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Detection of *Cryptosporidium* oocysts from out-patients of the Severance Hospital, Korea.

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Abstract: A total of 230 randomly collected formalin-fixed fecal samples (submitted to the Severance Hospital, Yonsei University) were selected for tests for human cryptosporidiosis. The stool specimens were examined for *Cryptosporidium* oocysts by acid-fast (AF) stain, auramin-rhodamine (AR) stain, and monoclonal antibody (mAb) OW3 fluorescence method specific for oocyst wall. Of the 230 stool specimens, 21% were identified by the AF method, 22% were identified by the AR method, and 10% were identified by the mAb fluorescence method, indicating that human *Cryptosporidium* infections have been existing in Korea.

Key words: *Cryptosporidium*, human stools, acid-fast stain, auramine, monoclonal antibody.

INTRODUCTION

Cryptosporidium infection in humans has been described only within the past dacade. A 1980 World Health Organization report on parasite-related diarrheas (WHO 1980) did not include *Cryptosporidium* sp. The onset of acquired immunodeficiency syndrome (AIDS) in the United States brought attention to its association with diarrheal illness when 21 patients with AIDS and cryptosporidiosis were reported to the Centers for Disease Control (CDC, 1982).

Human infection with *Cryptosporidium* sp. has been described in six continents but is most prevalent in developing countries, with children constituting the most susceptible portion of the population (Alpert et al., 1984). *Cryptosporidium* is now also recognized as a frequent cause of gastroenteritis in normal individuals, with diarrhea being the major symptom. In many areas, *Cryptosporidium* sp. is among the top three or four enteric pathogens identified (Hart et al., 1984; Sterling and Arrowood, 1993).

With the increased awareness that *Cryptosporidium* sp. can cause severe symptoms in humans, the development and implementation of many diagnostic techniques have been reported, including various concentration and staining methods (Cross and Moorhead, 1984; Zu et al., 1992). Clinical diagnosis of cryptosporidial infections has been primarily based on the detection of oocysts in stools.

Either overall prevalence of human *Cryptosporidium* infections or infection rate among patients has not been reported in Korea. The present study was initiated to detect *Cryptosporidium* oocysts in human stool specimens with fluorescent-monoclonal antibody (mAb)-based method and two conventional methods: acid-fast staining and auramine-rhodamine staining.

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MATERIALS AND METHODS

Specimens: Fecal samples (230 total) collected in 10% formalin were obtained from Severance Hospital, Yonsei University. These specimens were submitted for examination on a random basis, with many patients exhibiting no diarrheal illness. Fecal specimens were randomly ordered and coded for examination in a blind fashion. Replicate fecal smears of each unconcentrated, vortexed fecal sample were prepared on microscope slides, heat fixed, and assayed by the three oocyst detection methods described below. Fecal smears of specimens containing potassium dichromate ($K_2Cr_2O_7$) were rinsed with 0.025 M phosphate-buffered saline (PBS) (pH 7.2) and air-dried before proceeding with the assays.

Fecal smear examinations: Bright-field and fluorescence observations of fecal smears were performed at × 200 and × 400 magnifications. The entire smear was examined to verify the absence of oocysts. When smears contained many oocysts, only a portion of the smear was examined. Epifluorescence microscopy employed an Optiphot microscope (Nikon Inc., Garden City, N.Y.) equipped with a halogen UV light source, a 520-nm-wavelength barrier filter, a 510-nm-wavelength dichromic mirror, and a 450- to 490-nm-wavelength excitation filter.

Acid-fast staining of fecal oocysts: A commercially available acid-fast staining kit (VOLU-SOL; Medical Industries Inc., Las Vegas, Nev.) was applied as recommended to fecal smears. Briefly, the primary stain was applied at room temperature to the fecal smear for 2 min, and rinsed with tap H_2O. After the smears were dried, they were coated with a thin layer of immersion oil and observed by bright-field microscopy.

Auramine-rhodamine staining of fecal oocysts: Acid-fast staining with auramine-rhodamine was based on Truant auramine-rhodamine stain (Paik, 1980). The stain was prepared by combining 1.5 g of auramine O, 0.75 g of rhodamine B, 75.0 ml of glycerol, 10. 0 ml of liquified aqueous phenol (88% [wt/vol]), and 50.0 ml of distilled H_2O. Air-dried, heat-fixed fecal smears were stained for 15 min and rinsed with H_2O. Smears were decolorized for 2 to 3 min with 0.5% HCl (in 70% ethanol) and rinsed with H_2O. The smears were counterstained for 2 to 4 min with 0.5% potassium permanganate (in distilled H_2O), rinsed with H_2O, and air dried. Slides were examined by epifluorescence microscopy.

