

A Hospital-Based Serological Survey of Cryptosporidiosis in the Republic of Korea

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Abstract: The seroprevalence of cryptosporidiosis was examined using patients' sera collected from hospitals located in 4 different areas of the Republic of Korea. ELISA was used to measure antibody titers against *Cryptosporidium parvum* antigens from a total of 2,394 serum samples, which were collected randomly from patients in local hospitals; 1) Chungbuk National University Hospital, 2) Konkuk University Hospital, 3) local hospitals in Chuncheon, Gangwon-do (province), 4) Jeonnam National University Hospital, from 2002 through 2003. Of the 2,394 samples assayed, 34%, 26%, and 56% were positive for *C. parvum*-specific IgG, IgM, and IgA antibodies, respectively. Positive IgG titers were most common in sera from Jeonnam National University Hospital, Gwangju, Jeollanam-do, and positive IgM titers were most common in sera from Chungbuk National University Hospital, Cheongju, Chunchongbuk-do. The seropositivity was positively correlated with age for both the IgG and IgA antibodies but was negatively correlated with age for the IgM antibodies. Western blotting revealed that 92%, 83%, and 77% of sera positive for IgG, IgM, and IgA ELISA reacted with 27-kDa antigens, respectively. These results suggested that infection with *Cryptosporidium* in hospital patients occurs more commonly than previously reported in the Republic of Korea.

Key words: *Cryptosporidium parvum*, cryptosporidiosis, seroprevalence, ELISA, western blot

INTRODUCTION

Cryptosporidium parvum is a commonly occurring protozoan parasite of the gastrointestinal tract and has been identified as the agent responsible for numerous outbreaks of diarrheal illness [1]. This disease is self-limiting within a short-term period in immunocompetent hosts but can become severe in immunocompromised individuals, causing a chronic and debilitating disease [2-4]. Numerous studies have found that cryptosporidiosis is more common in diarrheal patients living in poorly developed countries compared to those living in America and Europe [5]. Epidemiological studies in the Republic of Korea have found approximate prevalences ranging from 1 to 11% in immunocompetent inhabitants [6-9] and a rate of 11% in immunocompromised (HIV-infected) patients [10]. However, there has been no report on cryptosporidiosis outbreak in the Republic of Korea.

Previous epidemiologic studies on cryptosporidiosis have

typically relied on the detection of oocysts in fecal samples [7-9]. However, fecal examination was not considered to be a useful method for the estimation of the endemicity of cryptosporidiosis in communities because the duration of oocyst excretion in infected patients is very short and transient. In addition, a large number of oocysts per gram of feces is needed for a positive detection result [11]. To evaluate exposure to the parasite, particularly in populations chronically exposed to *Cryptosporidium* through contaminated food or drinking water, antibody detection in sera is more sensitive than oocyst detection in stool samples, and hence, this method has been widely used in epidemiological studies in numerous countries [12-19].

Infection by *C. parvum* in humans and animals elicits the development of characteristic serum and mucosal IgG, IgA, and IgM antibody responses against parasite antigens detectable by enzyme-linked immunosorbent assay (ELISA), immunofluorescence assay, or western-blot analysis [14,16,18,20,21]. Although detection of specific serum antibodies should not be necessarily regarded as indicative of an active infection [22], some antigens identified by immunoblot analysis are recognized by IgG, IgA, and IgM serum antibodies of humans, and considered as excellent

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markers of infection [14,16,18,20,21].

In this study, we used ELISA technique to investigate the seroprevalence of cryptosporidiosis. In addition, we evaluated specific *C. parvum* antigens with serum samples showing positive ELISA titers using western blotting.

MATERIALS AND METHODS

Serum sample collection

Serum samples (n = 2,394) were collected from hospitals in 4 localities in the Republic of Korea, (1) Chungbuk National University Hospital, Cheongju, Chungcheongbuk-do (province) (n = 983), (2) Konkuk University Hospital, Chungju, Chungcheongbuk-do (n = 581), (3) local hospitals in Chuncheon, Gangwon-do (n = 340), and (4) Jeonnam National University Hospital, Gwangju, Jeollanam-do (n = 490) (Fig. 1). Surplus sera from routine serological tests conducted for other reasons were obtained from the same hospitals. The information on the immune status and clinical symptoms of patients were not collected. The sera were collected from September 2002 through June 2003 and stored at -80°C prior to testing.

