# Towards a US national estimate of the risk of endemic waterborne disease – sero-epidemiologic studies

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## ABSTRACT

Worldwide literature on serological methods and sero-surveys on waterborne pathogens has been reviewed. Outbreak investigation and research reports have also been examined to aid understanding of the serological response and transmission dynamics. The aim was to seek an estimate of seroprevalence and to determine if this could inform the US national estimate of risk for endemic waterborne infection associated with public water supplies. Antibody responses indicate infection, both symptomatic and asymptomatic, so probably give a truer indication of prevalence. Outbreak data can probably be regarded as the upper bound for seroprevalence estimations. Antibody is not necessarily protective *per se* but is a good indicator for at least partial resistance to symptomatic infection; absence of antibody will normally imply susceptibility. Pathogens transmitted by water are commonly transmitted by other routes. However, the fact that other transmission routes are more common does not detract from the potential protective effect of immunity when waterborne transmission occurs. Data indicate that seroprevalence varies widely, reflecting geographic, social and hygiene factors, but is generally greater where surface water sources are used rather than groundwater. Areas of low seroprevalence may expect a high attack rate in the event of contamination of their water supply.

**Key words** | *Campylobacter*, *Cryptosporidium*, ELISA, endemic infection, *E. coli* 0157, *Giardia*, molecular studies, noroviruses, outbreaks, sero-epidemiology, seroprevalence, waterborne pathogens, western blot

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## ABBREVIATIONS

| | |
|---|---|
| Ab | antibody |
| Ag | antigen |
| AHG | anti-human globulin (combined Ig classes) |
| BSA | Bovine serum albumin (used as blocking agent) |
| CMI | cell mediated immunity |
| ELISA | enzyme-linked immunosorbent assay |
| EITB | enzyme-linked immuno-electro-transfer blot syn. western blot |
| GW | groundwater |
| IFAT | immunofluorescent antibody test; also IIFA (indirect IFAT), IFA |
| Ig | immunoglobulin (syn. antibody); classes IgG, IgA, IgM, etc. |
| mw | molecular weight (mass) |
| p.i. | post-infection |
| SRSV | small round structured virus (e.g. Norovirus) |
| TSW | treated surface water |
| WB | western blot |
| MGF | minigel format |
| LF | large format |
| OD | optical density |



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## DEFINITIONS AND GENERAL ASPECTS OF IMMUNOLOGY

Epidemiology, at its simplest, is the study of the occurrence (number and frequency) and distribution of cases by person

(age, gender, etc), place and time, i.e. demographics. It also seeks to describe or statistically analyse the determinants of these (e.g. risk exposures or other causal factors). Cases are usually defined by an agreed set of clinical signs and symptoms although for gastro-enteric infections these are usually not pathognomonic and may be estimated by reference to more broadly defined criteria. Precise diagnosis often requires laboratory findings, including the isolation of the causative organism and/or the detection of specific antibody using methods capable of indicating recent infection. In some cases, this may be further enhanced by the isolation of an identical organism from the putative source of the infection or vehicle of transmission such as food or water (Tillett *et al.* 1998). Traditionally, epidemiology is associated with the study of epidemics and localized outbreaks (two or more associated cases). Epidemiologic study methods, however, can also be used for the study of sporadic (endemic) and pseudo-sporadic (diffuse, low-level outbreaks only identified as associated by such studies and by typing of isolates, particularly if these are unusual strains) infection that may share common features with outbreaks such as potential routes of transmission, including water (Casemore 1995; Meinhardt *et al.* 1996; Goh *et al.* 2004, 2005; Hunter *et al.* 2004). The occurrence of such background endemic or sporadic infection is of particular importance in the context of waterborne infection, which is often thought, erroneously, to involve only outbreaks. Large outbreaks usually follow a breakdown in, or sub-optimal operation of, water treatment processes. Low-level, intermittent penetration of treatment may lead to low-level intermittent transmission to consumers who may or may not suffer overt disease but who may show an antibody response. Waterborne pathogens are commonly also transmitted by the direct person-to-person route and indirectly via food, recreational water exposure, and other exposures. This makes interpretation of epidemiological findings problematic for estimating drinking water risk because an infected individual may have become infected from a variety of sources. Risk exposures may also differ significantly between different population groups and extrapolation of findings from one population group to another needs to be done with caution.

The outcome of the contamination of water supply and transmission to consumers depends on complex dynamics with various, usually ill characterised, factors–the organism, the exposed persons (and their levels of immunity) and the environment (Casemore 1994, 1995; Meinhardt *et al.* 1996). In order to better understand the natural history of waterborne infections and the complexities of the dynamics of transmission (see Figure 1), epidemiological investigations can be enhanced by serologic studies (Craun *et al.* 2001). The purpose of this paper concerns the national estimate of risk of endemic infection from water. However, reports on immunological studies and on the investigation of outbreaks are fundamental to the understanding of endemic infection and illness. In general terms, prevalence in outbreaks likely represents the upper boundary for endemic infection estimates.

Sero-epidemiology is a form of study in which the demonstration of antibodies to the organism of interest is used as a surrogate or marker for the distribution of infection, with or without overt disease. The samples (usually serum but sometimes other body fluids such as saliva and feces) that are analysed may be from those involved in an outbreak (cases and symptom-free "controls"). Others may be from groups (cohorts) defined by geography or other determinants, or representative sub-sets of whole populations (e.g. informed adult volunteers, anonomized blood donor samples or from patients investigated for other non-infectious conditions, referred to below as convenience samples). The purpose of sero-epidemiology is to investigate the frequency of sero-positivity in the group studied or, by extrapolation, to estimate the frequency in the population as a whole, and hence estimate the frequency of exposure to the organism. It may also be used to study the transmission or spread of the infecting agent in a defined cohort (e.g. within an outbreak area). Sero-prevalence is a measure of total infection levels at a point in time (point prevalence) or over a defined period (period prevalence), which may be normalized to a rate, e.g. cases per 100 000 population. It is important to recognize that circulating antibodies detected in serologic studies reflect infection *per-se*, with or without symptoms (i.e. clinical and sub-clinical or asymptomatic infection), at an often ill-defined point in time. Sero-incidence, on the other hand, is a measure of new acute cases in a particular location, at a point in time or over a defined period in time. This is often defined by sero-conversion, a change from sero-negative to sero-positive in a time series of samples.

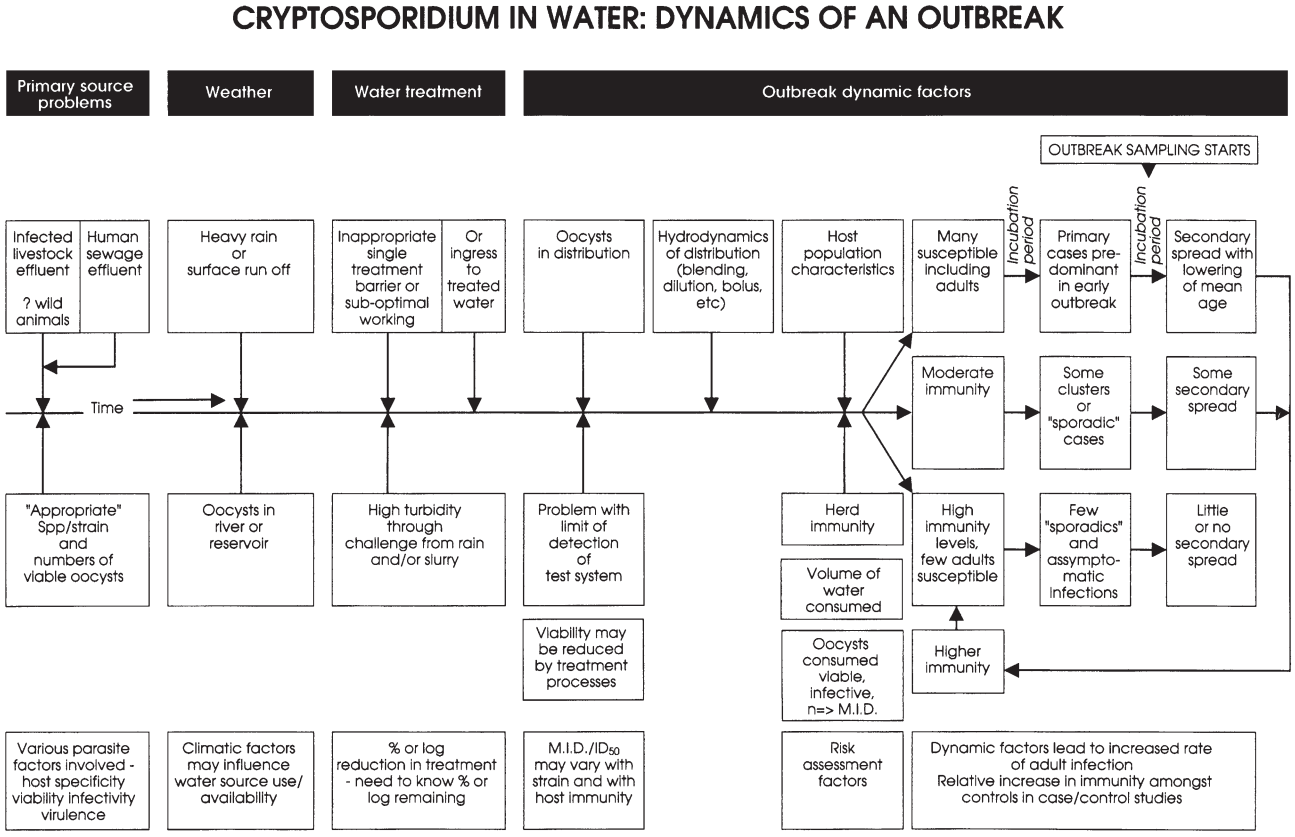


**Figure 1** | *Cryptosporidium* in water: dynamics of outbreaks. Oocysts in water are often derived from a variety of sources and host species, which may be of variable infectivity and pathogenicity for humans. Numbers and viability decline with dilution, age and environmental attrition, including water treatment processes. These will affect the likelihood of an infective dose being ingested. The risks for target populations vary according to several factors including quantities of water consumed and levels of immunity. For a given level of contamination with a pathogenic strain several outcomes are possible: (i) in an area of low seroprevalence the attack rate may be high, primary cases will tend to include an unusual number of adult cases; secondary cases occur more frequently in children so the age-specific rate will decline as the outbreak progresses. Although seroprevalence will be higher following an outbreak, this will decline again unless there is further input of infective oocysts. (ii)Where seroprevalence is moderate, outbreaks will tend to be smaller and secondary transmission less frequent but seroprevalence may be boosted by naive individuals with primary infection and sub-clinically infected exposed persons. Such immune exposed subjects if chosen as controls will tend to reduce apparent relative risk for water consumption. (iii) In areas with high endemicity and seroprevalence, outbreaks will be uncommon but visitors and other at-risk subjects will act as sentinels of high exposure levels. This dynamic compartmentalized model permits change of risk status depending on changes in oocyst input (numbers, viability and infectivity), antibody increase post-exposure and then declining, and recruitment of susceptibles.

Alternatively, the pattern of antibody classes (see below) may be used to indicate the probability of recent or past infection. Positive reactions to some antigens are associated with early or late stages of the immune response. In addition, in some infectious diseases, serum avidity (strength and speed of binding of an antibody with its homologous antigen) is also a useful marker to distinguish recent and past infection (Joynson *et al.* 1990; Roitt & Delves 2001; Garcia *et al.* 2004; Iturriza-Gómara *et al.* 2004).

## The nature of antibodies

Immunoglobulins (Ig) are present in the blood and other body fluids as part of the immune system's response to antigens present as components of infecting agents (see below). This may occur because of infection, passive transfer (e.g. from mother to fetus via the placenta), or immunization. Circulating (humoral) antibodies and secretory antibodies (in saliva and other body fluids) have a variable role in recovery and resistance to re-infection that is often poorly understood. Recovery and resistance may depend mainly or in part, according to the infecting agent, on cellular immune mechanisms such as cell-mediated immunity (CMI) or a combination of the two (Steiner & Guerrant 1996; Roitt & Delves 2001; Wyatt & McDonald 2004). However, if immunity (resistance) to re-infection is present then the presence of antibodies may provide a

useful marker for that. Importantly in the context of this report, the absence of antibodies makes it unlikely that there will be immunity and implies lack of exposure. However, it has to be recognized that infrequent exposure may lead to waning of the antibody to levels that are below the threshold of detection of the test(s) used. Such low-level antibody can be boosted by subsequent re-exposure to the same or antigenically related organisms (anamnestic response). Sero-prevalence may thus provide a useful surrogate for estimating the frequency of exposure to the infecting agent, provided that the tests are sufficiently sensitive and specific. Serology may also indicate the frequency of sub-clinical cases, for example in an outbreak, where sero-conversion or increased response (titre) is not associated with symptoms and recovery but nonetheless, does indicate recent infection. This may be of particular value where detection of the infecting agent is difficult.

Antibodies comprise several immunoglobulin classes, IgG, IgA, IgM, IgD and IgE, which have sub-classes and isotypes. Serological tests may detect whole antibody or individual classes or isotypes, depending on the test protocol. The function and significance of each of these differs but for the purposes of this report, the latter two classes, sub-classes, etc, have not been considered. IgM is usually detected early in infection and declines within a few months, often to undetectable levels, although exceptions have been described (see below). IgG often arises a little later but generally lasts for longer; if found in the absence of detectable IgM it is usually taken as evidence of past infection. The presence of IgM, with or without IgG, usually indicates recent infection. However, studies on *Cryptosporidium* infection, for example, have sometimes indicated prolonged ($\geq$12 months) IgM production, sometimes in the absence of detectable IgG. The reason for this is a subject of debate but it probably indicates ongoing exposure to the stimulating organism. IgA is present in the serum (humoral antibody) and is secreted into the mucosa, during which process its structural form changes to dimeric IgA ($IgA_s$ or sIgA) (Roitt & Delves 2001; Riggs 2002). Secretory IgA can be detected in faeces and saliva and behaves differently from the humoral (monomeric) form. It is of particular relevance to parasitic and other infections in the gut mucosa. Antibody studies are sometimes used to help characterise the pathology and pathogenesis of symptoms and recovery, and for diagnosis, rather than to measure sero-prevalence or sero-incidence. Nonetheless, such studies may also yield epidemiologically useful information. The particular importance of antibody studies epidemiologically is that they offer a truer reflection of prevalence than isolation of organisms and/or clinical diagnoses, both of which underestimate true prevalence (Hunter 1997*a* 2000; Craun *et al.* 2001; Frost *et al.* 2003*a*; Priest *et al.* 2005).

