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Determinants of antibodies to *Cryptosporidium* infection among gay and bisexual men with HIV infection

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SUMMARY

A cross-sectional serosurvey for markers of prior *Cryptosporidium* infection was conducted among homosexual or bisexual males infected with human immunodeficiency virus (HIV); of 262 individuals approached, 236 (90%) agreed to participate. Serological response to two *Cryptosporidium* antigens was measured using a Western blot assay. The intensity or detection of serological responses to two *Cryptosporidium* antigens was not associated with CD4 cell counts or tap water consumption. A number of sexual practices were related to increased serological response for only the 27-kDa marker, including having had sex within the past 2 years, having anal sex and having had a larger number of sex partners during the past 2 years. Attending a spa or sauna was related to serological response to both the 27-kDa and 17-kDa markers. Based on these results, activities related to sexual activity appear to be a significant risk factors for prior *Cryptosporidium* infection.

INTRODUCTION

Cryptosporidiosis in patients with the acquired immune deficiency syndrome (AIDS) who also have severely impaired immunity may be a devastating disease [1]. Not only can it cause chronic severe and intractable diarrhoea that greatly reduces the patient's quality of life, but in many patients it significantly shortens their life expectancy [2]. There are no reproducibly reliable palliative or curative therapies for this infection [3].

Reducing exposure to *Cryptosporidium* oocysts would seem to offer a practical public health prevention strategy. The organism is known to be transmitted through person-to-person and zoonotic routes and numerous outbreaks of cryptosporidiosis outbreaks have been well studied. Unfortunately however the relative importance of specific routes of transmission such as food, drinking water or person-to-person contact, for endemic *Cryptosporidium* infections in either immunocompetent or immunosuppressed persons is not well understood [4, 5]. Given the ubiquitous distribution of the parasite and the many potential routes of transmission, it is essential that those at highest risk of severe disease from infection be given effective recommendations for prevention based on knowledge of the most common routes of transmission [5]. If, for example, immunosuppressed persons focus their prevention efforts on uncommon routes of transmission, then prevention efforts will be misdirected.

Several factors make identification of routes of *Cryptosporidium* transmission difficult. First, labora-

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tory confirmed cryptosporidiosis is a relatively uncommon event, even among persons with AIDS [6]. Only approximately 2% of stools submitted to clinical laboratories are positive, resulting in a small number of cases available for studies and only a fraction of these individuals are infected with human immunodeficiency virus infection (HIV) [7]. A second factor is the difficulty of studying only symptomatic infections if many infections are asymptomatic. If asymptomatic *Cryptosporidium* infection occurs commonly, then many of the controls in a case–control study will also have been infected, making comparisons of the frequency of different exposures less sensitive.

Studies indicate that during the weeks following infection a serological response can be demonstrated to two particular groups of *Cryptosporidium* antigens of 15/17 Kda and 27 kDa [8]. The purpose of this study was to relate questionnaire data on possible risk factors among Australian individuals infected with HIV to serological responses to two *Cryptosporidium* serological markers.

MATERIALS AND METHODS

Study population

This was a cross-sectional study of individuals with HIV infection, living in Melbourne in 1997. Individuals were recruited from a hospital clinic or two community-based clinics during March–July 1997 and who lived in an area supplied with water from Melbourne Water Corporation. All were men who have sex with men or men who have sex with men and women. The study was approved by the local institutional research and ethics committee. Risk factors for exposure to *Cryptosporidium* were determined via a questionnaire adapted from those used during previous studies [9, 10]. The questionnaire asked about factors that may be associated with contact with faecal material including consumption of untreated tap water, use of swimming pools, sexual activity, overseas travel, exposure to young children, contact with animals or domestic pets, and contact with garden manure. Each question specifically asked about exposure during the last 2 years. The questionnaires were administered by one individual (CC), except for questions relating to sexual exposure which were self-administered. Venous blood samples were taken and placed in serum separating tubes. Samples were then centrifuged and stored at -20 °C.

