainless steel screen with a final mesh of 250 (61 μm porosity) to remove other debris. After sieving, the materials were suspended in PBS. Oocysts were counted by using a hemocytometer chamber to demonstrate the number of the oocysts of *Cryptosporidium* per gram (OPG).

RESULTS

For the detection of *Cryptosporidium* oocysts, fecal samples were collected from 201 calves which showed diarrhea. Twenty nine samples (14.4%) were positive for *Cryptosporidium* spp. by the MAFS, 23 samples (11.4%) were positive by commercial kit and 23 samples (11.4%) were positive by the IFA employing the mAb among examined samples.

When tested by both IFA and MAFS, 20 fecal samples were positive for *Cryptosporidium* oocysts whereas 169 fecal samples were negative (Table 1). If the MAFS is considered a standard method for oocysts detection, the IFA show 69% of sensitivity and 98% of specificity.

When tested by both IFA and commercial kit, 22 fecal samples were positive for *Cryptosporidium* oocysts while 177 samples were negative (Table 2). One sample tested by IFA was found to be false negative, when compared with the results by commercial kit. The sensitivity of IFA was calculated as high as 96%; the specificity 99% and the predictive

Table 1. Comparison of IFA and MAFS for detection of *Cryptosporidium* oocysts

IFA[a] result	No. of fecal specimens with following MAFS[b]		
	Positive	Negative	Total
Positive	20	3	23
Negative	9	169	178
Total	29	172	201

[a]IFA: indirect immunofluorescence antibody assay

[b]MAFS: DMSO-modified acid-fast stain.

value was also 99%.

Among the 23 IFA positive samples, 14 specimens (60.9%) were fewer than 10^3 OPG and 2 specimens (8.7%) were between 10^3 to 10^4 OPG. 10^4 to 10^5 OPG and 10^5 to 10^6 OPG oocysts detected in 3 samples (13.0%), respectively. One specimen (4.4%) showed the higher figure than 10^6 OPG (Table 3).

DISCUSSION

Clinical diagnosis of cryptosporidiosis is primarily based on the detection of oocysts from feces. Detection methods include floatation (Anderson, 1981), sedimentation (Zierdt, 1984; Baron *et al.*, 1989) and stain of fecal smears (Anderson, 1981; Garcia *et al.*, 1983; Bronsdon, 1984; Cross and Moorhead, 1984; Miller *et al.*, 1984; Pohjola, 1984; Smith *et al.*, 1989). All of these techniques permit a positive diagnosis when sufficient numbers are present for detection. Enzyme-linked immunosorbent assay (ELISA) is regarded as the quick and easy technique to perform the standardized test for *Cryptosporidium* antigens in stool samples for large-scale epidemiological studies (Anusz *et al.*, 1990; Robert *et al.*, 1990; Ungar, 1990). Some fecal samples, however, may contain only a few oocysts, making it difficult for the medical microbiologists or veterinary diagnosticians to decide whether one or two *Cryptosporidium*-like bodies seen in a stained fecal smear warrant of the positive diagnosis (Current, 1985; Cho *et al.*, 1993). To increase the sensitivity and specificity of diagnostic method of cryptosporidiosis, many scientists carried out the development of ELISA or IFA using the mAb for the detection of *Cryptosporidium* oocysts in feces. An IFA developed by Arrowood and Sterling (1989) and utilizing an IgM mAb was demonstrated to be both 100% sensitive and 100% specific compared with the Ziehl-Neelsen modified acid-fast stain. Also, Rusnak *et al.* (1989) demonstrated the 97% specificity of the IFA relative to the Ziehl-Neelsen modified acid-fast stain. Commercial kit (Merifluor) using the mAb fluorescent stain for *Cryptosporidium* oocysts was a reliable and specific, and the specificity was as high as 99.5% for Merifluor in a low-prevalence population (Baron *et al.*, 1989). These demonstrations showed that monoclonal antibody-based methods have provided for enhancing sensitivity and specificity over those of the conventional methods when the number of oocysts in stool specimens was few (Arrowood and Sterling, 1989; Baron *et al.*, 1989; Rusnak *et al.*, 1989; Smith *et al.*, 1989; Anusz *et al.*, 1990; Robert *et al.*, 1990). The method using the mAbs would also eliminate the possibility of false-positives and false-negatives those seen with routine staining methods for stool specimens (Arrowood and Sterling, 1989). These results confirmed that the mAb can be used for the diagnosis of cryptosporidiosis, although the IFA technique was the most expensive and lengthy of all the procedures that was easy in handling (MacPherson and McQueen, 1993).