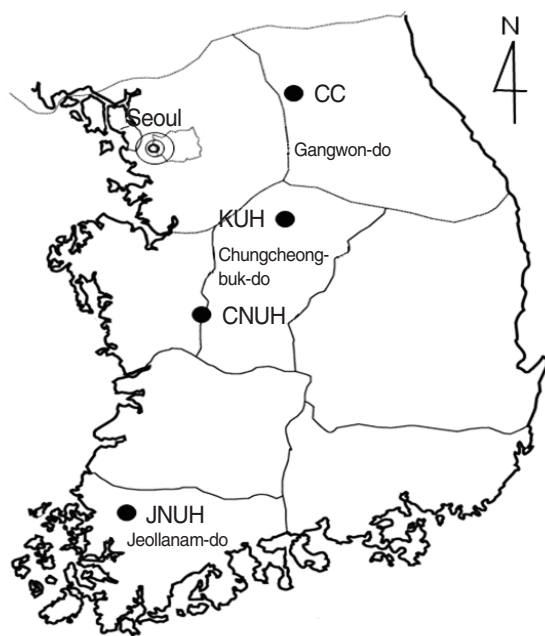


Fig. 1. Map showing the 4 surveyed areas (●) in the Republic of Korea. CNUH, Chungbuk National University Hospital, Cheongju, Chungcheongbuk-do (province); KUH, Konkuk University Hospital, Chungju, Chungcheongbuk-do; CC, local hospitals in Chuncheon, Gangwon-do; JNUH, Jeonnam National University Hospital, Gwangju, Jeollanam-do.

Oocyst crude antigen preparation

The oocysts of *C. parvum* (KKU isolate) were obtained from the feces of C57BL/6 female mice that were infected with oocysts after the induction of immunosuppression through the administration of dexamethasone phosphate disodium salt (Sigma, St. Louis, Missouri, USA) ad libitum in drinking water at a concentration of 10 mg/ml [23]. Mouse feces were examined using modified acid-fast staining to confirm oocyst shedding, collected in 2.5% potassium dichromate, and stored at 4°C. Oocysts were then purified through discontinuous Percoll gradient centrifugation [24]. Oocyst lysate was prepared by freezing and thawing of 10⁸ oocysts/ml in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic, and a pH of 7.4.) for 3 cycles in the presence of protease inhibitors (40 μg/ml bestatin, 10 μg/ml E-64, 1 mg/ml 4-[2-aminoethyl] benzenesulfonyl fluoride, and 0.7 μg/ml pepstatin), followed by sonication and centrifugation for the removal of particulate matter. Lysate was stored in aliquots at -80°C.

Enzyme-linked immunosorbent assay

ELISA was used to assess serum IgG, IgM, and IgA antibody titers against *C. parvum* antigens. Microtiter plates (96-well, Nunc, Rochester, New York, USA) were coated overnight at 4°C with oocyst lysate at a concentration of 2.5×10^5 oocysts/well. Coated plates were washed 3 times with PBS-T (PBS and 0.05% Tween 20) and blocked with 1% bovine serum albumin (BSA) in PBS for 2 hr at 37°C. Diluted serum (1 : 100 in 1% BSA in PBS) was added, and the plates were then incubated for 1 hr at 37°C. After 3 washes with PBS-T, peroxidase-conjugated goat anti-human IgG (γ-chain specific), IgM (μ-chain specific), and IgA (α-chain specific) antibodies (Sigma) (diluted 1 : 2,000 in 1% BSA in PBS) were added. The plates were then incubated for 1 hr at 37°C, washed 3 times with PBS-T, and a substrate solution containing ABTS (2,2'-azino-bis-[3-benzthiazoline-6-sulfonic acid]) (Sigma) was added. The reaction was terminated after 10 min by the addition of 5% sodium dodecyl sulfate (SDS) to the wells. The absorbance was then measured at 405 nm (A_{405} nm) using an ELISA microplate reader (MR 5000, Dynatech Laboratories, Virginia, USA). To control for plate-to-plate variation, the known *Cryptosporidium* spp. negative and positive serum samples were run on each plate. All samples in this study were run in duplicate. Negative controls consisted of sera from 10 infants, aged 6 months to 1 year, without any diarrheal episodes. The seropositivity cut-off was determined as the mean optical density plus 2 standard deviations of the negative control sera [12,25].