The batched sera were then shipped to the Southwest Centre for Managed Care Research (SCMCR) in Albuquerque, New Mexico where antibody assays to *Cryptosporidium* were performed. IgG serological response was determined using a Western blot assay [4, 8]. IgA and IgM were not measured because of the added cost and the anticipated short duration of their response. Prior studies compared enzyme-linked immunosorbent assay (ELISA) and Western blot assay results and found the Western blot results correlated better to known risk factors for *Cryptosporidium* infection [8, 11].

Western blot procedure

C. parvum oocysts from a calf (Iowa strain) were purified and the antigens extracted using methods previously described [12, 13]. Extracted proteins were treated with sodium dodecyl sulphate (SDS) without reducing agents and separated by SDS–PAGE (polyacrylamide gel electrophoresis) mini gel (15% acrylamide) in a continuous buffer system and electrophoretically transferred to polyvinylidene fluoride (PVDF) (14). The PVDF sheets with the transferred proteins were placed in a Bio-Rad Multi-Screen apparatus that isolates areas of the blot for analysis with different sera. Each isolated area was exposed to human sera at a 1/50 dilution in PBS/0·3% Tween. Bound human anti-*Cryptosporidium* antibodies were reacted with biotinylated mouse anti-human IgG antibodies (Zymed) (1/500 dilution) in PBS/0·3% Tween. Bound secondary antibodies were exposed to streptavidin alkaline phosphatase (GibcoBLR) and then visualized with 5-bromo-4-chloro-3-indolyl phosphate as substrate (Sigma) and nitro-blue tetrazolium as chromagen (Sigma). The positive control was pooled sera with strong serological responses and the negative control was sera from an individual with no prior serological responses to the two antigens.

The intensity of the serological responses to the two antigens (15/17 and 27-kDa) was analysed by an IS-2000 Digital Imaging System (Alpha Innotech Corporation, San Leandro, CA), calibrated using a grey scale card in the spot densitometry mode. The blot was imaged, stored as a tagged image file format (TIFF) file and digitally analysed. The 27-kDa and 15/17-kDa markers were manually selected for analysis. The base lines and limits were set manually prior to integration and both height and area were recorded. Maximum height was used in the analysis. Occasionally when two intensity peaks were observed for 15/17-kDa antigen, a single peak height for the

15/17-kDa marker was recorded. Results were expressed as the ratio of peak heights of the unknown serum to the positive control. For statistical testing, the square root of the band intensity ratio was used so that the distribution of the markers approximated that of the normal distribution. Statistical analysis included *t* tests, Mann–Whitney test and stepwise regression, conducted using SPSSPC® version 5.0.

As with previous studies, we used a serological response of $> 35\%$ of the positive control to indicate a high likelihood of recent infection [11]. This allowed the generation of odds ratio for infection. A sample was considered positive when either the 15/17 or 27-kDa band from the sample was $\geqslant 35\%$ of the positive control. Those samples where both bands were $< 35\%$ of the positive control were considered negative.

Data analysis

This study with 236 subjects of whom 104 (44%) were *Cryptosporidium* antibody positive had 80% power (at 2-sided 5% significance level) to detect odds ratios in the range approx. 2·1–2·4 for binary exposures prevalent in 25–75% of subjects. In addition there was 80% power to detect a trend in odds ratios of 1·6 per tertile increase in exposure.

Crude odds ratios were calculated using the software package Epi Info version 6. The following variables were assessed for association with *Cryptosporidium* antibodies: Age and CD4-cell count (categorized in approximate tertiles), water consumption in average number of glasses per day ($< = 1$, 1–5, $>$ 5), swimming in public pools (0, $< = 5$, > 5 times), number of sexual partners in last 2 years (0, 1, 2–9, $> = 10$), anal sexual practices (none, $<$ once per month, $> =$ once per month), attendance at spas or saunas (0, 1–5, > 5 times), handling of diapers or manure, contact with animals, and overseas travel destinations. Logistic regression was used to generate crude odds ratios for positive *Cryptosporidium* antibodies with subsequent adjustment for age and number of sexual partners together with 95% confidence intervals. All computations were performed using Stata® for Windows version 5.0.