## The nature of antigens

Antigens are substances, usually protein or protein complexes, that when introduced parenterally into the body provoke an immune response, including antibody production. They will subsequently react specifically with the antibodies so produced in some observable way (precipitation, agglutination, etc). In addition, various other molecules such as carbohydrate moieties (sugars) and lipids structurally associated with those proteins may subsequently react in test systems even though not necessarily capable alone of eliciting an immune reaction (partial or incomplete antigens, or haptens). Such molecules may also mask antigenic proteins in intact cells. The antigens traditionally used in laboratory tests often consisted of suspensions of microorganisms, parasites, etc, whole or crudely extracted chemically, by heat or other physical methods. More recently, antigens are, increasingly, well-defined molecular structures or sequences, including those extracted electrophoretically from crude preparations of target organisms or genetically engineered (recombinant) antigens in a microbial cell or virus. Some antigens are associated with structural molecules (e.g. cell wall structures, tubulins, flagellins, etc) or functional molecules (e.g. toxins, intracellular enzymes such as polymerases, etc) that may be biologically well conserved and present in a wide variety of organisms, not just closely related species. These, together with partial antigens, may cause cross-reactions that make interpretation of findings problematic. Other factors that may interfere with specificity of reactions include rheumatoid factor (RF). Those exposed to cattle may react with bovine serum albumin (BSA) which is sometimes used as a blocking agent in the enzyme-linked immunosorbent assay (ELISA) (Chart *et al.* 1998), and animal-derived sera used as reagents may react

inappropriately against target antigens or sera of other animal species used as reagents. Test reagents may also bind non-specifically and variably to the test vessel material, especially some plastics. These problems tend to be greater with ELISA and with methods in which crude target pathogen extracts have been used (Venkatesan & Wakelin 1993; Chart *et al.* 1998). It is less likely a problem if the precise molecular weight and other characteristics of the reacting antigen can be determined and the precise binding reactants identified, such as in western blotting (WB), especially with larger formats (LF WB) that permit the complete resolution of antigen complexes or families.

## Test modalities

Various test modalities are used to demonstrate antigen/antibody reactions. These include immunofluorescence (IFAT/IIFT), ELISA, and WB. The results from these different methods may differ both qualitatively and quantitatively from each other and thus may make comparisons of data from different studies difficult. Antibody classes may vary in valency, avidity, etc., and in the optimum conditions required for detection and quantitation. Enzymes used in ELISA differ in their optimum conditions of pH, temperature, and chemical constituents. Batch testing of large numbers of samples may lead to variations in reliability of individual results depending on position in the tray or stack of trays because of varying temperature. Even studies using notionally the same methods may differ depending on the precise antigen used, how the test system has been optimized, and operator variability. Different immunoglobulins having differing valencies need different test conditions to optimize the antigen/antibody reaction. In addition, different test systems may be optimized to maximize either sensitivity or specificity. As a general rule of thumb, maximum sensitivity (the greatest number of true positives) may need to have a broad specificity and may thus pick up more cross-reactions (false positives). Conversely, to maximize specificity (increase precision) these potential cross-reactions have to be excluded and some true (usually low-level) positives may be lost (false negatives). Generally speaking, the cruder the antigen (such as whole cell preparations), the more likely it is to suffer from cross-reactions. ELISA is more subject to "background noise" from such cross-reactions but has the advantage that it is generally easier to handle large numbers of samples and to automate the process. Sera can be readily titrated to provide an accurate quantitative result and a logical cut-off achieved mathematically (Cox *et al.* 2005). This approach has been used because of the difficulty of identifying true negative control samples (Cox, personal communication). The development of more precisely defined or genetically engineered antigens for some organisms has overcome some of these problems in recent years. WB is generally more sensitive and specific, at least in its larger format, but is technically demanding and expensive in both reagents and equipment and quantitation is often more subjective. Optical device methods have been developed to semi-quantitate WB reactions by measuring band density and/or size, but this is still likely less accurate than titration-based methods. Visual estimation of reaction strength is too subjective for comparative purposes.

## Microorganisms considered in this literature review

This review includes relevant studies in countries other than the United States and United Kingdom. The discussion is arranged according to organism and considers the study designs and results.

A wide variety of pathogens may be transmitted by the water route (Hunter 1997*b*; Craun *et al.* 2002; Hrudy & Hrudy 2004). The report is divided according to selected pathogens, which are addressed in order of their importance, in terms of prevalence (endemic and/or epidemic frequency of occurrence), and resistance to amelioration of risk by conventional water treatment, and hence their importance to the estimation of risk. These are *Cryptosporidium, Giardia,* other parasites transmitted by water in North America; viruses, particularly *Norovirus* (previously Norwalk virus) and related species known as small round structured viruses (SRSVs); bacteria, particularly *Campylobacter jejuni*, salmonella and *Escherichia coli* 0157. Some other species are included for completeness although the significance of these for risk estimation is likely minimal.

## *CRYPTOSPORIDIUM*—REPORTS ON THE SEROLOGICAL RESPONSE AND SERO-EPIDEMIOLOGY

This parasite has been the stimulus for numerous serological and related studies, partly because of its clinical impact and the lack of effective therapy, particularly on the immunocompromised. In addition, it has major impact on public health agencies and the water industry as a common waterborne pathogen, especially given its resistance to chemical disinfection such as chlorination. In addition, its relatively recent emergence has coincided with developments in molecular science that have enabled detailed studies to elucidate the natural history and transmission dynamics of infection, both sporadic (endemic) and epidemic.

### Early worldwide studies

Early studies were aimed generally at (i) establishing pathogenicity by demonstrating sero-conversion (part of the Koch–Henle postulates); (ii) studying pathogenesis and understanding the role of immunity in recovery as part of the natural history of the infection; and (iii) finding more widespread evidence of infection including otherwise undetected cases in outbreaks (sero-epidemiology). Implicit in these study reports, however, is the need to report on the development of suitable methodologies, some of which predated the current wider availability of potentially more precise molecular methods. They should therefore be seen as developmental rather than definitive.

The first reported study was by Tzipori & Campbell (1981), who looked at prevalence of antibodies in ten animal species, including humans. Numbers of samples examined were small. The method employed indirect IFAT with cryostat sections from infected lamb intestinal tissue as antigen. The results suggested widespread reactivity for all species tested and in individuals of each species (80–100% positivity). The method has not generally found favour and is unsuitable for more general sero-epidemiology.

Campbell and Current demonstrated antibodies in normal and immuno-deficient persons from North America with confirmed infection, also using indirect IFAT with AHG to detect specific antibody, and with infected mouse gut tissue sections as antigen (Campbell & Current 1983; Current & Bick 1989). Test sera were serially diluted to establish titres and some subjects were sampled over a prolonged period (2–400 days). Sero-conversion was demonstrated and antibody was shown to persist in some cases for more than one year. Two subjects showed a late increase in titre that was thought to result from subsequent re-exposure with a booster effect but showed no sign of clinical illness, an important observation in the context of this report.

Koch *et al.* (1985) studied nosocomial transmission using indirect IFAT to examine sera from 26 subjects exposed to an acute case of severe cryptosporidiosis (a patient with AIDS) and 18 not so exposed. They found evidence of person-to-person transmission in hospital personnel. Thirty-one percent of the exposed had antibody, compared with 17% of the non-exposed.

Using ELISA with sonicated oocysts as antigen, Ungar *et al.* (1986) demonstrated antibody responses to *Cryptosporidium* in immunocompetent and immunocompromised persons from North America. Both groups of subjects showed an antibody response, generally IgM first, followed by IgG. The results of serum sets from subjects in an underdeveloped country (21% positive) suggested moderately widespread infection. This study used a panel of sera from "presumed negative" subjects as negative controls but recognised the difficulty of this with a caveat when interpreting results; the antigen used was a crude (poly-antigenic) extract. These are important factors in assessing this and some later studies by others. Ungar and Nash (1986) used immuno-blotting (SDS-PAGE) to purify selected antigens, followed by EITB (WB) read with laser densitometry to study responses with IgG and IgM; they also used ELISA. They found that sera from infected individuals reacted with a low molecular weight (23 kDa) and additional high-mw (125–175 kDa) antigens, of which they thought that the 23 kDa antigen would prove useful (see later studies described below). Of particular note is that they found discrepancies in results with the different methods, in particular that ELISA reacted more broadly. Some sera shown to be positive by ELISA failed to show a positive reaction to the 23 kDa antigen by EITB while nine persons who were ELISA-negative and without demonstrable *Cryptosporidium* oocysts in stools reacted to that

antigen by EITB. Some of the discrepancies were thought to depend on differing antigen preparation methods for the different techniques. The results showed that undiagnosed infection was prevalent. The sero-epidemiology of infection in randomly selected sera from two Latin American populations was studied by Ungar *et al.* (1988) using ELISA with sonicated, whole, calf-derived oocysts as antigen. Again, "presumed negative" sera were used as controls despite the fact that their study showed that antibody was common in the absence of overt cryptosporidiosis. They found 64% had detectable specific IgG; 32% were also positive for IgM. This increased in the two to three year age group. IgG, and less often IgM, was found to persist for over 12 months. Ungar *et al.* (1989) looked for serologic evidence of infection in US Peace Corp volunteers serving in Africa, again using sonicated oocysts in ELISA. Sera were collected over a period from six weeks up to two years (time series or kinetic study samples). Positivity was assessed by reference to OD readings of a panel of sera from assumed negative (stool negative) persons. Thirty-two percent were IgG-positive initially, and an additional 5–14% became positive during the study period. Persistence of IgG and/or IgM was noted; in some cases, IgM persisted without IgG. The use of a panel of assumed negative sera would appear to be invalid given the frequency of positive findings in those not apparently suffering from cryptosporidiosis. The results showed that undiagnosed infection was prevalent. The use of the "negative" controls used in these studies will tend to raise the threshold for recognizing positive sera and may therefore reduce reported prevalence.

Casemore *et al.* (1986) and Casemore (1987) used IFAT with Percol®-purified whole oocysts (animal- and human-derived) fixed on multi-well microscope slides that permit screening serial dilutions to determine titres. This technique permits visualisation of the antibody/antigen binding site(s), in this case associated with the oocyst wall. Sero-conversion was demonstrated in confirmed cases during an outbreak in North Wales. Some high titre single serum responses were detected in previously undiagnosed cases (found to have been symptomatic on further investigation). IgG was found more often in non-outbreak (control) population groups who lived in rural areas when compared with an urban control group. No attempt was made to exclude low titre responses as there appeared to be no valid evidence to assume, as some others have done, that low titres reflect cross-reactions or non-specific reactions. Some differences were noted in titre and intensity of reaction when sera were tested against oocyst isolates from different host species (calf, lamb, human). A selection of positive test sera was also checked for positivity against *Toxoplasma* and rheumatoid factor (known to cause false-positive reactions with IgM); all were negative. Patel *et al.* (1997) detected antibody bands to a variety of molecular weight antigens in convalescent sera from patients with recent cryptosporidiosis. An experimental SDS-PAGE MGF WB phenotypic typing system for oocysts and antibody detection was used to look at isolates and sera from patients associated with waterborne outbreaks in the UK and comparing these with samples from non-outbreak areas (McLaughlin *et al.* 1998). Antibody to low molecular weight antigens of 6, 14 and 17 kDa was found in 88% of convalescent phase sera from confirmed cases. Nine months later, sera collected for other purposes from subjects not known to have been affected showed antibody to these low mw Ags in 32–49% of residents in the same area, in 15–17% of residents in an adjacent area that received some of the affected water, and ~7% in control subjects not resident that area. It was subsequently shown (Patel *et al.* 1998; Harrison *et al.* 2002) that this outbreak was predominantly due to *C. parvum* genotype 1, now *C. hominis* (Morgan-Ryan *et al.* 2002) although approximately one third were *C. parvum* (then genotype 2); some mixed infections were found. There is little published evidence of how responses compare when sera are tested with heterologous and homologous species or strains. However, recent studies in the USA suggest that responses differ quantitatively when sera are tested against the infecting and heterologous isolates and species (*C. parvum, C. hominis*), and that variable cross-reactions occur to the different isolates (C. Chappell, personal communication; Priest *et al.* 2006). It is thus important to bear this in mind in interpreting serological studies used for case and/or outbreak investigations if the infecting species has not been identified. It has been shown that the epidemiology, including risk variables, for sporadic infections differs between *C. parvum* and *C. hominis* (Hunter *et al.* 2004). A variety of species, genotypes and host-adapted strains may be present in water, some of which are

of doubtful pathogenicity to humans but may stimulate an immune response (Chalmers *et al.* 2002*a*, *b*; Chalmers & Casemore 2004; Ong *et al.* 2002; Ward *et al.* 2002; Mathieu *et al.* 2004; Zhou *et al.* 2004).

In the USA, Mead *et al.* (1988) identified and defined specific antigens that were recognized by sera from both animals and humans using WB. They used an antigen extracted from sporozoites by SDS-PAGE. The number of antigens recognized by sera increased with time post-infection. The major antigenic determinant appeared to be 20 kDa. The response began to decline in intensity after about five months unless re-exposure occurred. Some of the sera reacted with high mw (96–200 kDa) antigens. Mead *et al*. thought that the 20 kDa Ag corresponded to the 23 kDa Ag of Ungar & Nash (1986), the difference being an artefact of the purification processes used.

A large waterborne outbreak in North America in 1987, at Carrollton, GA, was reported by Hayes *et al.* (1989). In addition to fecal sample examination, paired serum samples and other random single samples were examined for the presence of specific IgG and IgM by the method of Ungar *et al.* (1986). The results suggested that background seroprevalence was higher than shown in other North American studies.

García-Rodríguez *et al.* (1989) used indirect IFAT to detect specific IgG and IgM for a seroepidemiology study in different population groups in Spain. They detected IgG in 6.9% overall but the rate was highest in rural settings and in children (15.6%); Ig M was found in a few cases.