Western blotting

Crude oocyst proteins from 10⁸ KJU isolate of *C. parvum* were dissolved in 9 × 8 cm sized gradient SDS-polyacrylamide gel (5 to 20% acrylamide). The proteins were then electrotransferred onto a polyvinylidene difluoride membrane (Immobilon P, Millipore, Bedford, Massachusetts, USA) that was cut into 2-mm-wide strips, with each strip incubated overnight at 4°C with a 1 : 100 dilution of serum in PBS-T. Bound antibodies were detected using the alkaline phosphatase-conjugated goat anti-human IgG (γ -chain specific), IgM (μ -chain specific), and IgA (α -chain specific) antibodies (Sigma). All antibodies were diluted 1 : 50-1 : 100 in 1% BSA in PBS. The bound antibodies were then visualized using the substrate 5-bromo-4-chloro-3-indolylphosphate (Sigma), with nitroblue tetrazolium (Sigma) as the chromogen in alkaline phosphatase substrate buffer. Development was then terminated by rinsing the strips with PBS, which contained 20 mM ethylenediaminetetraacetic acid (EDTA). All steps were carried out under continuous agitation.

Statistical analysis

The DBSTAT program (version 4.1, <http://www.dbstat.com>) was used for statistical analysis. The change of seropositivity

according to localities, age, and sex was tested for statistical significance using a 1-way ANOVA test. *P* values of < 0.05 were considered to be statistically significant.

RESULTS

Serum samples of 2,394 patients from 4 local hospitals in different locations were tested using the ELISA method for IgG,

Table 1. Seropositivity rates of cryptosporidiosis examined by ELISA in samples collected in the four local hospitals in different localities of the Republic of Korea

Hospital	No. examined/No. positive (%)		
	IgG	IgM	IgA
CNUH ^a	983/373 (37.9)	983/324 (33.0)	200/124 (62.0)
KUH ^b	581/128 (21.8)	581/151 (26.0)	200/88 (44.0)
LHCC ^c	340/113 (33.2)	340/39 (11.4)	152/105 (69.0)
JNUH ^d	490/197 (40.2)	490/113 (23.1)	199/104 (52.3)
Total	2,394/811 (33.9)	2,394/626 (26.1)	751/421 (56.1)

^aChungbuk National University Hospital in Cheongju-City, Chungcheongbuk Province; ^bKonkuk University Hospital in Chungju-City, Chungcheongbuk Province; ^cLocal hospitals in Chuncheon-City, Gangwon Province; ^d Jeonnam National University Hospital in Gwangju-City, Jeollanam Province.