RESULTS

Of the 262 individuals approached, 236 (90%) agreed to participate. All were men who have sex with men, or men who have sex with men and women. The mean age of the men was 39·5 years (range 22–72). The mean age of those who agreed to participate was not different from those who did (40·7 *vs.* 39·5 years).

The mean serological response to the two *Cryptosporidium* antigens was not associated with T-cell counts or tap water consumption ($P = 0{\cdot}59$). A number of sexual practices were related to increased serological response for the 27-kDa marker, including having had sexual within the past 2 years ($P = 0{\cdot}03$), having anal sex ($P = 0{\cdot}03$) and having had a larger number of sex partners during the past 2 years ($P = 0{\cdot}04$). Attending a spa or sauna was related to serological response to both markers ($P \leqslant 0{\cdot}05$).

The odds ratios for the presence of a 15/17-kDa and/or a 27-kDa serological response to *Cryptosporidium* above 35% of the positive control is shown in Table 1. In the crude analysis, age, and a number of activities relating to sexual activity in the last 2 years (number of sexual partners, any sexual intercourse, anal intercourse, anal insertive intercourse, and attending a spa or sauna in the past 2 years) were statistically associated with the presence of cryptosporidial antibodies (Table 1). No other factors were significantly associated with the presence of cryptosporidial antibodies. In particular the median number of glasses of unboiled tap water was similar for those with cryptosporidial antibodies compared to those without (3·6 *vs.* 4·5 glasses/day respectively).

The number of sexual partners remained significant when adjusted for age. Attendance at a spa or sauna remained significant when adjusted for age and the number of sexual partners (Table 1). Variables relating to the types of sexual activity (e.g. anal sex) were very closely correlated with the number of sexual partners (Table 2).

DISCUSSION

In this study of individuals with HIV infection, sexual activity was consistently associated with markers of prior *Cryptosporidium* infection. This is consistent with the known faecal–oral route of transmission for this organism. Drinking untreated tap water was not associated with the presence or intensity of cryptosporidial antibodies, despite this being a common source of transmission in published outbreaks [15].

Our study has a number of limitations that should be considered when interpreting the results. Our outcome measure was antibodies to *Cryptosporidium* because cases of diagnosed disease are too uncommon.

Table 1. *Characteristic of the men with and without cryptosporidial antibodies*

	Crypto antibody		Crude OR, 95% CI	Adjusted OR, 95% CI
	positive	negative		
Number (total $n = 236$)	104 (44·1%)	132 (55·9%)	—	—
Age (years)				
Mean (S.D.)	38·0 (8·6)	40·62 (9·7)	$P = 0·03$	
< = 35	48	49	1·0	—
35–45	38	43	0·9 (0·5, 1·6)	—
> 45	18	40	0·5 (0·2, 0·9)	—
CD4 cell count (/μl)				
Median (range)	220 (0–987)	234 (0–1000)	$P = 0·84$	—
< = 120	36	46	1·0	—
120–350	35	43	1·0 (0·6, 1·9)	—
> 350	33	43	1·0 (0·5, 1·8)	—
Tap water (per day)				
Median (range)	3·6 (0–16·0)	4·5 (0–22)	$P = 0·59$	—
< = 1 glass	21	34	1·0	1·0‡
> 1–5 glasses	47	45	1·7 (0·9, 3·3)	1·6 (0·8, 3·3)
> 5 glasses	36	53	1·1 (0·6, 2·2)	1·1 (0·5, 2·1)
Swimming in public pools*				
None	46	56	1·0	1·0‡
< = 5 times	29	27	1·3 (0·7, 2·5)	1·2 (0·6, 2·3)
> 5 times	27	48	0·7 (0·4, 1·3)	0·6 (0·3, 1·1)
Handling manure*				
No	59	74	1·0	—
Yes	45	58	1·0 (0·6, 1·6)	—
Overseas travel*				
None	67	84	1·0	—
To developing country	19	23	1·0 (0·5, 2·1)	—
To developed country	17	25	0·9 (0·4, 1·7)	—
Handling diapers*				
No	96	121	1·0	—
Yes	8	11	0·9 (0·4, 2·4)	—
Domestic animal contact*				
No	7	12	1·0	—
Yes	97	120	1·4 (0·5, 3·7)	—
Farm animal contact				
No	63	85	1·0	—
Yes	41	47	1·2 (0·7, 2·0)	—
Sexually active in last 2 years				
No	8	25	1·0	1·0‡
Yes	96	107	2·8 (1·2, 6·5)	2·3 (1·0, 5·6)
No sexual partners in last 2 years				
Median (range)	4·5 (0–150)	2·0 (0–1000)	$P = 0·008$†	—
None	8	25	1·0	1·0‡
1	25	33	2·4 (0·9, 6·1)	1·9 (0·7, 5·2)
2–9	31	38	2·5 (1·0, 6·4)	2·1 (0·8, 5·5)
10+	40	35	3·6 (1·4, 8·9)	3·0 (1·2, 7·6)
Anal sex				
No	32	59	1·0	1·0§
Yes	72	73	1·8 (1·1, 3·1)	1·1 (0·6, 2·2)