The immune response (IgG, IgA, and IgM) was studied in 15 stool-positive Filipino children by Laxer *et al.* (1990) using ELISA (modified from Ungar *et al.* (1986)), with purified oocysts as antigen; they also used IFA. In addition to sera, stool and duodenal fluids were also examined. Serum samples had a high (1/100) primary dilution that will likely reduce sensitivity but increase specificity. Total immunoglobulin levels and CMI markers were also measured. Antibody responses, including IgM, were shown to be marked quantitatively and qualitatively and maintained over time, which were thought to reflect the boosting effect of re-exposure. IgM (which is pentavalent) had the stronger binding capacity to surface antigens as determined by immune-electron microscopy. The relatively high screening dilution may have excluded many lower titre true positives.

A soluble (sonicated) oocyst antigen was used in ELISA by Gomez-Morales *et al.* (1992) to study sero-diagnosis (IgG, IgM) in Italian HIV-positive patients. Sera were diluted 1/50 for screening. Ninety-five percent of oocysts-positive cases were serologically positive (IgG and/or IgM) which persisted for up to a year in some; 5.3% of 300 presumed healthy subjects were also positive for IgG alone, suggesting that infection was common.

Lengerich *et al.* (1993) studied antibody in Wisconsin dairy farmers using an ELISA assay (Ungar *et al.* 1986). Results showed increased sero-prevalence compared with control subjects (44.3% cf 24%, RR = 1.9; 95% CI 1.1–3.2). No evidence was given of clinical episodes but in the experience of this author (DPC) over many years, no case of clinical infection has been seen locally in livestock farmers or adults living on farms, suggesting high levels of immunity (resistance) to clinical infection result from such exposure. A note of caution, however, is that the method used requires blocking non-specific binding with BSA, to which dairy farmers may have antibodies (Chart *et al.* 1998).

Cozon and his co-workers in France looked at the humoral and secretory antibody levels in HIV-AIDS patients and the relationship of antibodies to symptomatology using ELISA (Ungar *et al.* 1986) to detect specific IgG, IgA, and IgM in serum and secretory IgA in saliva (Cozon *et al.* 1994); CD4 + lymphocytes were also assayed. The persistence of symptoms in the presence of high titre antibody suggested that these are not sufficient to control infection, an observation of particular relevance to this report.

Newman *et al.* (1994) investigated transmission within 31 households in an urban community in Northeast Brazil using ELISA (modified from Ungar *et al.* (1986)). Tests for sensitivity and specificity were done; sera from "presumed low risk" (North American) children were used as "negative" controls (see the note above). Of 202 persons providing serum samples 191 (94.6%) had antibody (IgG and/or IgM). Interestingly, five (26%) young children developed cryptosporidiosis despite serologic (IgG + ) evidence of previous infection. As with Ungar *et al.* (1989), some showed persistence of IgM, sometimes in the absence of IgG, and the significance of this is discussed in this paper. Such persistence has also been noted previously (Casemore 1987; Ungar *et al.* 1989), and in some viral infections (Cox &

Medley 2003). A further report from Newman *et al.* (1999) described fecal studies on children in the same area, sampled over a four-year period, and confirmed evidence of possible recurrent or repeat symptomatic infection as well as asymptomatic cases. It was not possible however to be certain which of these recurrent stool positive cases resulted from persistent carriage, or re-infection with the same or different strains or species of *Cryptosporidium*; in either case, co-pathogens might have been responsible for some clinical episodes. An epidemiologic study of cryptosporidiosis in Peru found evidence that initial infection did not seem to protect against further clinical episodes in childhood although it was rare in adults (Bern *et al.* 2002).

Sero-epidemiology in children in Papua-New Guinea (PNG) was reported on by Groves *et al.* (1994), who used ELISA with purified, concentrated oocysts as antigen (Luft *et al.* 1987) to detect specific IgG and IgM. Test sera were diluted 1/100 (see comment re. Laxer *et al.* (1990)). Controls included known paired samples showing sero-conversion, a known negative, a high-titre positive and wells with all reagents except test serum, to allow construction of a standard reaction (OD) curve. A high level of reaction or sero-conversion was found in 24–38% of PNG children, compared with 8% of children and 5% of adults from Melbourne. These authors found IgG and IgM to develop within a few days, reaching a peak within 3–6 weeks, and returning to near baseline levels within 1–6 months. Poor or absent IgG response was found in some cases despite the presence of IgM, as found also by (Casemore 1987; Casemore *et al.* 1986). Re-exposure of one individual provoked an IgG response as reported by some others above (e.g. Ungar *et al.* 1986). There was an overlap in antibody levels in confirmed cases and some of the controls, underlining the problem of using sera from healthy persons as so-called negative controls.

Sero-prevalence in three communities in China, Brazil and Virginia, USA, were studied by Zu *et al.* (1994) using ELISA (Ungar *et al.* 1986). Positive rates were 42.3–57.5% in children in China, increasing with age after one year, and 50% in adults. In comparative populations studied, almost all children from a semi-urban population in Brazil became positive by their second year while the rate was 16.9% in a North American population from Virginia. It was thought that the difference between the communities in rates of acquisition of positive responses reflected differences in weaning age, hygiene practices, water quality and sanitation, family size, and the local environment, which is consistent with epidemiological findings (Casemore 1990; Casemore *et al.* 1997).

Studies by Kuhls *et al.* (1994) looked at seroprevalence from infancy to adolescence among 803 children of various ethnic groups in Oklahoma. They used ELISA (Ungar *et al.* 1986) with sonicated, calf-derived oocysts. Thirteen percent of those $<5$ years were seropositive; the rate was higher for those with a recent history of diarrhea and those attending day-care facilities, a known risk factor (Cordell & Addiss 1994). Thirty-eight percent of children 5–13 yr and 58% of adolescents (14–21 yr) were positive. Rates were higher for Black and Native Americans than white non-Hispanics. Interestingly, there was no significant difference between urban and more rural residents. In a later study but using similar methodology, Leach *et al.* (2000) looked at seroprevalence in children from various population groups along the Texas–Mexico border using ELISA to measure specific IgG and IgA. Overall, 70.2% were positive; prevalence was higher (82–89%) in the colonias and border communities compared with urban, non-border communities (46%). Risk factors identified included, in addition to young age and socio-economic status, the consumption of municipal water.

In São Paulo, Brazil, Braz *et al.* (1996) used IIF with purified oocysts to measure IgG and IgM. Oocysts were fixed on multi-well microscope slides and these were used to test two-fold test serum dilutions from 1/10; known positive and negative controls were used. Sixty-two percent of children found to be excreting oocysts were positive for both IgG and IgM. Children found negative for oocysts showed 20% positive for IgG and 40% for IgM, indicating likely infection not detected by fecal screening. In a group of HIV-positive adults with positive stools, 57% were positive for IgG and only 2% for IgM. The technique was viewed primarily as an adjunct to fecal study for diagnosis.

In Germany, Petry (1998) used a previously described ELISA test (Ungar *et al.* 1986), with mouse-derived freeze-thawed oocysts as antigen, to study the specific antibody status (IgG, IgM) of 495 persons of all age groups. Positive results were found in 15.4% despite a low incidence ($<2\%$) of fecal positives in diarrheal patients. Fifty percent of sera with high IgG (30 sera) were also positive for IgM,

suggesting recent infection. Sera were tested at 1/100 dilution and the authors suggest that the study may consequently underestimate true seroprevalence.

Miron *et al.* (2000) in Israel studied age-related seroprevalence in children by demonstrating acquisition of specific IgG and IgA by ELISA with sonicated oocysts as antigen, using previously described methods (Ungar *et al.* 1986; Kuhls *et al.* 1994). Serum from a confirmed case was used as a positive control; pooled sera from presumed negative children were used as a negative control. Test sera were diluted 1/100 (see previous comments). Sero-positivity (any Ig) varied from 50.9% to 95.6% with an overall prevalence of 65.6%. Like Casemore and colleagues (Casemore 1987; Casemore *et al.* 1986), they found that IgA was sometimes present in the absence of IgG, also reported by others with IgM. The study indicated high prevalence of the infection and that stool examination results underestimate this.

Steinberg and others studied seroprevalence of several waterborne infections in infants 6–36 months old in Guatemala (Steinberg *et al.* 2004). They used the second generation ELISA (see below, Priest *et al.* 2001) to measure *Cryptosporidium parvum*-specific IgG against the 27 kDa Ag. Antibody levels in 150 (28%) of the cohort samples increased rapidly from 27% at six months (presumably mainly maternal Ab) to 70% at 19–24 months, from when it remained at about that level. The authors expressed the view that serology was a useful tool for estimating prevalence and indicating need for intervention but felt that sero-incidence rates would also be useful.

Four studies by Frost and his co-workers examined sera from overseas locations. High levels of oocysts were reported to have been detected in drinking water in Sydney, Australia but without a concomitant rise in case numbers (Frost *et al.* 2000*d*). Sera were collected from blood donors (convenience samples) from Sydney and Melbourne, and these were assayed by MGF WB for IgG antibodies to 15/17 and 27 kDa Ags. Over half of sera in both groups had antibody with no statistically significant differences between the two groups. This gave further support to the view that the supposed oocyst detection and/or enumeration on Sydney drinking water was flawed. Frost and others looked for serological evidence of infection with *Cryptosporidium* in Italy using the same methodology (Frost *et al.* 2000*a*). The response rate in 100 Italian blood donors was higher (83% for the 15/17 kDa Ag; 62% for the 27 kDa Ag) than for four United States blood donor populations. The responses for the 15/17 kDa Ag were more pronounced (intensity of reaction has been used as a surrogate for titre). Responses were less pronounced than in sera collected six months after an outbreak in the US but higher than sera collected 2.5 years later. Increased reactivity was associated with increased age. Confirmed diagnosis of sporadic *Cryptosporidium* infection or of outbreaks is uncommon in the area but the results suggest that exposure is common. The findings suggest that the test may be of low diagnostic specificity for symptomatic infection but of high sensitivity for evidence of exposure. Egorov *et al.* (2003) examined sera collected in Russia, which were examined by the same methodology. Sixty-eight percent were positive for antibody to the 15/17 kDa Ag and 88% to the 27 kDa Ag. As with the previous study, increased age was associated with increased intensity of the reaction consistent with recurrent exposure. Swimming pool use was associated with increased positivity for both antigens while drinking water from shallow wells was associated with increase in the reaction to the 27 kDa Ag. Both of these practices are recognized risk factors for cryptosporidiosis (Casemore *et al.* 1997; Meinhardt *et al.* 1996). Blood donor sera collected from residents of two towns in New Zealand were studied by the same methods and risk exposures estimated by questionnaire responses (Duncanson *et al.* 2003). Over 60% of sera from both population groups were positive for antibody to one or both antigens and questionnaires gave positive associations for recognized risk factors. These four studies suggest that meaningful comparisons may be made between different population groups, and may help to identify or confirm likely risk factors.

Cox *et al.* (2005) studied seroprevalence of IgG to the immunodominant 27 kDa antigen in a Brazilian population using a recombinant form of the antigen, cp23 (see below, Priest *et al.* 1999), in ELISA and MGF WB (method of Frost *et al.* 1998*a*). In the ELISA test, a serially diluted high-titre human serum was included in each plate and given an arbitrary unitage. Positivity/negativity of test sera was determined by (i) normal frequency distribution of antibody concentrations (Cox *et al.* 1998*a*, *b*); and (ii) a cut-off point established at 10% of the positive control. Positivity for WB was by reference to the known positive control serum.

Sero-positivity was low in younger infants, increased to ~60% by five years and then 80% by age 10 years, after which the level remained constant. There was also evidence that antibody concentrations (titre) increased with age. The normal frequency method for determining positivity did not reveal an obvious cut-off value. There was a strong correlation between WB and ELISA ($r = 0.88$, $P < 0.005$) but sera with levels near the control cut-off threshold (10% of positive control) were more likely to be found positive when tested by ELISA. Overall, ELISA-positive levels were a higher percentage level (= titre) compared with the positive control. The study suggested that cp23Ag in ELISA may be the more sensitive method when detecting low levels of antibody.

## Discussion of early worldwide studies

These early, worldwide studies describe methodologies that were generally useful for further development and refinement. Some were aimed more at understanding the pathology of the infection and characterizing the immune response to it. In addition, however, some do provide useful comparative epidemiological data on epidemic (outbreak) and endemic (sporadic) infection dynamics and prevalence in a variety of settings. Most workers used ELISA, often based on the method described by Ungar and her colleagues (Ungar *et al.* 1986). Most used calf-derived oocysts, which were *Cryptosporidium parvum* (previously referred to as *C. parvum* genotype 2). However, some of the infections are likely to have been with *C. hominis* (previously *C. parvum* genotype 1) and possibly other species (Patel *et al.* 1998; Ong *et al.* 1999; Chalmers *et al.* 2002*a*, *b*; Xiao *et al.* 2004; Priest *et al.* 2006). Although these appear to share cross-reacting antigens, the precise difference in reactivity has not yet been clearly defined. The impact of these related species in inducing secondary or anamnestic responses is also unknown. Tests, such as ELISA, that may rely on crude antigen extracts are known to produce significant cross-reactions or "background noise" that makes interpretation difficult, especially of low titres. RF, BSA and other constituents such as reaction vessel plastics, may also interfere with the test (Venkatesan & Wakelin 1993; Chart *et al.* 1998). The studies by Ungar and Nash (1986) and Mead *et al.* (1988) used western blotting that enabled identification of important antigen molecules. This more specific approach has subsequently been greatly enhanced by the more extensive molecular studies reported from various workers described below. Répérant *et al.* (1994) found that antigens of mw 15–17 kDa and 21–23 kDa were major immunogenic molecules in different host species including humans, the larger of which seemed to correspond to the antigen identified by Ungar and Nash (1986).

## Recent North American studies

Several highly experienced research groups in North America have studied the immune response and seroprevalence. Some have further developed methodologies, particularly in the use of better-defined antigens, and significant insights have emerged with the use of these methodologies. Some of the reports described below predate these later developments and used previously established methodologies. They are included here to show the context of those developments.

