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Comparisons of ELISA and Western blot assays for detection of *Cryptosporidium* antibody

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SUMMARY

A seroprevalence survey was conducted using ELISA and Western blot (WB) assays for antibody to three *Cryptosporidium* antigens on 380 blood donors in Jackson County, Oregon. The purpose was to determine if either assay could detect serological evidence of an outbreak which occurred in Talent, Oregon 6 months earier. The ELISA, which tested for combined IgG, IgA and IgM, and the WB, which tested separately for IgG and IgA, detected an almost twofold increase in serological response for persons who consumed Talent drinking water during the previous 11 months. The increases, however, were statistically significant ($P < 0{\cdot}05$) only for the WB. The identification of serological evidence of infection, using sera collected 6 months after the end of the outbreak in a population not selected because of cryptosporidiosis-like illness, suggests that assays of *Cryptosporidium*-specific IgG and IgA may assist in estimating the magnitude of asymptomatic infections in the population.

INTRODUCTION

Cryptosporidium is a protozoan that can cause acute gastroenteritis of limited duration in immunocompetent individuals and severe chronic diarrhoea in immunocompromised individuals. It is transmitted through animal contact [1, 2], person-to-person contact [3–5], and contaminated food [6, 7] and water [8, 9]. Widespread occurrence of *Cryptosporidium* oocysts in raw [10, 11] and treated [12, 13] drinking water supplies throughout the USA has raised concern that low-level endemic waterborne *Cryptosporidium* infections may occur commonly.

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Epidemiological studies are hindered by the low incidence of laboratory-confirmed cryptosporidiosis. Surveys of clinical laboratory specimens show that infection is detected in < 2% of stools examined [14, 15]. However, serum *Cryptosporidium*-specific antibody levels remain elevated for weeks to months following infection. This suggests that seroprevalence surveys may better identify populations with elevated risks of infection [16, 17]. Anti-*Cryptosporidium*-specific IgA and IgG have been detected in humans using ELISA [18, 19], IFAT [20], or Western blot method [21]. Increases in the intensity of Western blot response for IgG antibody to two *Cryptosporidium* antigens (17- and 27-kDa) following infection were

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observed among infected Coast Guard cutter crew members [21] as well as among persons intentionally consuming *Cryptosporidium* oocysts [22].

Given the availability of the Western blot and ELISA assays for detecting *Cryptosporidium*-specific antibody and the cost differences between these assays, comparisons of findings are essential for planning future serological surveys for *Cryptosporidium*-specific antibody. This paper compares the ELISA assay for combined IgG, IgA, and IgM antibody and separate Western blot assays for IgG and IgA response. Findings were related to risk factors for infection, including residence in a city previously implicated in a waterborne *Cryptosporidium* outbreak [10, 11].

METHODS

Outbreak investigation

During January–June 1992, an outbreak of cryptosporidiosis was investigated in Jackson County, Oregon in which drinking water was implicated as a source of transmission [10, 11]. The outbreak involved residents and visitors to Talent, a small town located between Ashland and Medford in southern Oregon. Talent's drinking water is derived from a surface source and is both filtered and chlorinated. The source water was contaminated with human and animal sewage and the water filtration plant had operational deficiencies which compromised its efficiency [10]. As part of the Oregon Department of Health outbreak investigation, blood donors residing in Jackson County were recruited to donate blood for this study. Following approval of an ethical review board, a total of 403 donors agreed to participate, completed an informed consent and questionnaire, and contributed blood. The blood was drawn between the third week of October 1992 and the second week of November, 1992, approximately 4–6 months after the apparent end of the outbreak. These individuals were not selected because of any history of diarrhoeal illness, exposure to specific water systems, or other risk factors. The questionnaire asked donors to indicate the city in which they lived and whether since 1 January 1992 they had consumed Medford or Talent drinking water, consumed untreated drinking water from lakes or streams, been occupationally exposed to cattle, had children in their household who were in diapers or attended a day care centre, travelled outside the United States, swum in a public pool, or experienced diarrhoea lasting 4 or more days with three or more loose bowel movements per day. Although stored sera, collected prior to the outbreak, would have been useful to ascertain the levels of antibody responses prior to the outbreak, no such sera were available.

At the time of the blood donation, the sera were separated and frozen at −20 °C and in the fall of 1993, the sera were thawed and aliquoted for paired analysis. One sample from each pair was retained by the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia for Western blot analysis and the other was sent to the US Environmental Protection Agency Environmental Systems Monitoring Laboratory in Cincinnati, Ohio for ELISA analysis. A total of 23 samples either lacked questionnaire data or were misplaced during shipping or handling. Laboratory analyses of the remaining 380 samples were performed 1 July–31 December 1994 for the Western blot and 4 April–17 July 1994 for the ELISA.