Table 1. (*cont.*)

	Crypto antibody		Crude OR, 95% CI	Adjusted OR, 95% CI
	positive	negative		
Anal insertive				
None	33	63	1·0	1·0§
< 1/month	14	12	2·2 (0·9, 5·4)	1·7 (0·6, 4·3)
> = 1/month	57	56	1·9 (1·1, 3·4)	1·3 (0·7, 2·6)
Anal receptive				
None	41	67	1·0	1·0§
< 1/month	16	12	2·2 (0·9, 5·1)	1·7 (0·7, 4·1)
> = 1/month	46	52	1·4 (0·8, 2·5)	0·9 (0·5, 1·8)
Oral anal sex				
None	58	88	1·0	1·0§
< 1/month	17	16	1·6 (0·8, 3·4)	1·2 (0·5, 2·7)
> = 1/month	29	27	1·6 (0·9, 3·0)	1·2 (0·6, 2·3)
Attend spa/sauna				
None	34	61	1·0	1·0§
1–5 times	39	28	2·5 (1·3, 4·7)	1·9 (1·0, 3·8)
> 5 times	30	41	1·3 (0·7, 2·5)	0·9 (0·5, 1·9)

* Refers to the last 2 years.
† Mann–Whitney test.
‡ Odds Ratio adjusted for age.
§ Odds Ratio adjusted for age and sexual partners.

Table 2. *Median number of sexual partners for various categories of activity*

	Number in category	Median number sexual partners
Anal sex*		
No	90	1
Yes	145	6
Anal insertive*		
None	96	1
< 1/month	26	5
> = 1/month	113	6
Anal receptive		
None*	108	1
< 1/month	28	3·5
> = 1/month	98	10
Oral anal sex*		
None	146	1·5
< 1/month	33	10
> = 1/month	56	10
Attend spa/sauna*		
None	94	1
1–5	67	4
> 5	71	5

* Refers to the last 2 years.

However, studies based on antibodies are limited by the sensitivity and specificity of the assay. Laboratory studies suggest that the serological response to the 15/17 and 27-kDa proteins is specific for infection with *Cryptosporidium* [8, 16].

Studying antibody production can be an advantage particularly if a high proportion of infections are asymptomatic. This is because infected individuals who are asymptomatic are counted as cases in serological studies and not as controls which would occur in studies assessing clinical disease. It has been suggested that many cases of *Cryptosporidium* infection may be asymptomatic [17].

We used as exposure period of 2 years in the questionnaire for this study because antibodies have been shown to last for up to 2 years [9]. This is supported by a declining prevalence of antibodies with age. The lower prevalence of the 15/17-kDa marker which was nearly half that of the 27-kDa marker (26% *vs.* 39% over 35% of positive control) would suggest that the 15/17-kDa lasts a shorter time. If the 15/17-kDa marker lasts less than 2 years then it may not correlate well with the questionnaire data. This may be why more exposures in the last 2 years

were associated with the 27-kDa marker rather than the 15/17-kDa marker.