DuPont and colleagues (DuPont *et al.* 1995; Chappell *et al.* 1996, 1999) studied experimental infection dynamics and clinical response in a group of healthy young adult volunteers. The antibody pre-exposure status and responses to infection of volunteers were assessed by ELISA adapted from the method of Ungar *et al.* (1986) using a crude oocyst extract antigen. Some discrepant findings were thought to reflect insensitivity of the ELISA and that low-level antibody in some of the pre-exposure samples may have been missed. This has been the subject of subsequent debate (Frost & Craun 1998; Okhuysen *et al.* 1998; Chappell *et al.* 2001; Muller *et al.* 2001; Frost *et al.* 2003*b*; Priest *et al.* 2003). These well-characterized infections were used to study further the immune response (Okhuysen *et al.* 1998; Moss *et al.* 1998*a*, *b*; Chappell *et al.* 1999). Thus, Okhuysen and co-workers reported on susceptibility, oocyst excretion and serologic response to attempted re-infection with *C. parvum* one year after the original infectivity studies (Okhuysen *et al.* 1998). Antibodies (IgG, IgA and IgM) at 0 and +45 days p.i. were detected using ELISA as previously described (DuPont *et al.* 1995). Rates for diarrhea in this group were about the same, but the diarrhea was generally less severe. IgM responses were also less marked than after primary infection; IgG and IgA seroconversions increased although the responses did

not appear to correlate with the presence or absence of infection. Some of the apparent discrepancies may have been the result of lower oocyst excretion, below the threshold of detection. On the other hand it probably also reflects the low sensitivity of the ELISA used; it would perhaps have been useful had the serum samples been re-examined by WB. The infectivity of *C. parvum* in healthy adult volunteers with pre-existing specific IgG was also assessed (Chappell *et al.* 1999). These authors used the ELISA previously described (DuPont *et al.* 1995). Positive and negative control sera were included but it was not specified how their status was assessed. The positivity of test sera was defined as those giving 1.5 times the OD of the negative controls, and post-challenge change estimated using that baseline value. The authors noted decreased symptoms in these study subjects. They further suggested that a significant proportion of the population might experience protection from low-level exposure although it was accepted that it was not possible to determine whether that was directly related to the antibody or to other immune mechanisms such as CMI. Chappell *et al.* (1999) estimated that a population with a seroprevalence of 25% might experience a corresponding percentage reduction in clinical cases compared with a sero-negative population. A sensitive and specific and carefully controlled antibody test system is clearly a prerequisite for studies on infection dynamics.

Some of these workers and others (Kjos *et al.* 2005) have subsequently described the use of a recombinant 41 kDa (rCP41) antigen associated in its native state with the oocyst wall of *C. parvum* but not *C. baileyi* (Jenkins *et al.* 1999). An ELISA test was used for the detection of IgG and IgM, with inclusion of positive and negative control sera. They compared a crude oocyst-derived antigen to test a panel of sera, with highly concordant results (88%, $P < 0.0001$) for IgG but less well (79%) for IgM, which was more readily detected using native antigen. The sensitivity and specificity were assessed as 94.8% and 77.6% for IgG compared with 48.4% and 93.1% for IgM for the two antigens. The poor concordance for IgM detection did not appear to be related to low-level positive samples. They suggest that this test might provide a reliable and cost-effective method for assessing previous exposure to *Cryptosporidium*.

Moss *et al.* (1998*a*) also used sera from the volunteer infectivity study (DuPont *et al.* 1995; Chappell *et al.* 1996) to measure the immune response to 15, 17, and 27 kDa antigens of *Cryptosporidium* using WB and purified oocyst extracts as previously described. Possible prior exposure in the volunteers was assessed by ELISA (DuPont *et al.* 1995), which has been the subject of some debate (see above). This study showed that antibody level increase was more marked in symptomatic than asymptomatic subjects. In contrast, volunteers with pre-existing IgG to the 27 kDa Ag excreted fewer oocysts. Asymptomatic infected subjects showed higher initial (day 0) reactivity in IgG to the 17 kDa Ag and for IgM against the 27 kDa Ag. These results suggest that those with pre-existing antibody at the time of exposure may fail to show a change in antibody levels and to be less likely to develop symptomatic infection even though infected. These are critical observations in terms of this report. Some of these antigens, especially in the ~15–17 kDa and ~20–23 and 27 kDa ranges referred to separately by some authors, represent interrelated groups or families sharing common protein antigens with variable sugar and lipid moieties and with differing cleavage patterns (Mead *et al.* 1988; Priest *et al.* 1999, 2001; Riggs 2002). The response to the 27 kDa can be detected using a CP23 recombinant antigen (Priest *et al.* 2001; Smith *et al.* 2001). These antigens are associated with the sporozoite surface and probably merozoites, and may thus have potential involvement in any protective effect.

Antibodies have also been detected in fecal fluid, including from experimentally infected volunteers (Kapel *et al.* 1993; Dann *et al.* 2000). It was suggested that this might be more sensitive than detection of humoral antibody but this has been disputed (Muller *et al.* 2001).

Moss *et al.* (1994, 1998*a*) reported on studies related to the massive outbreak in Milwaukee in 1993 (Roy *et al.* 2006), in particular, the serological response of cases that occurred among the crew of a US Coast Guard cutter that had filled its water tanks from the contaminated supply. They used ELISA and EITB (WB) with proteins extracted from calf-derived oocysts that had been sonicated, freeze-thawed to disrupt them, followed by clarification, and then purified by SDS-PAGE. The method distinguished reactions with 15 kDa, 17 kDa and 27 kDa antigens; IgA was measured against the 17 kDa Ag, IgM to the 27 kDa

Ag and IgG to all three. Changes in intensity of reaction in paired serum samples measured by EITB for these antigens were useful as diagnostic markers when compared with stool results and the clinical picture. Of the 10 confirmed cases, only four showed IgG responses. Results for ELISA did not always identify confirmed cases and did not correlate well with EITB results. EITB gave significantly better correlation with risk (quantity of water consumed) than ELISA, and EITB was considered potentially useful as an epidemiological tool. McDonald and colleagues (McDonald *et al.* 2001) studied the specific antibody response among children residing in Milwaukee during the outbreak there. They used ELISA to study the response to the 17 and 27 kDa antigens (Priest *et al.* 1999). The former was partially purified (Triton X-114 treated) native oocyst-derived antigen while the latter was a recombinant antigen. Controls included WB-confirmed negative samples that were used to calculate OD values indicating a positive result. Hence, the cut-off is more likely accurate than assumed negatives. Sensitivity and specificity were estimated to be >90% relative to WB. The positivity rates increased from 15% to 82% and 17% to 87% for each antibody respectively over a five-week period. Increases noted from children in adjacent areas were smaller but nonetheless suggested more widespread infection than had been thought, as has been noted by others (Pollok *et al.* 1998).

Priest and his colleagues (Priest *et al.* 1999) used two antigens, of mw 17 kDa and 27 kDa (McDonald *et al.* 2001), in an ELISA to detect IgG specific to those antigens. Positive responses to the antigens in the ELISA were re-examined by WB, which showed sensitivity and specificity of ≥90%. These new ELISAs were more sensitive and specific than those using crude oocyst antigens. The IgG levels in sera collected during outbreaks were 2.5-fold higher than non-outbreak sera. This improved test protocol should therefore be of value in epidemiologic studies. The confirmation of the lower sensitivity of the older test format may confirm that this is a source of discrepant findings in some of the earlier studies, including the volunteer infectivity studies. These authors make the point that an ELISA is needed that gives the sensitivity and specificity of LF WB.

Priest *et al.* (2001) extended the above studies to assay IgG in time-series (longitudinal) serum samples from patients with confirmed cryptosporidiosis. These sera were collected from cases in outbreaks in British Columbia, Canada (see below). The two groups in Atlanta and Vancouver compared results and determined inter- and intra-laboratory consensus on results for comparison of the methods, critical for determining robustness. They compared the two ELISAs previously described with large format (LF) and minigel format (MGF) WB. The LF WB is said to be both sensitive and specific but is generally too cumbersome and expensive for large-scale screening; the MGF is simpler and uses ten-fold less antigen and is therefore cheaper. The MGF compared well with the LF WB for the 17 kDa Ag but failed to detect nearly a third of samples positive for the 27 kDa Ag. Other studies have noted that one third to a half of sera positive by LF WB may have a response only to the larger mw antigen (Frost *et al.* 1998*a*; Priest *et al.* 1999). In addition, MGF is less able to resolve antigens close in size although this may be of little significance in comparing cohort surveys where the same method has been used. The authors conclude, however, that the MGF WB in its current form may significantly underestimate seroprevalence. The ELISAs were generally found to be both sensitive and specific and to provide a convenient test format for large-scale screening. Some discrepancies were found between ELISA and WB. The results for the smaller mw native (Triton-extracted) antigen were somewhat lower than in WB with sequential sera, especially for later specimens (>92 days) but many of these subsequently became negative by WB. Thus, they appeared to result primarily from lower sensitivity with borderline positive samples. The authors feel that the ELISA test using the 17 kDa is still of value for detecting recent infection, while the 27 kDa (Cp23) antigen test is a valuable tool for assessing past exposure. It should be noted that using multiple test formats always results in some discrepant results, as is often also the case for the same test protocol used in different laboratories. ELISA is prone to a variety of inherent errors and great care is required in performance and the use of the necessary quality controls (Venkatesan & Wakelin 1993). The ELISA format described by these authors addresses these problems. In particular, the authors believe that the ELISA tests minimize the effect of cross-reactions from other organisms, many of which are derived from non-protein epitopes. Carbohydrate moieties,

alone or in combination with lipids or proteins, are likely to be important determinants in the immune response to *Cryptosporidium* although they may have a broader reactivity than protein epitopes alone (Luft *et al.* 1987). Antibody to both antigens appeared to have half-lives of about twelve weeks and reach a constant baseline by about one year.

In Canada, Isaac-Renton *et al.* (1999, 2003) and Ong *et al.* (2005) reported on sero-prevalence in British Columbia, and Ong *et al.* (1999) reported on the molecular epidemiology of outbreak isolates. A comparison was made of prevalence of antibodies to *Cryptosporidium* and *Giardia* in three communities with different types of water supply (Isaac-Renton *et al.* 1999). In the first study, *Cryptosporidium*-specific IgG in 1944 sera were tested by MGF WB to detect reactions to the 15/17 kDa and/or 27 kDa antigens. The overall rate for *Cryptosporidium*-specific antibody was 50.5%; rates varied by season and between communities/water supply types. In one of the communities that experienced an outbreak of cryptosporidiosis, reaction to both 15/17 kDa and 27 kDa antigens appeared to be the best marker for recent infection. Subsequent re-examination of these sera by the second generation ELISA test (Priest *et al.* 2001) used in the following study showed that the miniblot significantly under-estimated positive sera (82.6% cf 50.5%). In the later study, Isaac-Renton *et al.* (2003) and Ong *et al.* (2005) used ELISA to study the IgG Ab response to the 27 kDa recombinant antigen with arbitrary units to determine cut-off for ascribing positivity. Sera from more than 4000 pregnant women in six communities in British Columbia were collected over a 24-month period. Seroprevalence was high (85% overall, range 77–92%) in all of the districts, but with significant differences between communities. Two of the communities had waterborne outbreaks during the study period and showed sharp rises in positive rates that declined over the following 2–3 months. Significantly, in the context of this report, one of these outbreaks was confirmed to be due to *C. hominis* and the other to *C. parvum*. This test has been shown to be more sensitive and specific than ELISA using the 17 kDa native Ag and is useful in detecting past infection (Priest *et al.* 1999, 2001; McDonald *et al.* 2001). The ELISA results remained significantly better than miniblot even if the arbitrary cut-off was increased by 50%. The authors expressed the view that the test was more useful for the type of epidemiologic study described here; it also appeared to be significantly better than oocyst detection rates for estimating the size of outbreaks. It is well known that detection of oocysts in feces, as with *Giardia* cysts, is very inefficient (Frost & Craun 1998b). They point out, however, that a proportion of the antibody responders may be asymptomatic; the significance of such infections for potential secondary transmission is unknown. In the experience of the author of this report, such cases excrete only small numbers of oocysts at the limit of detection and probably do not represent a significant risk of transmission in most case where domestic and personal hygiene standards are reasonable (Casemore 1989). The studies were subjected to rigorous control and statistical analysis, which showed that the modified ELISA transferred well between laboratories and was robust. There was no evidence of cross-reactivity with *Toxoplasma* and *Giardia;* some studies have suggested a degree of cross-reactivity between *Cryptosporidium* and *Eimeria* spp (Ortega-Mora *et al.* 1992) but this is probably of little significance in human subjects. This is the most comprehensive and robust study to date and confirms the value of the second generation (CDC) ELISA for mass population (seroprevalence) studies. However, discrepant results have been reported using this ELISA, which is a subject of debate (Frost *et al.* 2003*b*).

Several groups have used these more advanced methods to study HIV-infected subjects. Caputo and colleagues looked at the antigenic determinants of antibody to *Cryptosporidium* among gay and bisexual men in Melbourne, Australia, with HIV infection, using MGF WB with 15/17 and 27 kDa Ags (Caputo *et al.* 1999). Details are given of the way in which the test was calibrated. A positive test indicative of recent infection was indicated when the test serum result was $>35\%$ of the positive control. The 27 kDa Ag response gave the most reliable indication of exposure to risk factors over a two-year period. The same group followed up subjects after one year and found 34% to have seroconverted; seroconversion correlated with patterns of sexual activity (Friedman *et al.* 2001). Spencer *et al.* (1997) studied seroprevalence and the prevalence of cryptosporidiosis in HIV-infected persons in New York. They used the ELISA method described by Ungar *et al.* (1986) to measure specific IgG and stool examination for evidence of previously undetected current infection (patients with previously diagnosed *Cryptosporidium* infection were excluded from the study). Seroprevalence was 20.3%; evidence by fecal

oocyst detection of newly recognized current infection was found in 3.6% but the authors note that this is likely an underestimate given the poor sensitivity of oocyst detection methods. Eisenberg, together with Priest and colleagues (Eisenberg *et al.* 2001), used the new ELISA (Priest *et al.* 1999, 2001) for detecting the serologic response in 11 HIV-infected persons with confirmed cryptosporidiosis. They found that the test was reliably predictive of infection and could provide an effective epidemiologic tool to monitor *Cryptosporidium* infection in immunocompromised patients. Frost *et al.* (2005*a*, *b*) looked for evidence of protective immunity associated with a strong serologic response among HIV-infected individuals. Such an association was demonstrated between the 27 kDa antigen and a reduced risk of diarrhea in those not exhibiting weight loss; the association was not present in those with diarrhea plus weight loss. The authors suggest that this demonstrated protective immunity against the effects of cryptosporidiosis although it is not evidence that the antibody itself is protective. Their data suggest that the test can be an effective tool to monitor *Cryptosporidium* infection in immunocompromised patients. These studies suggest that HIV-infected subjects may well respond serologically in much the same way as those with intact immune function, a factor that may well be significant for sero-epidemiologic surveys and risk evaluation. However, the severity and persistence of the infection underlines the critical nature of the CMI response in immunity to *Cryptosporidium*.