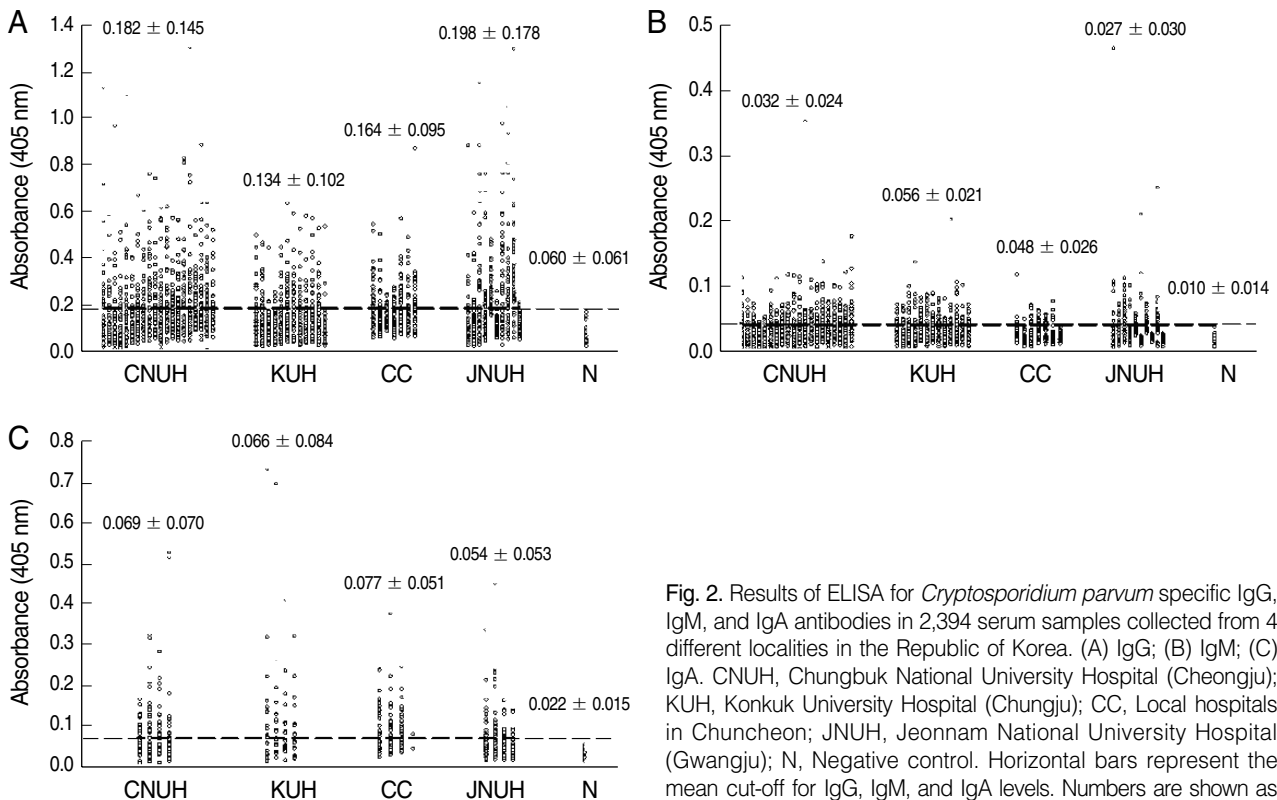


Fig. 2. Results of ELISA for *Cryptosporidium parvum* specific IgG, IgM, and IgA antibodies in 2,394 serum samples collected from 4 different localities in the Republic of Korea. (A) IgG; (B) IgM; (C) IgA. CNUH, Chungbuk National University Hospital (Cheongju); KUH, Konkuk University Hospital (Chungju); CC, Local hospitals in Chuncheon; JNUH, Jeonnam National University Hospital (Gwangju); N, Negative control. Horizontal bars represent the mean cut-off for IgG, IgM, and IgA levels. Numbers are shown as mean OD ± standard deviation.

IgM, and IgA antibodies against *C. parvum* crude antigens. The overall prevalence of antibodies against *Cryptosporidium* spp. was 33.9% for IgG, 26.1% for IgM, and 56.1% for IgA (Table 1). Positive IgG and IgM titers were most common in sera from Jeonnam National University Hospital, Gwangju ($P < 0.0001$) and Chungbuk National University Hospital, Cheongju ($P < 0.0001$), respectively. However, the seropositivity rate for IgA did not significantly differ with the locality. The mean optical density (OD) measurements of IgG, IgM, and IgA were the highest in patients from Jeonnam National University Hospital in Gwangju, Chungbuk National University Hospital in Chungju, and local hospitals in Chuncheon, respectively (Fig. 2).

As age was increased from 0 to 9 years and from 60 to 69 years, the percentage of subjects positive for IgG and IgA antibodies increased from 7.7% to 41.7% and from 15.1% to 79.5%, respectively, while that for the IgM antibody decreased from 31.7% to 18.1% (Fig. 3A). In subjects older than 70 years of age, the seropositivity decreased for all 3 antibody types tested (Fig. 3A). The positive rate of sera having IgG, IgM, and IgA antibodies together were 0% for ages 0-9, 10% for ages 10-19, and 48% for ages 60-69 (Fig. 3B).