Preparation of the reference antigen

C. parvum oocysts were obtained from a calf [23] and isolated and purified using methods previously described [24, 25]. Washed and purified oocysts on ice were sonicated (50 watt, 80% output Vitrosonic, Virtis Company, St Louis, MO) until > 95% of the oocysts were disrupted. Proteins were extracted from purified oocysts and the concentration was determined using Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). For the Western blot, extracted proteins were treated with sodium dodecyl sulphate (SDS) without reducing agents as previously described [26]. The SDS-treated proteins were separated by polyacrylamide gradient gel electrophoresis (SDS–PGGE) using 3–25% gradients in a discontinuous buffer system [27].

ELISA method

An ELISA, similar to those previously described was developed to detect anti-*Cryptosporidium* antibody [28]. Optimal concentrations of reagents were determined by checkerboard titration. Flat bottom polystyrene 96-well plates (Immulon 2, Dynatech Laboratories, Chantilly, VA) were coated with 100 μl of sonicated oocysts preparation (200 ng/well) in 0·15 M PBS, pH 7·4, (BupH™ Modified Dulbecco's Phosphate Buffered Saline lot no. 931011001, Pierce, Rockford, IL) containing 0·02% thimerosal overnight at 4 °C. Unbound antigens were removed by washing with 0·15 M PBS, pH 7·4 containing 0·1% Tween 20

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(washing buffer). Coated plates were blocked with 250–300 μl of 1% goat casein (Sigma Chemicals, St Louis, MO) in 0·15 M PBS, pH 7·4 for 60 min at 37 °C on a shaker at 250 rpm. When coated and blocked, plates were not used immediately. Plates were washed three times, sealed, then kept at −20 °C until needed. All pre-coated plates were used within 3 months. To analyse serum samples, 100 μl of appropriate (1/1024) test serum in freshly prepared solution (0·5% goat casein and 0·01% thimerosal) was added in triplicate and incubated for 45 min at 37 °C on a shaker. At the end of the incubation period, plates were washed five times. Then 100 μl of goat anti-human Ig conjugated with horseradish peroxidase (goat anti-human IgM-HRP at 1/50000 [lot no. 03-224004], IgG and IgA-HRP at 1/30000 dilution [lot no. 931109027]; Pierce, Rockford, IL) diluted in washing buffer was added. Then plates were incubated for 30 min at 37 °C on a shaker. At the end of the incubation period, plates were washed 7–10 times to remove unbound conjugate. The substrate (TMB Peroxidase Substrate Kit; Solution A lot no. 48124B and Solution B lot no. 48527A; Bio-Rad, Hercules, CA) was added (100 μl/well) and allowed to develop for 10 min at room temperature on a rocker. The reaction was stopped with the addition of 100 μl of 2 N H_2SO_4. The absorbance was measured within 10 min after the addition of the stop solution using a microplate reader at 450 nm (MR 600 Dynatech Laboratories, Chantilly, VA).

Pooled known negative sera with no serological response (12 replicates) and pooled sera with a strong positive response (56 replicates each of strong and weak response) were used as controls on each plate. All positive samples were titrated and borderline samples (weak positive) were reanalysed. Randomly selected samples (10% of the total samples) were retested to check for reproducibility of the assay.

To evaluate the performance of the ELISA, statistical quality control charts were reviewed. Three plates failed to satisfy the control limits and the samples in these plates were reanalysed. Samples were classified using pooled control values. Inter-plate variation was considered as a random factor and tested using an ANOVA model.

Western blot

Separated proteins were then electrophoretically transferred to polyvinylidene difluoride sheets (Millipore, Medford, MA) using methods previously described [21]. After cutting the sheets into 2 mm-wide strips, the transferred proteins were exposed to human serum at 1/200 dilution for total IgG and 1/50 for IgA. Bound human anti-*Cryptosporidium* antibodies were reacted with biotinylated mouse anti-human IgG or IgA. Bound secondary antibodies were exposed to streptavidin alkaline phosphatase and then were visualized with 5-bromo-4-chloro-3-indolyl phosphate as substrate and nitro blue tetrazolium as chromagen. Each Western blot included positive control serum, collected from a confirmed cryptosporidiosis patient with a strong, serological response, and negative control serum collected from a person whose stool was negative for oocysts and whose serum was not reactive to the antigens. Both were from the Carrollton, Georgia outbreak [29].