Frost and colleagues conducted a number of studies of seroprevalence in North America. A comparative study (Frost *et al.* 1998*b*) estimated seroprevalence to what are described as three antigens (15, 17 and 27 kDa) by both ELISA (Ungar & Nash 1986), and by LF WB (Moss *et al.* 1994). The WB tested for IgG and IgA, while the ELISA tested for combined IgG, IgA and IgM (AHG). Antigen was SDS-PAGE-purified, sonicated oocyst extract. Sera were from subjects without a known history of cryptosporidiosis, from Talent, in Jackson County, Oregon, four to six months after a waterborne outbreak there. Pooled known negative and positive sera were used as controls in the ELISA; single known positive and negative sera were used as controls in the WB. ELISA tests were read by plate reader and WB strips were scanned and assessed for positivity electronically by reference to the control reactions, thus decreasing subjectivity. Positives in the ELISA were those that were $\geq$25% of the positive control, and $\geq$35% for WB. The rationale for these limits and other statistical aspects are discussed. The authors report that tests showed poor separation of the 15 kDa antigen group from the 17 kDa antigen group. However, these are now believed to represent the same antigen group. Various potential risk factors were included in the questionnaires used. The numbers of subjects with the main risk exposure histories were small. Both tests detected an almost two-fold increase in positives for those with a history of consumption of Talent water but the differences were statistically significant only for WB. The outbreak investigation was particularly significant in the context of this report in that it appeared to demonstrate protection (negligible attack rate or undetected asymptomatic infection) in the population who had been regularly exposed to surface water-derived supply compared with a nearby population who had not previously been so exposed but who were temporarily supplied from the suspect source. In a further study, Frost *et al.* (1998*b*) reported on a two-year follow-up to the same outbreak using paired sera collected at six months and 2.5 years later. The sera were examined by MGF WB to the 15/17 and 27 kDa Ags. Intensity of the former remained largely unchanged, while those to the latter declined to 54% over the follow-up period. Reaction intensity was used as a measure thought to approximate to titre. Increased levels were noted in Talent residents suggesting high prevalence of endemic infection and/or re-infection. The inability of MGF WB to resolve some antigens in the same size range as key antigens may be of little significance in this context where the change over time for the same group is being measured. The 27 kDa Ag response is known to persist somewhat longer than that for the smaller mw antigens but the MGF WB may be less sensitive with this antigen.

The possible effect of enhanced water treatment on seroprevalence was investigated by Frost *et al.* (2000*b*) in an unnamed city in the northeastern USA. The water, which was surface source-derived, had been chlorinated only, but filtration was introduced in spring, 1997. The assay method was MGF WB as used previously by these authors. Serum samples were collected from college student volunteers, one month prior to (107 samples) and five months after (225 samples) the introduction of filtration; most volunteers

were female. Nineteen percent of the first sera showed reaction to the 15/17 kDa Ag, changing to 24% of the second sera. For the 27 kDa Ag the figures were 27% and 41% respectively; the increase in reactions with the larger mw antigen was significant ($P = 0.02$) and may have been an underestimate given the decreased sensitivity of this test for reactions with this antigen. The 15/17 kDaAg response tends to last for up to six months; the 27 kDaAg reaction tends to persist for more than 12 months. The results suggested an increased rate of exposure over the study period. Statistical analysis of risk factors suggested that swimming and consumption of untreated surface water were predictive for increased intensity of response. The study period covered the summer months when these exposures might be expected to increase. It would have been helpful had the second serum samples been collected one year after the first for the following reasons:

(i) to avoid this period of confounding risk exposures;
(ii) to allow for the expected decline in antibody levels resulting from infection prior to the change in water treatment;
(iii) for the seasonal exposures to be approximately the same, including such factors as the impact of spring period conditions on the source water. Seasonal changes in incidence and risk factors are well recognized (Casemore *et al.* 1997). It is not possible to assess such factors in the study area, other than the likely increased exposure to surface waters during summer, a recognized risk activity for *Cryptosporidium* infection.

Frost and colleagues also conducted a serological study on 89 convenience sera collected from anonymous persons in Collingwood, Ontario, during and following an outbreak there in 1996; 80 sera were also obtained from persons in Toronto for comparison (Frost *et al.* 2000*c*). Sera were assayed using the same methodology as before. The occurrence of an outbreak had been questioned because of the differing age distribution of cases between affected residents and visitors. A higher proportion of sera from Collingwood had a detectable response and the mean intensity was higher than in the Toronto sera. The mean intensity for Collingwood sera was highest in sera collected during the eight-week period following the initial case reports. Cases were mainly in visitors, in elderly nursing home residents of Collingwood and in local children but not in other local adults. This is consistent with the findings in a waterborne outbreak in the south-west of England, in which the faulty water supply had been associated with a previous outbreak, almost certainly resulting in increased resistance to symptomatic infection in residents other than young children and those only recently resident, including retirees from other areas (Harrison *et al.* 2002).

Frost (1998) reported on a study of blood donor sera from two cities in the northwest of the USA, one with a surface-derived water supply and the other groundwater. The sera were tested for IgG to the 15/17 kDa and 27 kDa Ags in a MGF WB test format. Sera from the former group had significantly more positive reacting sera (21–31%) compared with the latter (11–23%). Frost *et al.* (2001) examined 200 convenience (blood donor) sera from each of two cities in southwest USA, which have contrasting water sources expected to lead to differing risk indexes. The sera were tested as in the previous report. There was an increased positive rate and higher levels of reactivity among the population with a surface-derived water supply than among the other population group who had a groundwater-derived supply. Surprisingly, the higher risk group did not show decreased antibody reactions if they consumed bottled water and this requires further study to explore possible confounding risk factors. The epidemiologic differences between the populations represented by the sample donors, including differing rates of reported infection, are discussed.

Frost *et al.* (2002*a*) compared seroprevalence in convenience samples from two study groups from two midwestern US cities; two sets of serum samples were collected nine months apart. One of the cities was supplied with drinking water from surface water and the other from groundwater sources. Sera were examined for specific IgG to the 15/17 kDa and 27 kDa Ags, by MGF WB (Okhuysen *et al.* 1998; Frost *et al.* 2000*c*) for evidence of differing exposure rates. Initial samples showed higher rates of antibody (54% *vs.* 38%, RP = 1.39, CI 1.21–1.60) in those from the surface water supply city. Follow-up sera from those with no baseline response to the 15/17 kDa Ag (indicative of recent infection) showed an increased frequency of seroconversions (33% surface water cf 11% groundwater at 20% of positive control

cut-off). Those with an initial reaction showed increased intensity of responses to both antigens. Although it is not possible to exclude all possible confounding risk exposures, the results strongly suggest that a surface water-derived supply leads to a significantly increased rate of exposure, as might be expected. This may not be expressed clinically and the authors reasonably argue that mild, self-limited or subclinical infection is a more likely outcome of exposure in this setting.

A further study by Frost *et al.* (2003*c*) examined differences in serological responses to *Cryptosporidium* antigens among residents of areas with surface and groundwater sources. Nearly 500 recruits (urban adult blood donors and college students) provided convenience samples, of which 270 also supplied follow-up samples (90–180 days post-initial sample) for kinetic studies. Sera were analysed by MGF WB as previously described by these authors to detect responses to the 15/17 and 27 kDa Ags. Intensity of the reaction was estimated photometrically using pixel density to give a proxy for titre. Participants were questioned about a variety of potential exposures. Participants from the surface water area had a higher seroprevalence to the 15/17 kDa Ag (72.3% vs. 52.4%, $P < 0.02$) and to the 27 kDa Ag (82.6% vs. 72.5%, $P < 0.02$). Shorter residence in the higher risk area or consumption of bottled water was associated with lower seropositivity rates. Seroconversion between paired samples to the 15/17 kDa Ag was more common in the surface water area. Use of private wells was also associated with higher seroprevalence to the 15/17 kDa Ag. It is notable that even in the groundwater areas seroprevalence rates were high (>50%) but it is not possible, as with most of the studies described, to draw any conclusions about the likely source of those infections or how often this involved acute symptomatic infection.

Sera from 1356 NHANES III participants were analyzed by Frost *et al.* (2004) for specific IgG. Samples were drawn from seven areas with varying population characteristics, water supplies, etc. They used MGF WB with the antigens and methods previously described by this group. Intensity of reaction was compared by geography, age, sex, race/ethnicity, income, and hepatitis A virus seropositivity. Results showed that Hispanics, blacks and females had higher seropositivity; the first two of these may be proxies for socio-economic factors, the latter to increase exposure to infants and children. Significant differences were seen with different geographic areas that may reflect differing water sources, including catchment control. Increasing positivity was also noted with increasing age ($\sim$ 70% by age 70). The authors suggest that this finding is likely a true reflection of recurrent exposure to *Cryptosporidium* rather than the result of anamnestic boosting from cross-reacting antigens from other species. They suggest that this is more likely true with WB than with ELISA although this is less likely true with the second generation ELISA. While specificity has been less reliable with ELISA using a crude oocyst extract as antigen, the much-improved second generation ELISA appears to be more dependable.

Frost *et al.* (2005*a*) conducted a study designed to estimate the importance of protective immunity as an indicator of decreased risk for acquiring cryptosporidiosis. Serum samples and enteric illness records were obtained over a two-year period from 326 people. These were supplied with drinking water from either unfiltered surface sources or a groundwater source; filtration was initiated at one of the groundwater sources during the study period. Analytical methods used were as previously described. Subjects with strong responses to the 15/17 kDa Ag (acute phase reaction) had <65% of the risk of 1–3-day episodes of gastrointestinal illness and <40% of the risk of ≥4-day episodes compared with those who had a moderate response. Water source, treatment intervention, and very weak responses were unrelated to illness events. The authors conclude that endemic *Cryptosporidium* infections are a common cause of gastrointestinal illness in those with a moderately strong response to the 15/17 kDa Ag. Further, they conclude that users of surface-derived drinking water are more likely to have such antibody and will thus be at lower risk of more severe illness. While this is the finding of a number of studies, the use of serologic findings as an indicator of immunity (i.e. resistance) is a matter of debate. However, it is consistent with epidemiologic findings as a marker for resistance. The difference in responses associated with the different supplies during the two stages of the study and in relation to water treatment change, is unclear. While acute, primary cryptosporidiosis tends to be associated with more extended illness, i.e. ≥ 4 days (Casemore 1989), it is unknown what the cause of illness

was in these subjects. In addition, risk level estimate has to be predicated on infection (asymptomatic as well as symptomatic) and not just clinical illness. In a further study by Frost and colleagues (2005*b*), serological responses in convenience samples from women in Hungary were analysed according to water source, using MF WB. Results from those consuming riverbank-filtered water indicated protection from infection by this method of natural filtration. It should be noted, however, that some outbreaks in the UK have been attributed to use of bank-side abstraction (Harrison *et al.* 2002) or migration through a river-associated aquifer possibly due to drought induced fissures (Willocks *et al.* 1998). The distance between surface water and the well, and the nature of the subsoil or aquifer are thus critical.

## Discussion of *Cryptosporidium* sero-epidemiology

Some of the studies described above were generally designed to investigate infectivity and/or pathogenesis but also yield useful epidemiologic information incidentally. Others were designed specifically to generate epidemiologic data (see Table 1). In both cases, many of the methods used are inherently developmental. The differing test modalities described, the antigens used, the choice of controls, their differing levels of development and optimization make it fundamentally important not to compare too closely the absolute values or levels of antibodies detected. A given test system, if operated in the same way in different times or places, may be used to compare trends for positivity but may not be used reliably to compare quantitative values (titre or strength of reaction, or the precise proportion of the test population found to be positive). Where different modalities are used, or similar tests are optimized differently then comparison, especially quantitative, is problematic. Comparison of trends and epidemiological interpretations may however be possible. The benefits and drawbacks of each method need to be recognized, reflecting the continuously developing nature of the test systems and antigens used. In a useful review, Frost *et al.* (2003*a*) have collated information comparing antibody responses according to water sources. This showed that, despite the geographically variable results, surface water-derived supplies tend to be associated with raised seroprevalence and hence increased rates of infection but not necessarily clinical disease. Prior immunity may result in misclassification of infected but asymptomatic persons as uninfected (Casemore 1995; Hunter 1997*a*, 2000; Craun *et al.* 2001). When confirmed cases and other symptomatic persons (who may have been true but unconfirmed cases) have been excluded from potential control groups then the remaining cohort will likely contain an increased proportion of subjects who though exposed have not developed symptoms because of pre-existing immunity. Thus, such misclassified cases introduce bias in case-control studies that will tend to reduce the statistical association with water because such immune subjects may have consumed water from the same supply as cases.

Water may contain a variety of different *Cryptosporidium* species and sub-species or strains (Ong *et al.* 1999; Chalmers *et al.* 2002*a*, *b*; Ward *et al.* 2002; Xiao & Ryan 2004; Zhou *et al.* 2004) that may or may not be pathogenic for humans or may be of low virulence or low viability resulting from environmental attrition, including water treatment processes. By definition, outbreaks must depend upon the presence of more pathogenic species or types. However, less pathogenic isolates may be capable of initiating an antibody response or boosting pre-existing antibody and may be responsible for confusing serological findings. Casemore & Jackson (1984) first noted that there appeared to be an urban (non-zoonotic) cycle of infection that was subsequently borne out by the identification of *C. hominis* (*C. parvum* Genotype 2). Hunter *et al.* (2004) noted that risk factors for acquiring infection with *C. parvum* and *C. hominis* differ in addition to the role of animals. However, serology is unable currently to distinguish the infecting species or sub-type. It has been noted in a number of epidemiologic studies that a history of regular consumption of raw vegetables is negatively associated with cryptosporidiosis, a so-called protective effect. However, it is not known if this is the result of such low-level exposure resulting in immunity, other non-specific effects, or confounding (Casemore *et al.* 1997; Hunter *et al.* 2004; Roy *et al.* 2004).