Western blot analysis was used to investigate specific antigens reacting with the sera, thus showing positive antibody titers against *C. parvum* crude antigen through ELISA. The 27-kDa antigen was recognized in 91.9%, 83.3%, and 77.4% of sera positive for IgG, IgM, and IgA antibodies by ELISA, respectively (Table 2). The smaller antigen (15/17-kDa) was detected in 28.7% and 17% of IgG and IgA ELISA positive sera, respectively. However, the IgM-positive sera by ELISA did not react with the 15/17-kDa sized antigen (Table 2). No sera showed only a positive band to the smaller antigen, but sera showing positivity to the 15/17-kDa antigen were also positive to the 27-kDa antigen. Negative

sera in ELISA also showed negative results in the western blotting analysis (data not shown).

DISCUSSION

A number of studies have investigated on serum antibody responses to *Cryptosporidium* spp. in individuals residing in both developed and developing countries [12,25,26]. Antibodies against *C. parvum* had previously been identified in 20% and 16% of study populations in Peru and Venezuela, respectively [26], and in 17-70% of the US population [12,25]. In southern Europe, 62-83% of studied populations were detected to have the IgG antibody to *Cryptosporidium* antigens [27]. The present study showed that IgG seropositive rate for *C. parvum* was 34% in the hospital patients in the Republic of Korea. It was impossible to correlate with specific diseases having higher titers for cryptosporidiosis, since we did not collect the information of the disease name of the individual patients whose sera were examined for cryptosporidiosis. Previously, 10-20% of cryptosporidiosis was reported from out-patients at the Severance Hospital by immunofluorescence assay in the Republic of Korea [6]. Several studies using fecal examination also reported various prevalence rate of *C. parvum*. The prevalence of *C. parvum* was 7.9% in in-

Table 2. Results of western blot analysis performed with sera showing high ELISA positive titers to *Cryptosporidium parvum* crude antigen

Antibody	No. of sera with			Total no. of sera either one or both
	Tested	27 kDa	15/17 kDa	
IgG	87	80 (91.9)	25 (28.7)	80 (91.9)
IgM	60	50 (83.3)	0 (0.0)	50 (83.3)
IgA	53	41 (77.4)	9 (17.0)	41 (77.4)

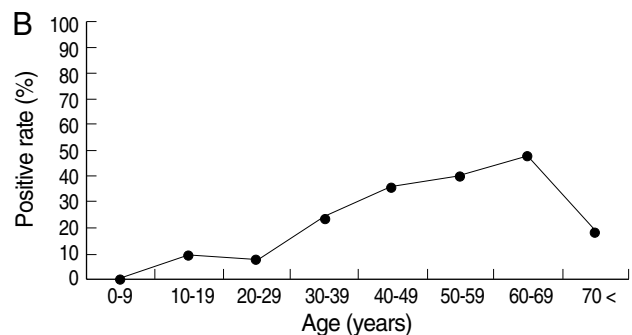
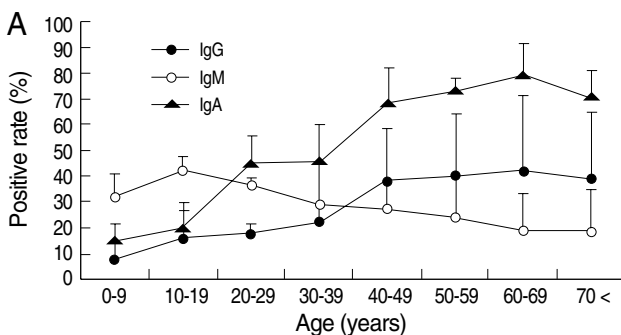


Fig. 3. Age distribution of *Cryptosporidium parvum*-positive sera examined by ELISA. (A) Age-prevalence of IgG, IgM, and IgA antibodies against *C. parvum*. Data are shown as mean and standard deviation values. A total of 2,134 serum samples were examined for IgG and IgM antibodies, and 751 were examined for the IgA antibodies. (B) Prevalence of positive sera having IgG, IgM and IgA antibodies together, by age.