After development, a tagged image format file (TIFF) was created on the dried strips from each Western blot using a ScanJet IIc scanner and DeskScan II softwarwe (Hewlett–Packard, San Diego, CA). Scanner values on antigen groups were obtained used the TIFF and SigmaScan software (Jandel Scientific, San Rafael, CA). Based on a grey-level scale, 0 (white) to 256 (black), the highest scanner value obtained in a 3 pixel-wide line drawn perpendicular through each antigen group was used to obtain data. To eliminate human error, a macro was created to import scanner values into Excel™ software which selected the highest value for each antigen group. Band intensity values were expressed as the ratio of the scanner value of the unknown serum to the scanner value of the positive control serum. Separate band intensity values were obtained on IgG response to the 27-, 17-, and 15-kDa antigen groups and on IgA responses to the 17-kDa antigen group.

Statistical analysis

Statistical analyses of both ELISA and Western blot results were conducted using SPSSPC™ version 5.0. A positive response was defined as a serological response at 35% or higher of the positive control for the Western blot and 25% or higher for the ELISA. For Western blot, 35% was selected because tests on paired sera collected over time showed that many individuals maintained responses up to 30% for extended periods of time whereas many individuals with responses of ⩾ 35% declined, over time, to ⩽ 35% (unpublished data). A lower level of positivity (25%) for the ELISA was selected because very few individuals in this study had ELISA responses of

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Table 1. *Percent positive ELISA and Western blot responses* by age*

Age (n)	ELISA	IgG 15	IgG 17	IgG 27	IgA 17
15–29 (41)	7·3	13·9	19·5	43·9	14·6
30–39 (107)	10·3	12·2	16·8	40·2	24·3
40–49 (128)	21·1	25·5	28·1	49·2	36·7
50–59 (104)	20·9	30·0	32·7	54·8	31·7
Total (380)	15·5	21·8	25·5	47·8	29·4
P-value (χ^2)	0·054	0·011	0·039	0·181	0·027
P-value (ANOVA)	0·041	0·060	0·052	0·588	0·003

* P-value (ANOVA) is based on analysis of variance tests of the square root of the sample response (optical density or intensity level) to that of the positive control.

$\leqslant$ 35%. Differences were statistically analysed using the χ^2 test. To determine whether the selection of these specific levels for positivity (35% and 25%) altered the statistical findings, parallel analysis of variance (ANOVA) tests were performed using the square root of the normalized intensity level divided by the positive control. The square root transformation was performed so that the distribution of the marker responses approximated the normal distribution. Results of both statistical tests (χ^2 and ANOVA) are presented. Correlation coefficients between the marker responses (ELISA and different Western blot markers) were also calculated using the square root transformed ratio.

RESULTS

Of the 380 participants, 16 (4·2%) resided in Talent, 233 (61·3%) in Medford, and 131 (34·5%) elsewhere. Twenty-six (6·8%) participants had consumed Talent drinking water since 1 January 1992 and 19 (5·0%) currently or formerly lived in Talent or currently worked in Talent but did not report Talent water consumption. Between 1 January 1992 and the time of the blood donation, 40 (10·5%) participants had children in day care, 27 (7·1%) had contact with cattle through their occupation, 77 (20·3%) had swum in a public pool, 53 had travelled outside the USA, 49 (13·9%) had consumed untreated drinking water from lakes or streams. Only 24 (6·3%) reported having diarrhoea lasting 4 or more days with three or more loose bowel movements per day. Seventy-nine donors (20·8%) had lived in the area for less than 3 years whereas 205 (53·9%) resided there for 20 or more years.

Two Western blots (50 results) showed poor separation of the 15-kDa antigen group from the 17-kDa antigen group, and thus, data on IgG responses to 15-kDa group were not obtained on 50 participants. Both ELISA and Western blot results were otherwise available for each individual. Results for the IgG 15-kDa group are therefore based on a smaller sample size than for other markers.

Questionnaire data

Gender of the participant, having household children in day care or diapers, swimming in a public pool, travelling outside the USA, consumption of untreated water from streams or lakes, duration of residence in the area, and having diarrhoea lasting 4 or more days were not related to higher ELISA or Western blot responses ($P > 0{\cdot}1$). An increase in average responses with increasing age of the donor for each marker was observed except for the IgG 27-kDa marker ($P = 0{\cdot}59$). This increase was statistically significant using both the χ^2 and ANOVA tests for the IgA 17-kDa marker (Table 1) ($P < 0{\cdot}05$). Persons with occupational exposure to cattle ($n = 27$) showed higher average marker responses, however statistical significance was achieved only for the IgA 17-kDa and the IgG 15-kDa markers (Table 2). Differences in the mean responses of the 26 donors who drank Talent water and the 344 who did not were statistically significant for all Western blot markers but not for the ELISA (Table 3).