Large format WB is generally highly specific and sensitive; however, the test is expensive, technically demanding, labour-intensive and is less useful in quantitation of responses, and is not suitable for mass screening

**Table 1** | *Cryptosporidium* seroprevalence levels in US studies 1989–2005

| Site | Population | Age range | Water supply | Method(s) | *n* | Antibody frequency | Comments | Reference |
|---|---|---|---|---|---|---|---|---|
| Carrollton GA | (i) Residents | Adult | (i) TSW | Ungar ELISA | (i) 86 | (i)Ill/well 76%/56% | Outbreak investigation | Hayes *et al.* (1989) |
| | (ii) Unexposed controls | | (ii) NS | | (ii) 20 | (ii) 35% | | |
| Wisconsin | (i) Dairy farmers | ≥50 yr | NS | Ungar ELISA | (i) 70 | (i) 44.3% | Suggests increased infection rate without illness in farmers. | Lengerich *et al.* (1993) |
| | (ii) Non-farmers | | | | (ii) 50 | (ii) 24.0% | | |
| Oklahoma | Hospital patients Convenience samples | <5 yr – 21 yr | NS | Ungar ELISA | 803 | 13–58% | Rate increased with age. | Kuhls *et al.* (1994) |
| Virginia | Hospital patients Convenience samples | <1–29 | NS | Ungar ELISA | 172 | 16.90% | Rate increased with age; <1 yr negative. | Zu *et al.* (1994) |
| New York | HIV clinic patients | Adult | NS | Ungar ELISA | 137 | 20.30% | 3.6% stool positive | Spenser *et al.* (1997) |
| Jackson County Oregon | Blood donors | Adult | TSW | (i) Ungar ELISA | 380 | (i) 15.5% | 6 mo. post-outbreak | Frost *et al.* (1998*a*) |
| | Convenience samples | | | (ii) MGF WB | | (ii)21.8–47.8% | | |
| Two cities NW USA | Blood donors | Adult | (i) TSW | MGF WB | 500 per site | (i) 21–31% | | Frost *et al.* (1998) |
| | Convenience samples | | (ii) GW | | | (ii) 11.23% | | |
| NE USA | College students | Young adult | TSW | MGF WB | (i) 107 | 15–17/27 kDa | (i) Chlorinated only | Frost *et al.* (2000*c*) |
| | | | (i) pre- | | (ii) 225 | (i) 19%/27% | (ii) Plus filtration confounders – see text | |
| | | | (ii) post-Filtration | | | (ii) 24%/41% | | |
| Milwaukee | Convenience samples | Children 6 mo–12 yr | TSW | Second generation ELISA | (i) 218 | 15–17/27 kDa | Evidence of increased infection rate in adjacent area | McDonald *et al.* (2001) |

**Table 1** | (*continued*)

| Site | Population | Age range | Water supply | Method(s) | *n* | Antibody frequency | Comments | Reference |
|---|---|---|---|---|---|---|---|---|
| | (i) outbreak area<br>(ii) adjacent area | | | | (ii) 335 | (i) 15–82%/ 17–87%<br>(ii)10–43%/ 22–46% | | |
| SW USA - | Blood donors | Adult | (i) TSW | MGF WB | 200 per site | 15–17/27 kDa | TSW group showed increased intensity of reactions | Frost *et al.* (2001) |
| (i)Las Vegas | Convenience samples | | (ii) GW | | | (i) 49%/55% | | |
| (ii)Albuquerque | | | | | | (ii) 36%/52% | | |
| Two cities | Blood donors | Adult | (i) TSW | MGF WB | (i) 462 | (i) 54% | Further detailed analysis of repeat samples and according to mw of Ag in paper. | Frost *et al.* (2002*a*) |
| Mid-West USA | Convenience samples | | (ii) GW | | (ii) 503 | (ii) 38% | Some evidence of lower illness rates in TSW area | |
| Four cities in Iowa and Wisconsin | Blood donors & college students | Adult | (i) TSW | MGF WB | (i) 184 | 15–17/27 kDa | Greater frequency of increased intensity of response in repeat samples in group (i). | Frost *et al.* (2003*a*, *c*) |
| | Convenience samples | | (ii) GW | | (ii) 309 | (i) 72.3%/82.6% | Higher prevalence in sub-populations users of private wells | |
| | | | | | | (ii) 52.4%/72.5% | | |
| USA – 7 sites | NHANES III | 11 yr– >71 yr | Various – see paper cited | MGF WB | 1356 | (i) 15–17 kDa | Positive trend with age. | Frost *et al.* (2004) |
| | Participants | | | | | 13.4–67.9% | Geographically and socio-economically variable responses. | |
| | | | | | | (ii) 27 kDa 21.3–81.5% | | |

**Table 1** | *(continued)*

| Site | Population | Age range | Water supply | Method(s) | *n* | Antibody frequency | Comments | Reference |
|---|---|---|---|---|---|---|---|---|
| 3 sites in NW USA | 3 cohorts divided by water type and two phases | Various | Cohorts A,B: TSW | MGF WB | 522 from 326 persons | All sera | Enteric illness records analysed in relation to water supply suggested surface water use protected against illness. | Frost *et al.* (2005*a*, *b*) |
| | | | Cohort C: GW | | | (i) 15–17 kDa 52% | | |
| | | | | | | (ii) 27 kDa 73% | | |

Abbreviations used: NS – not stated; TSW – treated surface water (see reference cited for details); GW – groundwater; Ungar ELISA – see Ungar *et al.* (1986); MGF WB – minigel format western blot; for details of 15–17/27 kDa see papers cited.

exercises. Minigel WB is easier to use but suffers from some lack of discrimination between antigens of similar mw and relatively poor sensitivity for some antigens that may in part be due to the small amount of antigen used. Nonetheless, trends in rates can be compared where that method has been used. It has yielded very useful epidemiological information and increased understanding of outbreak dynamics, including the impact on risk evaluation of previous exposure and immunity. ELISA often has problems with specificity, background "noise", and robustness, but is relatively cheap, can be automated, readily permits accurate, objective quantitation by titration, and is easy to use for mass screening provided the problem of specificity is adequately addressed. However, it may lack some sensitivity to the 15–17 kDa Ag and produces some false-positive reactions (Frost *et al.* 2003*b*) and the significance of this needs to be assessed. Its more widespread use depends upon the availability and cost of the defined antigens. In both systems, QA, QC and validation for sensitivity (maximum detection of true positives) and specificity (minimum of false positives) are essential. Maximizing sensitivity may lead to inclusion of false positives (cross-reactions); maximizing specificity may result in the loss of weak true positives, potentially overlapping with false positive reactions which usually give weak reactions. Evaluation requires a "gold standard", which is usually LF WB using time series samples from confirmed cases with known dates of onset. True negative samples are often difficult to identity for common infections except by testing by the consensus most sensitive method such as LF WB. Establishing a "negative" cut-off for some tests such as ELISA is problematic except by consensus based on experience with the test but this inevitably runs the risk of excluding low-titre true positives. Simply assuming that convenience samples from presumed asymptomatic individuals are negative is unacceptable practice. Methods to be used for epidemiological studies need to be readily transferable between laboratories while maintaining QA/QC performance.

A note of caution: the studies above uniformly used *C parvum* (previously *C. parvum* genotype 2) as antigen source, often the Iowa strain. It is still unclear how the results are affected where *C. hominis* (previously *C. parvum* genotype 1) or other species have been the etiologic agent.

In some cases, the two species and others were likely present in the water and/or in infections in humans (Patel *et al.* 1998; Tanriverdi *et al.* 2003; Mathieu *et al.* 2004; Priest *et al.* 2006). It is unclear how important the anamnestic (booster) response is (including from other species or genera with cross-reacting antigens, although some studies have looked at a limited range of related species) in producing high titre responses that might be interpreted as indicating recent infection, especially where subjects are exposed to concurrent or episodic multiple waterborne pathogens. Indeed, it can reasonably be argued that frequent exposure through intermittent low-level contamination, including with different *Cryptosporidium* species, will lead to infection that is not expressed clinically and that mild, self-limited or sub-clinical infection is more likely the outcome of exposure. In such situations, outbreaks are less common but sporadic cases in the previously unexposed will be common, including infants and visitors; the immunocompromised will be particularly at risk. In a longitudinal study of *Cryptosporidium* species-specific IgG in Peruvian children, Priest *et al.* (2006), using *C. parvum*-derived antigens, detected antibody responses during infections with *C. parvum, C. felis, C. meleagridis,* and four different sub-types of *C. hominis*. There was however, a reduced serological sensitivity (73% positivity) in *C. hominis* infections. The significance of this for seroprevalence studies needs to be further evaluated.

The infectivity of different isolates of *C. parvum* is known to vary (Okhuysen *et al.* 1999; Teunis *et al.* 2002). Many oocysts in water may be of low infectivity through poor viability, or low pathogenicity or virulence for man. Some of these may be capable of initiating limited infection and stimulating or boosting immunity. High levels of herd immunity may suppress secondary (person-to-person) transmission (Fine 1993; Casemore 1994, 1995). Whether humoral (circulating) and/or secretory antibodies in the gut are protective is still unclear (Current 1989; Heyworth 1992; Riggs 2002; Wyatt & McDonald 2004). Some antibodies are known to neutralize surface antigens on zoites that are associated with attachment, which would intuitively suggest a potential protective mechanism. Conversely, HIV-positive patients can have chronic disease in the presence of circulating antibody. In terms of risk estimation, it can be said that while antibody *per se* may not be protective it acts as a marker for increased resistance, which is likely dependent more on CMI although that mechanism may be enhanced by the presence of antibody on surface epitopes.

A further note of caution concerning the test antigens selected for use in antibody assays is that these have been evaluated primarily against adult sera; the response to them in children may be different and this has not been fully evaluated (R. Morris, personal communication).

## *GIARDIA*—REPORTS ON THE SEROLOGICAL RESPONSE AND SERO-EPIDEMIOLOGY

*Giardia* was first described by Leeuwenhoek (1632–1773), and re-described and named in the 19th century separately by Giard and Lamble. It was generally considered medically insignificant by clinicians until the 1960 s, mainly because of the frequency with which it could be found in apparently asymptomatic subjects. It is the most common enteric protozoan parasite worldwide. It is widely associated with the water route of transmission in the US, and by the food route, as well as in the day-care setting; some 5000 persons are hospitalized annually in the US with giardiasis (Craun 1990; Shandera 1990; US EPA 1998; Gardner & Hill 2001). Curiously, in the UK *Giardia* is uncommonly associated with the water route although it has been shown to be a risk factor for sporadic infection in a study in the Southwest of England (Stuart *et al.* 2003). The importance of the water route of transmission in the US became apparent in the 1960 s with the recognition of cases among travellers returning from Leningrad in Russia and outbreaks centered on Aspen, CO (Shandera 1990). In an outbreak in New Hampshire in 1977, associated with a contaminated water supply, it was noted that there was a high rate of asymptomatic infection, suggesting prior exposure and immunity to clinical infection (López *et al.* 1980). The infection has been commonly regarded as a zoonosis but the natural history and etiology is now known to be more complex (Thompson 2004). Infection is generally non-invasive, remaining localised within the small intestine. Innate mechanisms and humoral and cell-mediated immune responses have been identified over many years in humans and in animal models (Farthing 1989, 1990; Janoff

& Smith 1990; Faubert 2000; Wyatt & McDonald 2004). Primary infection in the immunocompetent will usually be symptomatic although this varies with the species or strain involved. Subsequent reinfection is commonly asymptomatic and may persist in the presence of antibody. Infection in the immunocompromised is often symptomatic and very persistent, particularly in those with hypogammaglobulinemia. The latter suggests that humoral antibody has an important role in clearance and recovery. Conversely, chronic infection is found in the presence of circulating antibody. Infection in AIDS patients does not seem to be especially persistent or severe although this view may be a reflection of the availability of specific treatment (Faubert 2000). The diagnosis of giardiasis is usually by detection of cysts (and sometimes trophozoites) in stools but this is known to be insensitive, often requiring multiple samples from known cases to detect the parasite (Farthing *et al.* 1987). Parasite antigens can also be detected by other methods such as ELISA. Antibodies can be detected by a variety of means, using serum, saliva or fecal fluid. The diagnostic value of this is uncertain but it may be of epidemiological value for indicating exposure.

### *Giardia* antigens

The clinical presentation, severity and immune response differ with the different species of *G. lamblia* (syn. *G. duodenalis; G. intestinalis*) capable of infecting humans, their sub-types (assemblages), and other variants, and the frequency of exposure (Nash 1997; Janoff & Smith 1990; Hunt 1999; Faubert 2000; Lane & Lloyd 2002; Thompson 2004). Cysts and trophozoites are thought to contain many antigens, some of which are restricted to one or other life cycle stage; some maybe dependent on *in vivo* or *in vitro* exposure of the parasite to immune and non-immune components (e.g. bile) of the gut milieu (Taylor & Wenman 1987; Reiner & Gillin 1992; Udezulu *et al.* 1992; Palm *et al.* 2003; Adam & Nash 2004). Some antigens are immunodominant, while others appear not to be directly involved in clearing of or resistance to the parasite. Some antigens detected in tests are not specific to *Giardia*, i.e. are cross-reacting with antigens from other organisms; false positive reactions appear to be common in unexposed subjects (Moore *et al.* 1982; Jokipii *et al.* 1988; US EPA 1998; Faubert 2000). In addition, *Giardia* undergoes surface antigenic variation that may aid the parasite's persistence (Nash 1997). Analyses of antigens from different isolates has revealed a number of important molecules ranging in size from 14 to 225 kDa in crude extracts of trophozoites and 21 to 49 kDa in cysts. An antigen of mw 31 kDa seems to be common to many isolates and is probably the structural antigen referred to as giardin (Taylor & Wenman 1987). Other antigenic molecules identified include heat shock proteins, lectins, tubulins, etc. (Farthing 1992; Janoff & Smith 1990; Faubert 2000). Antigens of 30 to 34 kDa, 57 and 82 to 88 kDa have been reported to be consistently recognized (Farthing 1989; Char *et al.* 1991; Janoff & Smith 1990), some of which are restricted to either trophozoites or cysts.