The second main limitation of our study was its size. It is a relatively small study which did not have the statistical power to detect small or uncommon risk factors. For categorical variables, it had an 80% power to detect an odds ratio of 2·5 or more for risk factors that were present in 20% of the control group ($P = 0{\cdot}05$). The study had greater precision for numerical variables as was evident from the confidence interval around the difference in the median number of glasses of untreated drinking water consumed ($-0{\cdot}7$ to 1·4 glasses/day). Therefore, this study does not rule out a small contribution from drinking water.

It is evident from our study that infection with *Cryptosporidium* is more common than clinical disease. Our study suggests that the annual incidence of infection in this group is about 20% if antibodies last about 2 years and the prevalence is 40%. Clinical cryptosporidiosis in Melbourne, however, is uncommon [6]. In Melbourne, 85 cases of cryptosporidiosis occurred between 1991 and 1997 among individuals with HIV infection. During this time, there were about 300 individuals with HIV infection who had AIDS living each year, corresponding to about a 1 in 20 annual chance of clinical cryptosporidiosis [6].

The relatively high incidence of antibodies to *Cryptosporidium* found in our study has been confirmed in other studies of both individuals with and without HIV infection. Soave and colleagues using the ELISA assay found 25 (20·3%) of 137 individuals with HIV to have *Cryptosporidium* antibodies but did not identify any statistical association with potential risk factors (18). In one study in the USA, the prevalence among blood donors with a surface water supply (Las Vegas) was 40% compared to 30% with a ground water supply (Albuquerque). No risk factors for the presence of antibodies were identified in this study [19].

We did not identify drinking water as a risk factor for the presence of cryptosporidial antibodies in this study in Melbourne. A number of issues should be considered in interpreting these results. The first is the very high quality of Melbourne's drinking water which is sourced from a highly protected catchment with no human habitation or agriculture. The retention time in the large reservoirs is up to 2 years. The water is lightly chlorinated but does not undergo any additional treatment, such as filtration. During 1997, Melbourne Water Corporation collected 225 samples each containing 20 litres of which two had *Cryptosporidium* detected at 0·1 occysts/litre (personal communication, Melbourne Water Corporation).

We did not find a relationship between the serological response to cryptosporidium and CD4 lymphocyte count which is surprising in view of the impaired ability of individuals with HIV infection to mount humoral immune response (20). The proportion of those with CD4 lymphocytes who of less than 50 who had prior exposure to cryptosporidium (44·4%) was very similar to those with CD4 counts $\geqslant 50$ (44·0%). The median number of sexual partners was actually greater in those with more than 50 CD4 lymphocytes than those with < 50 CD4 lymphocytes (4 compared to 1) so it is possible that cryptosporial antibodies are not lost in advance HIV infection.

It is difficult to separate out which specific sexual activity most strongly correlates with cryptosporidial antibodies. This is because almost all activities are closely correlated with one another and each one would by their nature be assumed to carry some risk. A much larger study that was capable of stratifying by each behaviour would allow the different behaviours to be clearly ranked.

In this study, we did not identify drinking water as a risk factor for *Cryptosporidium* antibodies but feel very strongly that these results should not stop individuals with HIV from boiling or treating their drinking water. This is because our results are only relevant to Melbourne's water supply and also because the consequences of infection may be profound in this patient population. Our results do indicate however that reducing sexual activities that are likely to result in exposure to human faecal contamination may reduce the incidence of this infection.