In the present study, commercial kit, IFA employing the mAb C6, and MAFS revealed

Table 2. Comparison of IFA and commercial kit for detection of *Cryptosporidium* oocysts

IFA result	No. of fecal specimens with commercial kit[a]		
	Positive	Negative	Total
Positive	22	1	23
Negative	1	177	178
Total	23	178	201

[a]Merifluor *Cryptosporidium* kit (Meridian Diagnostics Inc., Cincinnati, Ohio, USA)

Table 3. Results of IFA performed on fecal specimens collected from calves known to be positive for *Cryptosporidium* oocysts

Total	No. of oocysts per gram of feces				
	$<10^3$	$>10^3$-$<10^4$	$>10^4$-$<10^5$	$>10^5$-$<10^6$	$>10^6$
23	14	2	3	3	1

that 11.4%, 11.4% and 14.4% of the 201 calves showing diarrhea. Detection rates of *Cryptosporidium* oocysts by IFA technique using the mAb produced in our laboratory were agreed to the specificity (96%) and sensitivity (99%) that of the commercial diagnostic kit. Thus, this suggested that the IFA technique using the mAb produced in our laboratory may prove useful for diagnostic analysis of bovine fecal samples and others.

In outbreaks where *Cryptosporidium* is the dominant pathogen, the duration of diarrhea depends on various factors, such as the level of environmental infection, which determines the intake of infective oocysts, the pathogenicity of the infecting isolate, host susceptibility, and age at the time of primary infection. In field investigations, the true role of the parasite as a cause of diarrhea can be determined only if exclusion of other known enteropathogenic agents from the fecal samples can be demonstrated by recognized diagnostic methods. Infection has been confirmed in calves as young as 4 days and as old as 4 weeks, but analysis of numerous reports indicates that oocyst excretion and diarrhea occur seldom in the calves under 7 days old and tend to peak at approximately 2 weeks of age. Because the life cycle of *C. parvum* in calves is only about 4 days, clinical infection in a calf as young as 4 days would indicate heavy environmental infection in the calving area (Dubey *et al.*, 1990). In transmission experiments, oocyst numbers of the order of 10^5 to 10^6 given to conventionally reared calves that had at least one feed of their dams' colostrum, and which were known to be free of viral and bacterial enteropathogens before oocyst dosing, caused acute diarrhea, oocyst number of the order of 10^6 to 10^7/g of feces were excreted, which is in line with natural infections (Current, 1985; Dubey *et al*, 1990). The most outstanding sign of cryptosporidiosis in calves is diarrhea associated with profuse shedding of infective oocysts (in the order of 10^5 to 10^7/g of feces)(Current, 1985; Wee *et al*, 1995). In the present study, IFA employing the mAb C6 revealed that 23 (11.4%) of the calves showing diarrhea were positive for *cryptosporidium*. Of 23 IFA positive samples, 4 samples (5%) showed cryptosporidial oocysts more than 10^5 OPG. Therefore, it is concluded that the calves showing cryptosporidial oocysts more than 10^5 OPG in the feces were highly associated with clinical cryptosporidiosis.

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=초록=

야외 송아지 설사변에서 작은와포자충 검출에 대한 평가

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작은와포자충이 송아지에서 설사에 직접적으로 영향을 미치는지를 알아보고자 야외에서 설사를 발현하는 4개월 이하의 송아지 분변 총 201건를 수거하여 DMSO-modified acid fast 염색법 (MAFS), commercial kit(Meridian Diagnostics, Cincinnati, Ohio) 그리고 본 실험실에서 만든 단세포군항체(C6)를 이용한 간접형광항체법(IFA)으로 작은와포자충의 검출을 시도하였다. 작은와포자충의 검출은 MAFS에서 29건(14.4%), commercial kit와 IFA에서 각각 23건씩(11.4%) 검출되었다. 작은와포자충 오오시스트의 검출에 사용된 진단기법간의 진단일치율을 비교하였던 바 MAFS를 기준으로 하였을 때 IFA는 민감도 69%, 특이성 98%였다. Commercial kit와 IFA진단법에서는 양성 22건, 음성 177건이 서로 일치하였으며, commercial kit를 기준으로 할 때 IFA의 민감도는 96%, 특이성 99% 그리고 진단일치에 대한 기대치는 99%였다. 따라서 본 실험에 사용된 IFA는 commercial kit와 일치율이 높아 진단목적으로 사용될 수 있을 것으로 생각된다. 한편, IFA에서 작은와포자충이 검출된 23건의 분변에 대해 분변 g당 작은와포자충 오오시스트의 수(OPG)를 측정하였다. 14건이 10^3 이하의 OPG수준을 나타냈으나 10^5 이상의 OPG도 4건이었다. 작은와포자충에 감염되어 설사가 발현될 때의 OPG의 수준이 10^5 이상임을 감안해 보면, 이번 조사된 송아지 설사변 201건 중 4건(5%)은 작은와포자충에 의한 설사로 분석되었다. 이러한 결과를 종합해보면, 국내에서 발생되는 송아지 설사 원인중 약 5%는 설사와 관련되는 다른 원인체와 관계없이 작은와포자충 단독감염만으로도 임상적으로 설사가 발현되고 있는 것으로 판단되었다.

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