habitants of 2 areas in Seoul and Chollanam-do (in positive rate), 3.3% in inhabitants of 4 provinces (Gangwon-do, Chungcheongbuk-do, Jeollanam-do, and Gyeongsangnam-do), and 1.5% in residents of 25 coastal islands in Jeollanam-do [7,28,29]. In addition, the positive rate of Chuncheon city and Chungju city in the previous study were 2.2% and 1.1% using stool examination, respectively [28]. The serum IgG positive rate in this study, however, was 33% in Chuncheon city and 22% in Chungju city. The overall seropositive rate of 2 local hospitals in Chuncheon and Chungju cities in the present study was significantly higher than those of previous data in these 2 cities ($P < 0.0001$).

The results of the higher seroprevalence rate in the present study suggest that previous data based on fecal examinations underestimated the prevalence of human cryptosporidiosis in the Republic of Korea. However, there is a possibility of higher infection rate of hospital patients than normal population, because former population could be more vulnerable to infection than healthy population. Using the fecal examination method, Jeollanam-do has previously been reported as the highest *Cryptosporidium* endemic area [7,9]. In the present study, this area proved to be a bona fide *Cryptosporidium* endemic area, with the highest IgG positive rates by ELISA in patients from Jeonnam National University Hospital, Gwangju, Jeollanam-do. It has been reported that the IgG and IgA titers change more frequently after a second challenge, whereas IgM conversion is more frequent after the primary challenge [31]. Therefore, it seems to be that reinfection of *Cryptosporidium* occurs more frequently in Gwangju, Jeollanam-do than the other 3 areas.

In addition, the young population (10-19 years) in the present study showed an approximate 16% seropositivity by IgG ELISA. This is a similar level compared with the seropositive rates of young adults in the US (20%), but much lower compared with levels reaching 70% in rural China and 90% in Brazil [30]. We found that the seropositivity rate for the IgM antibody was the highest (42%) in those aged 10-19 years. This result is similar to the findings by Kuhls et al. (1994), who reported an even higher seropositivity rate (58%) in those aged 14-21 years, which suggests that younger subjects are more susceptible to *C. parvum* infection. The explanation for the higher seropositivity in younger subjects (especially teenagers) is not clear, but this may be influenced by physiological, immunological, and behavioral characteristics. The age-associated increases in IgG and IgA responses indicated that infections accumulated with age. However, all antibody responses decreased in those older than 70 years, which may have been due to weakened immunity of these subjects.

Previous studies have shown that 2 antigen groups of *C. parvum* (15/17- and 27-kDa) induce specific antibody responses in infected hosts [14,16,18,20,21]. Both of these low-molecular-mass antigens are located on the surface of the sporozoites and associated with protection from symptoms during infection [14,32]. Antibodies against these 2 antigens have the potential to serve as useful markers for past infection with *C. parvum* [21]. In the present study, western blotting with sera exhibiting ELISA positive titers to *C. parvum* crude antigen showed that 92% of the IgG-positive serum samples were positive for the 27-kDa antigen. In addition, IgM and IgA positive sera showed high positive rates for the 27-kDa antigen (77-84%). In contrast, only a very low percentage of IgG and IgA positive sera (17-29%) showed a positive result for the 15/17-kDa antigen. This result is not consistent with the previous report showing that both 17-kDa and 27-kDa antigens reacted with the positive ELISA sera with at least 90% sensitivity and specificity [21].

The present study also showed that the 15/17-kDa antigen does not induce an immune reaction in primary infections of *C. parvum* because no IgM antibodies reacted to this antigen. It is not clear why the reactivity of ELISA positive sera to 15/17-kDa is so much lower than 27-kDa antigen in this present study. In addition, the IgA antibody was known to respond to the 17-kDa antigen [33], but only 17% of IgA positive sera responded to 15/17-kDa antigen in the present study. These results suggested that the 27-kDa antigen is more useful for epidemiological investigations of *C. parvum* infection than the lower antigen group. In conclusion, the seroprevalence of cryptosporidiosis among the hospital patients suggested that infection with *C. parvum* in this population could be more common than previously estimated in the Republic of Korea.

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