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REFERENCES

1. Colford JM, Tager IB, Hirozawa AM, Lemp GF, Aragon T, Petersen C. Cryptosporidiosis among patients infected with Human Immunodeficiency Virus: factors

related to symptomatic infection and survival. Am J Epidemiol 1996; **144**: 807–16.
2. Anonymous. Classification system for human T-Lymphotropic virus Type 111/lymphadenopathy-associated virus infections. MMWR 1983; **35**: 334–9.
3. Ungar BLP. *Cryptosporidium* and Cryptosporidiosis. In: Broder S, Merigan TC, Bolognesi D, eds. Textbook of AIDS medicine. Baltimore: Williama & Wilkins; 1994: 323–43.
4. Addiss DG, Arrowood MJ, Bartlett ME, et al. Assessing the public health threat associated with waterborne cryptosporidiosis: report of a workshop. MMWR 1995; **44** (No RR-6): 1–20.
5. USPHS/IDSA Guidelines for the prevention of opportunistic infections in persons infected with Human Immunodeficiency Virus: disease-specific recommendations. Clin Infect Dis 1995: **21** (suppl 1): S32–S43.
6. Stuart RL, Hellard ME, Jolley D, et al. Cryptosporidiosis in patients with AIDS. Internat J STD & AIDS 1997; **8**: 339–41.
7. Hellard ME, Fairley CK. Gastroenteritis in Australia: who, what, where, and how much? Aust NZ J Med 1997; **27**: 147–9.
8. Moss DM, Chappell CL, Okhuysen PC, et al. The antibody response to 27-, 17-, and 15-kDa *Cryptosporidium* antigens following experimental infection in humans. J Infect Dis 1998; **178**: 827–33.
9. Ungar BLP, Mulligan M, Nutman TB. Serologic evidence of *Cryptosporidium* infection in US volunteers before and during peace corps service in Africa. Arch Intern Med 1989; **149**: 894–7.
10. Goldstein ST, Juranek DD, Ravenholt O, et al. Cryptosporidiosis: An outbreak associated with drinking water despite state-of-the-art water treatment. Ann Intern Med 1996; **124**: 459–68.
11. Frost FJ, de la Cruz AA, Moss D, Curry M, Calderon R. Comparison of ELISA and Western blot assays for detection of *Cryptosporidium* antibody. Epidemiol Infect 1998; **121**: 205–11.
12. Moss DM, Mathews HM, Visvesvara GS, Dickerson JW, Walker EM. Purification and characterization of *Giardia lamblia* antigens in the feces of Mongolian gerbils. J Clin Microbiol 1991; **29**: 21–6.
13. Arrowood MJ, Sterling CR. Isolation of *Cryptosporidium* oocysts and sporozoites using discontinuous and isopycnic percoll gradients. J Parasitol 1987; **73**: 314–9.
14. Moss DM, Lammie PJ. Proliferative responsiveness of lymphocytes from *Cryptosporidium parvum*-exposed mice to two separate antigen fractions from oocysts. Am J Trop Med Hyg 1993; **49**: 393–401.
15. Kramer MH, Herwaldt BL, Craun GF, Calderon RL, Juranek DD. Surveillance for waterborne-disease outbreaks – United States, 1993–4. MMWR 1996; **45** (SS-1): 1–33.
16. Moss DM, Bennett SN, Arrowood MJ, Hurd MR, Lammie PJ, Wahlquist SP. Kinetic and isotypic analysis of specific immunoglobulins for crew members with cryptosporidiosis on a U.S. Coast Guard cutter. J Euk Microbiol 1994; **41**: 52S-5S.
17. Frost F, Craun GF, Calderon R, Hubbs SA. So many oocysts, so few outbreaks. J AWWA 1997; **89**: 8, 10.
18. Spencer KL, Soave R, Acosta A, et al. Cryptosporidiosis in HIV-infected persons: prevalence in a New York city population. Int J Infect Dis 1997; **1**: 217–21.
19. Frost F, Muller T, Rodman J, Calderon R, Craun G. Study of serological responses to two *Cryptosporidium* antigens in Las Vegas, Nevada and Albuquerque, New Mexico. In: Water Quality Technology Conference; 1997 Nov 9–12; Denver, CO: AWWA; 1997: 1–13.
20. Terpstra FG, Al BJ, Roos MT, et al. Longitudinal study of leukocyte functions in homosexual men for HIV: rapid and persistent loss of B cell function after HIV infection. Eur J Immunol 1989; **19**: 667–73.