### Serological study reports

As with *Cryptosporidium* studies, various methodologies have been used to detect and measure the immune response to *Giardia*, particularly IFAT and ELISA but also complement fixation, lectin-based tests, immunodiffusion and indirect haemagglutination, etc; WB has been much less frequently used. Some have advocated detection of secretory IgA in saliva by ELISA for seroprevalence studies (Al-Tukhi *et al.* 1993; Hashkes *et al.* 1994). Many of the early antibody studies were aimed at characterizing the immune response and/or the pathogenesis of symptoms, and investigate resistance and susceptibility to re-infection (Ridley & Ridley 1976; Farthing 1989, 1990; Janoff & Smith 1990; Faubert 2000; Gardner & Hill 2001). The interpretation of serological findings, the importance of the different responses in producing resistance to infection, and the significance of the various antigens involved continue to be a matter for debate (Farthing 1992; Heyworth 1992; Nash 1993). Some workers have shown that specific antibody levels may indicate more severe infections associated with enteropathy and/or malabsorption (Ridley & Ridley 1976; Farthing 1989, 2003; Faubert 2000; Frost & Craun 1998b), although others have not corroborated this finding (Faubert 2000). Such variable findings may reflect infections with different species and strains. Chronic, asymptomatic or mildly symptomatic infections may persist for months or even years in the presence of circulating antibodies (Faubert

2000). The mechanism of protection afforded by maternal antibody, other non-specific factors in milk, and relative protection from the environment for breast-fed infants is a matter of debate (Miotti *et al.* 1986; Nayak *et al.* 1987; Sterling *et al.* 1991; Zu *et al.* 1992; Walterspeil *et al.* 1994; Tellez *et al.* 2003). Some have shown significant benefit while others have suggested that other non-specific mechanisms might be equally important. Mucosal (local gut-derived) specific sIgA and/or mucosal secretory IgM may be involved, with the complement pathway and opsonization, probably in concert with CMI mechanisms, in resolution or protective immunity (Heyworth 1990; Palm *et al.* 2003). Hypogammaglobulinemic patients with IgG and IgA deficiency tend to get persistent infection, implying that antibody may be important in clearance (Palm *et al.* 2003). However, diagnostically, serum IgA is a less useful indictor of active infection in acute cases as many infected subjects fail to show a humoral IgA response; serum IgM is probably more useful as an indicator of recent infection (Farthing 1989; Janoff & Smith 1990; Nash 1993; Faubert 2000).

## Early worldwide studies

Early reports on *Giardia* serology tend to suffer from the fact that they are based on tests using crude cyst or trophozoite antigens and varied test protocols, often with little or no evaluation of performance. Tests often did not distinguish the Ig classes. Nacapunchai and others found that several of these methods had poor sensitivity and/or specificity (Nacapunchai *et al.* 1986). However, studies on populations in developing countries in the 1970 s and 1980 s did indicate that antibodies were highly prevalent (Ridely & Ridely 1976; US EPA 1998; Farthing 1989; Janoff & Smith 1990; Faubert 2000). Exposure to *Giardia* begins early in life, with rapidly increasing rates in infants and young children; high rates of carriage were found in all age groups in apparently asymptomatic subjects (Gilman *et al.* 1985, 1988; Miotti *et al.* 1986; Nacapunchai *et al.* 1986). Newly acquired infection in infants in these populations is frequently symptomatic, compared with asymptomatic carriage in older family members (Janoff & Smith 1990). As noted with *Cryptosporidium*, visitors from developed countries often act as sentinels for this high prevalence with high rates of symptomatic infection. The attack rate falls with age in these groups, suggesting a role for the development of immunity (US EPA 1998; Farthing 1989).

Goka *et al.* (1986) described the sero-diagnosis of 52 patients and control subjects in India and the UK. Testing was by detection of specific IgM and IgG using an ELISA test with frozen trophozoites as antigen; sensitivity and specificity were estimated at 96%. IgM response was consistent with current infection, the response tailing off in as little as 2–3 weeks in three patients who provided sequential samples. Tests for IgG were unable to distinguish cases from controls with a history of previous infection. However, Gandhi and others found that the ELISA in their hands was specific but very insensitive for IgM, antibody being detected in only 24/73 confirmed cases, one of 37 control patients with other parasitic infections and none of 29 healthy controls (Gandhi *et al.* 1989). The results suggested that IgM detection might not be suitable for the more prolonged infection seen in their patients, compared with the shorter duration infections in the patients studied by Goka. This finding is consistent with the demonstration by Vinayak & Kumkum (1989), using AHG in an indirect ELISA with a trophozoite plasma membrane and 56 kDa Ags. This showed that lower antibody titres were found in persistent than non-persistent acute infection. Char *et al.* (1993) also showed a lower IgA response, in an ELISA with a 57 kDa Ag *Giardia* heat shock antigen, in persistently infected children in The Gambia. This suggests that the more severe symptoms may be immunogenic (Farthing 2003).

Miotti and his co-workers investigated age-related acquisition of IgG sero-positivity in four diverse populations in the USA and South America, using an ELISA test system with trophozoites as antigen (Miotti *et al.* 1986). The specificity of the test was confirmed by blocking with monoclonal antibody to the giardia antigen. No attempt was made to study stool positivity rates. Rates in South American adult subjects and Apache Indians in Arizona showed rates of 44–48%, while adults living in Baltimore, Maryland, showed only 18% positivity ($P < 0.01$) and with lower levels of reactivity ($P < 0.001$). Children living in different countries and settings showed widely varying rates of acquisition, the fastest rate being in Peru. The results are consistent with levels of hygiene and environmental conditions in the respective communities. This was also shown in a study by Gilman and others in Lima, using stool examination, which showed that children rapidly

became re-infected following anti-giardial treatment (Gilman *et al.* 1988).

Taylor & Wenman (1987) in Canada sought to characterize the immunologic response to *Giardia* in 16 confirmed cases, age range 3 to 76 years, who had acquired their infections in Canada or while travelling abroad; three of the patients had chronic infection associated with common variable hypogammaglobulinemia and no detectable IgA. They also used sera from 10 healthy control subjects. Antigens were obtained from cultivated trophozoites by SDS-PAGE and then used in WB. A majority of the sera reacted with a range of antigens, but particularly with a 31 kDa polypeptide Ag, which was thought to be surface disc-derived protein referred to as "giardin", also described by others.

Char *et al.* (1991) in India and the UK studied the serum antibody response in children to an immunodominant 57 kDa Ag purified by SDS-PAGE in WB. All sera from confirmed cases showed specific IgG but not IgM although IgM reactivity was found with other mw antigens but these were also often recognized by control sera. Sera from nine of ten patients had detectable IgA to the 57 kDa Ag.

Ljungström & Castor (1992) studied the immune response to *Giardia* in a waterborne outbreak in Sweden involving 1400 cases in a previously largely unexposed population estimated at ~3000 persons. The outbreak resulted from sewage contamination of the drinking water supply over a defined period of about one week. Serum samples were obtained from 352 exposed persons and tested by IFAT for IgG and IgA. Results were correlated with fecal microscopy; "negative" controls consisted of serum samples from 428 asymptomatic persons. IgG and/or IgA were found in 68% of microscopically confirmed cases and 22% of exposed but microscopy-negative persons, compared with 10% of controls. Antibody titres were generally low and in 32% of microscopy-positive cases antibody could not be detected. A further molecular study by this group looked at the response to non-variable antigens (Palm *et al.* 2003). Background titres in control subjects were low. They suggest that antibody aids clearance and enhances resistance to further clinical infection. Hypogammaglobulinemic patients with decreased levels of IgG and IgA are prone to develop persistent infection (Janoff & Smith 1990).

Soliman *et al.* (1998) studied serum antibody in symptomatic and asymptomatic Egyptian children using axenic trophozoites (WB strain) as antigen source with IFAT, ELISA and WB. IFAT showed significant titre differences between the two groups. By ELISA, IgA and IgM levels, but not IgG, were higher in the symptomatic subjects. Total *Giardia*--specific IgG was the same in both groups but isotypes IgG1 and IgG3 levels were higher in the symptomatic group. The significance of different antibody isotypes, which this study measured, is discussed. Results by WB failed to show a clear difference between the groups.

## Serological studies in North America

Although giardiasis is the most commonly reported waterborne infection in North America (US EPA 1998; Craun *et al.* 2002) there are few seroprevalence studies from there. Smith *et al.* (1981) used an ELISA test to examine sera for antibodies in subjects in Washington, DC, and found 14% to be positive. Lopez *et al.* (1980) described an outbreak in a community in which those who had a history of exposure to surface water had a significantly higher rate of asymptomatic infection, suggesting acquired immunity to symptomatic disease but not reinfection. Istre *et al.* (1984) investigated an outbreak of waterborne giardiasis at a mountain resort that also showed evidence of protective immunity in local residents in that local residents were less frequently symptomatic than were visitors. Smith *et al.* (1981) used intact trophozoites in an ELISA test to examine sera from patients and controls from Colorado and elsewhere in the USA. The study was aimed partly at developing a reliable and reproducible test protocol. They found that 81% of 59 symptomatic patients and 12% of controls had detectable IgG antibody; 11 of 15 patients tested serially had detectable antibody from 2 weeks to 15 months after treatment. Prevalence of antibody in 197 convenience samples from Washington, DC, was 14%. A further study used ELISA to measure IgG, IgM and IgA in sera from 29 AIDS patients with acute giardiasis and other groups, including immunological normal patients with and without giardiasis (Janoff *et al.* 1988). Responses were further characterised by WB (method of Janoff *et al.* 1988). This study showed significantly depressed antibody responses ($P < 0.0001$) in the AIDS patients. All 25 immunocompetent subjects with giardiasis had good IgA and IgM responses. It was not established whether this was related

to IgAs levels (IgA2). Their IgG response was less marked than in AIDS patients with more persistent *Giardia* infection whose symptoms were not related to the parasite, although the latter group had a poor IgM response. WB showed that, although there were many reactive bands, all positive sera reacted to antigens between 30 and 34 kDa also identified by other workers as important immunodominant antigenic molecules. Of particular interest to this report, Birkhead *et al.* (1989) studied serum IgG, IgA and IgM levels in an ELISA with trophozoite-derived soluble protein antigen(s). Subjects included 24 convalescent persons and 20 non-residents following a waterborne outbreak in a rural trailer park in Vermont. Residents showed higher levels of IgG and IgA but not IgM. Nine residents showed higher mean levels of IgA, which increased relative to their consumption of tap water. This study indicated that anti-giardial IgA might be useful in the investigation of outbreaks.

Miotti *et al.* (1986) used ELISA to look at age-related antibody acquisition in subjects in an inner city area of Baltimore, Maryland, and three other locations described above. They found 18% of Baltimore subjects had detectable IgG antibodies to *Giardia*, significantly less frequent than in subjects from other groups studied from areas with poorer hygiene and environmental conditions. This is broadly comparable with the rate in the previously described study. Stool isolation rate for the same area was 0.9% over a five-year period, underlining the poor sensitivity of microscopy for diagnosis and prevalence studies.

Sullivan *et al.* (1987) conducted serological studies on patients with giardiasis and others in a Midwestern city in the USA. Using IFAT, they showed that symptomatic patients tended to have higher titres and titres remained high for a prolonged period in those chronically infected, suggesting that antibody was not associated with clearance of the infection. Indochinese refugees, HIV-positive patients and other symptomatic individuals generally had higher mean titres than healthy controls but there was a broad overlap.

The anti-*Giardia*-specific antibody response was evaluated as a diagnostic tool in children with suspected infection (Sullivan *et al.* 1991) and compared with stool microscopy and jejunal mucosal biopsy. High titre ($\geq$1/800) specific IgM had a sensitivity of 63%, specificity of 93%, and predictive value of 85% and correlated better with active infection than IgG or IgA. Sensitivity may have been improved by using a lower cut-off but this might be offset by decreased specificity.

Several sero-epidemiologic studies have been reported from Canada. Isaac-Renton *et al.* (1994) investigated the use of serology in an outbreak in a locality in British Columbia that had had another outbreak five years before. They used an ELISA with a soluble antigen derived from axenized, cultured trophozoites from the strain recovered from water samples and an ATTC reference strain (WB). Sera were collected from 51 cases, 21 non-cases in the affected area, and from 35 controls from two different groups. Full demographic details of study subjects are described. Sera were analyzed for *Giardia*-specific IgG, IgA and IgM. Forty-three (84%) of the cases gave significant positive antibody reactions in one or more Ab class. In contrast to the study by Birkhead *et al.* (1989), levels of IgG were more elevated than IgA; IgM levels were elevated but less frequently, as reported by other workers. Use of the heterologous isolate did not affect the results. The study, including isolation rates, confirmed that those resident at the time of the previous outbreak were significantly less likely ($P < 0.001$) to suffer symptoms on re-exposure. The authors point out that serologic studies can be useful in non-endemic communities, giving a better estimate of case numbers than stool examination, especially where there is delay in investigation but are likely to be of limited value in endemic areas. Isaac-Renton *et al.* (1996) conducted a 24-month longitudinal study of two discrete water catchment communities in British Columbia with *Giardia* cysts in their water supplies. There had been a waterborne outbreak in one of the two communities some five years before this study. Over the two-year period, there were no outbreaks but evidence suggested an endemic background level of infection but with amelioration in the area that had previously experienced an outbreak. Serologic studies, using the previously described ELISA, on 1122 samples showed 37% had IgG levels above the cut-off value; some had raised IgM levels. Differences were noted in serologic rates reflecting water treatment differences and the previous history of an outbreak in one area. Sera from the latter group showed that 64% had no detectable antibody and this community was thought to be at risk should further significant contamination occur. Isaac-Renton *et al.* (1999) studied three communities

with different water source types [(i) deep wells, (ii) protected watershed surface water, (iii) a surface water known to be contaminated] by estimating sero-prevalence rates against *Cryptosporidium* (see above) and *Giardia*. Using the previously described ELISA, seroprevalence for *Giardia* antibodies was 30.3% overall (590/1944). Curiously, group (i) had a rate of 19.1%, group (ii) 34.7%, and group (iii) 16.0%; the rate for (ii) dropped significantly over the study period but not in the other two areas ($P < 0.001$) although the reason for this difference was not known, it suggests perhaps that group (ii) had experienced an unrecognized outbreak prior to the study period.