Monoclonal antibody reagent: The monoclonal reagent, OW3, was employed. The mAb OW3 to *Cryptosporidium parvum* was originally developed from Dr. Charles R. Sterling's laboratory of the University of Arizona and has been commercialized by Meridian Diagnostic Inc., Cincinnati, Ohio,U.S. A. For the production of OW3, oocyst walls were isolated by sonicating 5×10^6 intact oocysts, shocking freed sporozoites with distilled water, and washing in 0.025 M phosphate-buffered saline to remove debris. Spleen cells of adult RBN/Dn mice immunized on days 0, 14, and 28 were fused on day 32 with FOX/NY mouse myeloma cells by use of polyethylene glycol. Hybrid cells were grown in 24-well culture plates.

One hybridoma producing an immunoglobulin M monoclonal antibody OW3, as determined by double diffusion in agar against an isotype-specific goat anti-mouse immunoglobulin, was positive for oocysts by indirect immunofuorescence assay. After cloning, this hybridoma was injected into pristane-primed mice to produce ascites tumors. The ascites fluid was purified, tested, and divided into portions for use.

Oocysts detection with monoclonal antibody (fluorescence): Mouse antibody (5 ul of 1:100 dilution) was applied to fecal smears in 50-μl volumes. The slides were incubated at room temperature for 15 min in a humid chamber, rinsed three times with PBS (for over 9 min). The fluorescein isothiocyanate-labeled anti-mouse antibody (Kirkegaard and Perryy Laboratory, Inc.) (5 μl) was added to each well and incubated at 37° C for 20 min. The slide was rinsed four times in PBS, mounted with PBS-glycerol (1:1) (pH 8.0), and covered with cover slips. Slides were observed by epifluorescence microscopy.

RESULTS

Fluorescent mAb-based method, acid-fast staining, and auramine-rhodamine staining were employed for the present study. A total of 230 randomly collected formalin-fixed human fecal samples submitted to the Sevrance Hospital of Yonsei University were examined for the detection of *Cryptosporidium* oocysts. Of the 230 stool specimens, 48 samples (21%) were identified by the AF staining, 50 samples (22%) were identified by the AR staining, and 23 samples (10%) were identified by the mAb fluorescence method as having *Cryptosporidium* oocysts (Table 1). The stool specimens were diagnosed positive in the AF staining (Fig. 1, A) if there wer spherical organisms stained red 4 to 6 μm in diameter and diagnosed positive in the AR staining (Fig. 1, B) if there were organisms showing green to yellow under epifluorescence microscopy for cryptosporidial infections. *Cryptosporidium* oocysts were round and easily visible (4 to 6 um), showing apple-green fluorescence against a dark background free of nonspecific fluorescence in the mAb-based method (Fig. 1, C) under epifluorescence microscopy.

DISCUSSION

Increased interest in *Cryptosporidium* as a causative agent of diarrhea in humans has led to the development of various techniques for concentrating and detecting parasites. Prior to 1980, diagnosis of human cryptosporidiosis depended on identifying the *Cryptosporidium* oocyst in biopsy samples of intestinal tissues processed for light or electron microscopy (Bronsdon, 1984; Current and Garcia, 1991). Invasive, time-consuming procedures, however, are no longer necessary now that several techniques have been developed to identify *Cryptosporidium* sp. oocysts in fecal specimens from animals and humans.

The most widely used are the modified acid-fast staining, auramine-rhodamine staining, negative staining, and Sheather's sugar floation techniques (Baxby *et al.* 1984, Cross and Moorhead 1984, Garcia *et al.* 1983). All of these techniques permit a positive diagnosis when sufficient numbers are present for

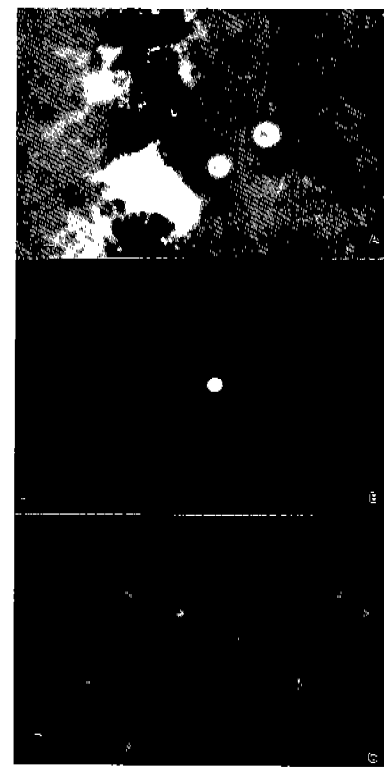

Fig. 1. *Cryptosporidium* oocysts in human stool specimens stained by acid-fast stain technique (A), auraminc fluorescent technique (B), and indirect immunofluorescent technique using a commercially available monoclonal antibody (C), respectively. Bar, 5 μm.