To determine the relationships between ELISA and different Western blot marker responses, Pearson correlations between responses were determined for the 330 donors with results for all serological markers (Table 4). Each marker showed statistically significant correlations with each other marker ($P < 0{\cdot}001$). In general, both the ELISA and Western blot IgA correlations with Western blot IgG markers were lower than correlations between Western blot IgG markers.

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Table 2. *Percent positive ELISA and Western blot responses* by whether the donor's occupations brought them into contact with cattle during the past 11 months*

	ELISA	IgG 15	IgG 17	IgG 27	IgA 17
Yes (27)	25·9	38·1	37·0	59·3	48·1
No (344)	14·7	20·6	24·6	46·9	28·0
P-value (χ^2)	0·120	0·061	0·152	0·215	0·027
P-value (ANOVA)	0·039	0·012	0·109	0·055	0·000

* *P*-value (ANOVA) is based on analysis of variance tests of the square root of the sample response (optical density or intensity level) to that of the positive control.

Table 3. *Percent positive ELISA and Western blot responses* by whether the donor consumed drinking water during the past 11 months*

	ELISA	IgG 15	IgG 17	IgG 27	IgA 17
Yes ($n = 26$)	26·9	39·1	65·4	73·0	46·2
No ($n = 321$)	14·6	19·7	21·8	45·5	27·1
P-value (χ^2)	0·097	0·029	0·000	0·006	0·038
P-value (ANOVA)	0·065	0·072	0·002	0·021	0·004

* *P*-value (ANOVA) is based on analysis of variance tests of the square root of the sample response (optical density or intensity level) to that of the positive control.

Table 4. *Pearson correlations between ELISA and Western blot responses*

	IgG 17	IgG 27	IgA 17	ELISA
IgG 15	0·66	0·66	0·45	0·52
IgG 17		0·61	0·53	0·42
IgG 27			0·42	0·41
IgA 17				0·28

$n = 330$; all correlations $P < 0{\cdot}001$.
* Responses are expressed as the square root of the ratio of the sample response (optical density or intensity level) to that of the positive control.

DISCUSSION

Both ELISA and Western blot assays detected elevations in antibody responses for persons who had been occupationally exposed to cattle or had consumed Talent drinking water. However, the Western blot results showed greater statistical separation between persons with and without cattle or Talent drinking water exposure. Because only 27 people had been occupationally exposed to cattle and 26 had consumed Talent water during the first half of 1992, the number of individuals with exposures of interest was very small. With additional cases, statistical significance might have been achieved for the ELISA assay.

There is uncertainty over the relative importance of various risk factors for transmission of *Cryptosporidium* infection, including the risk from oocyst contaminated municipal drinking water. In fact, several water utilities and public health agencies have funded special disease surveillance programmes to better estimate the magnitude of waterborne *Cryptosporidium* risk. However, the limited completeness of illness-based disease surveillance programmes and the likely high fraction of asymptomatic infections may result in either biased or misleading findings from such studies. Serological techniques may provide an alternative which can better characterize the magnitude of the risk of *Cryptosporidium* infection and evaluate the importance of various risk factors. Because, in this study, there was no evidence that the city of Talent was experiencing an outbreak of cryptosporidiosis at the time of this survey, the identification of serological evidence of infection among Talent residents, using sera collected 4–6 months after the end of the outbreak, suggests that the serological technique had either detected evidence of the prior outbreak or had detected evidence of an ongoing occurrence of infections/reinfections. Ongoing serological surveillance may be able to determine if the outbreak had ended and if the intervention programme had succeeded. Failure to

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detect an increased risk among residents of Medford may have been due to the decline of marker intensity following the outbreak. Alternatively, since the epidemiological evidence implicating the Medford drinking water was less convincing, a waterborne outbreak might not have occurred there [30].

Additional studies are needed to better characterize the intensity and lifespan of the different serological responses to infection and to better determine if antibody cross-reactions with infections by other organisms occurred. Since most studies which have used the Western blot to estimate antibody prevalence to *Cryptosporidium* antigens have occurred in the USA studies in other populations are needed to assess the baseline prevalence of antibody response and to investigate cross-reactions with other infectious agents. If prior infection is protective against illness from subsequent infections, it would also be important to know whether the lack of a serological response places one at greater risk of illness from subsequent infections. Furthermore, reasons for persistently high serological responses in certain individuals need to be better understood.

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