Table 1. Detection of human *Cryptosporidium* infection by acid-fast staining, auramine-rhodamine staining, and fluorescent mAb-based method

Number of specimens examined	Number of specimens that were positive by		
	AF staining	AR staining	IF technique (mAb)
230	48(21%)	50(22%)	23(10%)

detection. Some stool samples, however, may contain only a few oocysts, making it difficult for the medical microbiologist or the veterinary diagnostician to decide whether one or two *Cryptosporidium*-like bodies seen in a stained fecal smear warrant a positive diagnosis (Current, 1985; Sterling and Arrowood, 1993).

Increased sensitivity can be achieved, however, by using an immunofluorescent assay using a monoclonal antibody. The mAb-FITC conjugate has been widely used to confirm questionable *Cryptosporidium* infections in both humans and animals. Specificity of the mAb OW3 for oocysts has been verified by Garcia *et al.* (1987). The OW3 immunofluorescence assays were observed to be 100% sensitive and 100% specific compared with the acid-fast method (40.6% sensitive and 52.0% specific) and the auramine-rhodamine method (93.8% sensitive and 85.7% specific) (Arrowood and Sterling 1989, Garcia *et al.* 1987). Some specimens previously considered negative by the acid-fast method were positive by the monoclonal antibody technique (Garcia *et al.* 1987). The method with monoclonal antibodies would also eliminate the possibility of false-positives and false-negatives that are seen with routine staining methods for stool parasites (Arrowood and Sterling 1989).

Based on the foregoing, the present study employed a mAb-based method and two conventional methods (acid-fast staining and auramine-rhodamine staining) to detect *Cryptosporidium* oocysts in the feces. Our findings indicate that human *Cryptosporidium* infections have been existing in Korea. However, it has not been known how these patients were infected. Immunofluorescence using a mAb, AF, and AR stainings revealed that 10%, 21%, and 22% of the 230 out-patients were found to have stools positive for *Cryptosporidium*, respectively. We do not know the overall prevalence of human cryptosporidiosis in Korea since the present study was confined to stools of the patients and stools of healthy individuals were not examined. It was not known that age distribution of the patients and whether or not they showed gastrointestinal symptoms at the time they visited the hospital. However, our findings indicate that human cryptosporidiosis has been existing in Korea. It has not determined how these patients were infected with *Cryptosporidium parvum*. Drinking water and animals have been suggested to play an important role in transmitting *Cryptosporidium* to humans.

Navin summarized 14 prevalence studies in persons with *Cryptosporidium* (Navin, 1985). Stool positivity rates varied from 1.1% (15/1317) in Canada (Anon, 1984) to 11.1% (8/72) in Rwanda (Bogaerts *et al.*, 1984). In industrialized countries, overall prevalence was 2.1% (487/23735). Stool positivity rates varied from 0.4% (6/1710) in Portland, Oregon (Skeels *et al.*, 1986), to 4.4% (41/935) in Galway, Ireland (Corbett Feeney, 1987), and 5% (7/140) in Lismore, Australia (Parker et al., 1985). In developing countries, the overall rate of stool positivity was 8.5% (532/6295) (Rahman et al., 1985; Pape et al., 1987;Mata, 1986). Prevalence among individuals varied from 2.6% (19/735) in Manila (Cross *et al.*, 1985) to 16.7% (138/824) in Haiti (Pape *et al.*, 1987). Prevalence of cryptosporidiosis among patients with gastrointestinal symptoms was high even in industrialized countries. In southern Sweden, 3% of patients with acute diarrhea had cryptosporidiosis (Atterholm et al., 1987). In Australia, rates for patients with gastroenterites varied from 2% (10/515) to 7% (26/369) (Navin and Juranek, 1984). The report from a New York City medical center in which 11.9% (15/126) of patients undergoing upper gastrointestinal endoscopy and endoscopic retrograde cholangiopancreatography had duodenal aspirates positive for *Cryptosporidium*. Testing of individuals visiting a health center in Finland for reasons other than acute illness found asymptomatic cryptosporidiosis in 9.5% (6/63) of persons who had long-term contact with cattle (Pohjola et al., 1986).

Cryptosporidium was the most common enteric pathogen recovered from the stools of malnourished children with diarrhea in Kingston, Jamaica; of malnourished children 24% (14 of 59) were positive for *Cryptosporidium*, compared with 3.5% (6 of 256) of well-nourished children (Crawford and Vermund, 1988). In a children's hospital in the occupied West Bank, *Cryptosporidium* was the

most common enteric pathogen in patients admitted with diarrhea and undergoing a comprehensive laboratory assessment, 13.5% (30/221). In southern India, *Cryptosporidium* was found in 13.1% (89/682) of patients less than 4 years old with acute diarrhea and in 9. 8% (41/418) of healthy controls (Mathan et al., 1985). In Liveria, *Cryptosporidium* was identified in the stools of 8.4% (20/237) of children with diarrhea (Hojlyng *et al.*, 1986). The average prevalence of *Cryptosporidium* was 7.3% in children with diarrhea in Brazil, Venezuela, Ecuador, Chile, and Costa Rica (Mata, 1986).

Cryptosporidiosis has been reported in individuals with varying degrees of impaired immunity (Petersen, 1992; Petersen, 1993). As of April 4, 1986, 3.6% (696/19,182) of AIDS patients reported to the CDC were infected with *Cryptosporidium*. In 1987, 34 AIDS patients with cryptosporidiosis seen at UCLA Medical Center since 1981 were described (Crawford and Vermund, 1988). *Cryptosporidium* was diagnosed in most of the patients toward the end of their course of disease. In the third world countries, *Cryptosporidium* infection may also be more common among AIDS patients (Quinn *et al.*, 1986). In a report from Haiti, 41% (11/27) of AIDS patients were found to have *Cryptosporidium*-positive stools (Malebranche *et al.*, 1983;Petersen, 1992).

The present study indicates that human cryptospridiosis has been existing in Korea although overall prevalence of *Cryptosporidium* infections was not determined. It has not been known how these patients were infected with *Cryptosporidium parvum*. Drinking water and animals have been suggested to play an important role in transmitting *Cryptosporidium* to humans. A recent WHO report presents data to suggest that drinking surface water was the source of an ourbreak of cryptosporidiosis in New Mexico, USA (1988). *Cryptosporidium* oocysts, in fact, have been identified in high numbers from potable and waste waters of periurban pueblo joven communities in South America. Preliminary studies being conducted in Arizona and elsewhere indicate the presence of *Cryptosporidium* in a high percentage of our surface waters (> 25%) and in virtually all effluent dominated waters tested to date. It thus seems hardly surprising that waterborne spread of *Cryptosporidium* has been suggested. Ma et al.(1985) suggest that cryptosporidiosis may be acquired through consumption of contaminated food or water.

Acquisition of *Cryptosporidium* infection via drinking water was suspected in a 1986 outbreak of the disease in England that affected 104 persons; oocysts were identified from cattle on farms adjoining the reservoir area, and from surface water (Fayer and Ungar, 1986). Animals such as mice, house rats, pigs, and cattle in Korea were found to be infected with *Cryptosporidium* sp.(Lee *et al.*, 1991). Infected animals have been implicated as the source of human *Cryptosporidium* infections. Further, the fact that these organisms can be excreted in the feces of one animal host and cause infection when ingested by another host suggests that members of this genus can be transmitted in any of a number of way (Fayer and Unger, 1986; Fayer *et al.*, 1990; Sterling and Arrowood, 1993). Might we expect to find similar environmental contamination within Korea?

In the case of *Cryptosporidium*, however, there is still much to be learned about the epidemiology of human disease caused by members of this genus. It has been little more than a decade that *Cryptosporidium* has been implicated in human disease, and present consensus is that this genus is a ubiquitous pathogen and infects a broad range of vertebrates including humans. Based on the foregoing, one might reasonably predict a rather high level of environmental contamination, especially where sanitary conditions are lacking. Infection with this organism, therefore, will probably prove to be more widespread than heretofore recognized.

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=국문초록=

연세대학교 세브란스병원 환자에서의 *Cryptosporidium* 오오시스트 검출률

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국내의 *Cryptosporidium* 인체감염 실태를 조사하기 위하여 연세대학 세브란스 병원을 찾은 230명의 외래 환자 분변을 수거하였다. Acid-fast 염색, auramine-rhodamine 염색과 *Cryptosporidium parvum* oocyst에 특이적인 단클론 항체를 이용한 동정법을 이용하였다. 230명의 환자 중 48명(21%)이 AF 염색법에 의하여, 50명(22%)이 AR 염색법에 의하여, 그리고 23명(10%)이 단클론 항체를 이용하는 형광현미경법으로 각각 *Cryptosporidium*에 감염된 것으로 조사되어 국내에서도 *Cryptosporidium* 인체 감염이 존재하고 있는 것으로 나타났다.

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