### Discussion of *Giardia* serology

Given the high prevalence of this parasite, surprisingly little has been reported on prevalence of *Giardia*-specific antibodies in the USA. However, the studies described above suggest that useful information:

- can be gained from studies on the natural history and dynamics of transmission of giardiasis;
- can help to identify areas of high prevalence that may not be apparent from clinical diagnostic rates and which may be attributable to water;
- can identify communities at risk from occasional water contamination events.

With reference to risk estimation, the evidence generally supports the view that while antibody may not protect against infection *per se*, reinfection in an antibody-positive person is less likely to be symptomatic (US EPA 1998). The potential for risk from zoonotic sources has perhaps been overestimated as research indicates that many animal types are not infective for humans other than those belonging to assemblages A or B (Lane & Lloyd 2002; Thompson 2004).

## SEROLOGIC ASPECTS OF OTHER WATERBORNE PROTOZOAN PARASITES

### Entamoeba histolytica

Although waterborne outbreaks have occurred in the USA, these are very uncommon (Marshall *et al.* 1997; Tarleton & Petri 2004). Many older studies have been invalidated by the separation of the so-called non-pathogenic variant as a species, *Ent. dispar* (Clark 2004). Little is known of the prevalence of antibodies to these and other classic species in the USA and such data would be of little relevance to the risk estimate.

### Emerging parasites

Several parasite species have emerged in recent years as waterborne protozoan pathogens, although all have other routes of transmission. These include:

- *Toxoplasma*. Toxoplasmosis is occasionally waterborne in North America but this is uncommon (Benenson *et al.* 1982; Bowie *et al.* 1997; Dubey 2004). Infection is usually transmitted by means of oocysts from infected cat feces, directly or via the soil or water, and from tissue stages via consumption of raw meat. Seroprevalence in the USA has been estimated at ≥20% in NHANES and other studies and appears to be stable at that level (Bowie *et al.* 1997; Jones *et al.* 2001 2003). Methodology and interpretation are well defined in the USA (Garcia *et al.* 2004). In Brazil, widespread infection, estimated through seroepidemiology, is associated with consumption of contaminated water and commonly results in ocular toxoplasmosis (Bahia-Oliveira *et al.* 2003).
- *Cyclospora* This parasite has emerged as an enteric pathogen in recent years and is thought to be commonly waterborne in some developing countries. The requirement for maturation of oocysts in the environment means that direct person-to-person spread is unlikely. Evidence was found in a study in Peru to suggest that primary infection protects against further clinical episodes (Bern *et al.* 2002). Age-specific rates supported an environmental route for the infection. In the USA, it appears to be primarily foodborne and is uncommon in children although waterborne transmission has been reported in the USA occasionally (Herwaldt 2000; Sterling & Ortega 2004). The failure to recover and purify oocysts in large numbers from human feces or to develop an animal model means that serological studies have not yet been developed.
- *Microsporidia* This large and ubiquitous group of parasites has emerged in recent years as a cause of

infection, mainly in the immunocompromised. Concerns have been expressed about its potential role as a waterborne pathogen but little is known about this yet (Dowd *et al.* 1998; Franzen & Müller 1999). Serological tests have been developed but little is yet known about the prevalence of antibodies in the USA (Hollister *et al.* 1991; Van Gool *et al.* 2004).

While antibody studies to these parasites may be of general epidemiologic interest, it would be of doubtful value to the national risk estimate.

## SEROLOGIC ASPECTS OF WATERBORNE VIRUSES

### Introduction

It has long been recognized that a variety of viruses may be transmitted by the water route, including noro-, entero-, adeno- and rotaviruses, and some hepatitis viruses. Waterborne outbreaks of what later became known as Norwalk virus have been recognised since the 1920 s (Spenser's disease), long before the etiologic agent was identified. In the USA, work in the 1970 s and 1980 s by Kapikian, Greenberg, Kaplan and others led to the identification of the Norwalk virus (now norovirus) as one of the most important waterborne infections. Various other members of a group of related viruses, known as Norwalk-like or small round structured viruses (SRSVs), were also identified. The group is now known to be a complex of genotypic and antigenic subtypes classified as members of the *Caliciviridae* (Fankhauser *et al.* 1998; Atmar & Estes 2001; Von Bonsdorff & Maunula 2003; Gallimore *et al.* 2003). Other viruses sharing similar epidemiology include the sapoviruses and astroviruses. These may account for as little as 3% of outbreaks in which the etiologic agent and route of transmission is identified (Glass *et al.* 2000). However, many more outbreaks are reported in which an etiologic agent is not identified, but with SRSV-like symptoms and which are associated with water (Frost *et al.* 2002*b*).

The perceived epidemiology of rotavirus has been skewed by the concentration on infection in infants and young children but it is more widespread in adults than is usually recognized, in developed as well as developing countries (Casemore 1987, unpublished data; Cox *et al.* 1998*a*, *b*; Cox & Medley 2003; Iturriza-Gomara *et al.* 2004). Transmission is usually assumed person-to-person and other potential routes have been little explored.

All of these viruses are more commonly transmitted by the direct person-to-person route and via food, environmental/recreational water exposure, etc. (Appleton 2000; Lopman *et al.* 2003; Widdowson *et al.* 2005*a*). The multiple routes make it difficult to estimate the proportion due to water as the only positive indication of source or route is in well-defined outbreaks that are investigated epidemiologically using case-control studies. As with the parasites described above, endemic areas may mask high rates of environmental transmission and this reduces the apparent contribution of waterborne transmission or level of association (relative risk) in epidemiological studies. Visitors from non-endemic areas act as sentinels, thus suggesting the importance of immunity in resistance to symptomatic infection. However, primary infection leads to short-term immunity lasting only weeks or months and little or no immunity to re-infection with homologous as well as heterologous types although there is considerable cross-reactivity in testing (Appleton 2000; Matsui & Greenberg 2000; Schaub & Oshiro 2000).

### Serological studies with the *Caliciviridae* (norovirus and associated SRSVs)

As with the parasite studies described above, many studies are developmental and this makes comparison and interpretation difficult. More recently, the introduction of molecular methods in particular has enhanced the ability to distinguish morphologically similar species and sub-types, while the development of recombinant capsid-protein antigen (baculovirus-expressed norovirus-like particles) has made serology more precise (Atmar & Estes 2001). As with parasites, detection of fecal (secretory) antibodies may be useful diagnostically although not much used for seroepidemiology (Okhuysen *et al.* 1995). However, their use has also shown the antigenic as well as genetic diversity, which makes interpretation of findings problematic unless studies are done using homologous virus antigens (Hale *et al.* 1998; Pelosi *et al.* 1999; Lopman *et al.* 2002). For the purposes of this report, no attempt is made to distinguish

the members of this group, which share similar epidemiologic features, except for Hepatitis E virus (HEV) which is dealt with separately (see below). Volunteer and other studies have shown a mixed response in SRSV-specific antibody-positive subjects, some being resistant to reinfection while others have had symptoms on reinfection, thus indicating a variable degree of protective immunity and that several exposures might be required for protection (Matsui & Greenberg 2000; Schaub & Oshiro 2000; Lopman *et al.* 2002). Gray and others in the UK estimated seroprevalence at nearly three quarters in >3000 subjects (Gray *et al.* 1993); age-related rates were 75% in infants <6 months, followed by a dip to 25% (suggesting the effect of maternal antibody) and then progressively increasing, reaching 74% in the teens, rising to 80–94% in adults.

Gray *et al.* (1994) conducted volunteer studies with norovirus. They found little difference in initial IgG titres between those who were symptomatic following challenge, and those who were not. An IgM response was subsequently found in all infected volunteers. In those who were symptomatic, there was a marked increase in IgA and IgG titres but this was variable in those without symptoms. This study suggests that antibody is often not protective but the immunological difference between those who did and those who did not develop symptoms is not clear.

In Europe, seroprevalence has been found to be high in all age groups. For example, a study of >1000 sera in France using NVL (recombinant baculovirus-derived) antigen found high prevalence in all age groups, similar to those in the UK; >60% in infants <6 months, followed by a dip and then progressively increasing, reaching a peak of >80% in the teens; overall rate 74.1% (Nicollier-Jamot *et al.* 2003). Lopman and others reported that the test system tended to be restrictive in indicating infection with heterologous strains. A similar study in Italy found a seroprevalence of 91% (Pelosi *et al.* 1999). Others have reported, however, that antibodies have been found in human subjects to bovine strains, the significance of which is not yet clear (Widdowson *et al.* 2005*b*).

Seroepidemiologic studies have been reported from developing countries. For example, O'Ryan *et al.* (1998) studied risk factors for acquisition of antibody to Norwalk and Mexico viruses in Chilean individuals in two geographic locations. The main determinants were lower socioeconomic status and increasing age; other factors varied with location and setting. Smit *et al.* (1999) in South Africa conducted a seroepidemiologic study of genogroup I and II infections; seropositivity ranged from 81–99%, indicating the high prevalence of these infections. Steinberg *et al.* (2004) and others in Guatemala found a similar age-related pattern but, as might be expected, with a more rapid progression from 27% at 6–12 months to 94% at 25–30 months.

In North America, Payment *et al.* (1994) studied seroprevalence and the possible impact of point-of-use filtration of subjects' drinking water. They used a native (stool-derived whole virus) antigen in a blocking (indirect) ELISA test system. Seropositivity increased with age from 55% in those aged 9–19 years, increasing steadily to 100% at 60 + years. Changes in serum titre (seroincidence) were seen in 8.7–24.11% in different study seasons, but these changes did not appear to be related to waterborne infection.

Noel *et al.* (1997) reported on the correlation of patients' immune response to four different genetically characterised SRSV antigens in 23 outbreaks in the US, 1990–95, to estimate the distribution of these viruses. Recombinant-expressed capsid proteins were used in a direct ELISA to determine whether the responses were strain-specific. The results suggested that significant differences were present in responses to heterologous and homologous antigens, resulting from the distinct antigenic diversity of different SRSVs, and suggested that additional expressed antigens were needed.

## Other viruses

A variety of other viruses may be transmitted by the water route, including astroviruses, rotaviruses, Polioviruses, Coxsackieviruses, Echoviruses, and hepatitis A (HAV), and E (HEV), and they may be found in surface and groundwaters along with animal-derived species or types. HAV is transmitted fecal–orally by the water route as well as directly from person to person. HAV is of relatively low endemicity in the US with the exception of certain socially deprived groups (Poovorawan *et al.* 2002). Improved knowledge of hepatitis viruses has enabled the recognition of further types for which water is an important route of transmission, especially HEV. HEV is often asymptomatic but can be fatal in pregnant women (Poovorawan *et al.*

2002). Such cases are uncommon in the US but antibody has been found in up to 28% of healthy subjects in the US in some surveys (Meng *et al.* 2002). Highest rates have been found in veterinarians; a closely related virus is known to be present in pigs in the US, and that may have been transmitted to humans (Meng *et al.* 1997; Schlauder *et al.* 1998). HEV may be more common in developed countries than is generally appreciated (Aggarwal & Krawczynski 2000; Clemente-Casares *et al.* 2003). Little is known of its seroprevalence in the USA. Enteroviruses are generally ubiquitous, even in areas with high levels of hygiene and water quality, although sub-types vary in frequency of reporting geographically and temporally. Antibody studies generally reflect this ubiquity (Payment 1991; Cox & Medley 2003). Data from a study by Jiang and others supports a protective role for antibody to rotavirus, or as a correlate of protective immunity (Jiang *et al.* 2002). Some rotaviruses may be zoonotic (Iturriza-Gómara *et al.* 2004). Groundwater as well as surface water supplies have been associated with viral infection in the USA (Craun *et al.* 2002).

### Discussion of virus seroprevalence

Fecal contamination of water sources, both surface and groundwater, may lead to transmission of viruses as well as other pathogens and through 1971–1998 there were 51 outbreaks in the US involving recognized viruses and 347 of undetermined etiology, many of which will have been viral (Craun *et al.* 2002; Frost *et al.* 2002*b*). In Europe, Norovirus was responsible for >85% of all non-bacterial gastroenteritis outbreaks in 1995–2000 (Lopman *et al.* 2003). Serology has provided considerable help in better understanding epidemiologic aspects of these infections. Progress in molecular-based studies has enhanced the validity of serologic findings but also highlight the problem for serology, and interpretation of results, because of the multiplicity of enteroviruses, SRSVs and small round non-structured viruses (SRVs), including animal types that are of doubtful significance in humans. Serologic studies do indicate, however, the very high prevalence of these virus infections, both symptomatic and asymptomatic, in industrialized as well as developing countries. Common antigens may result in cross-reactivity and anamnestic responses, which complicates interpretation of findings. The protective effect of antibody is generally short-lived and antibody requires boosting by re-infection to be protective. It has to be noted that water, although undoubtedly implicated in some sporadic cases and outbreaks, is probably a minor route of transmission compared with person-to-person spread, directly or via food, in industrialized communities. It is difficult to see how serologic data can be reliably applied to estimating risk from this route.

## SERO-EPIDEMIOLOGIC STUDIES OF BACTERIAL INFECTIONS

A variety of bacterial pathogens may be transmitted by the water route. Species that have been reported in the USA with some frequency include campylobacter species (mainly *Campylobacter jejuni*), salmonella, pathogenic *Escherichia coli* (e.g. *E. coli* 0157), *Helicobacter* (Hunter 1997*b*; Craun *et al.* 2002; Hrudey & Hrudey 2004). Species reported as occasional or localized waterborne pathogens (e.g. *Plesiomonas, Aeromonas*), water-associated species (e.g. *Leptospira*, *Legionella*), and the classical waterborne pathogens that cause typhoid and cholera, occur much less frequently in industrial countries including the USA and thus will not be addressed in this report. Most bacterial pathogens that are addressed here are ubiquitous and more commonly associated with foodborne transmission. Standard treatment, particularly chlorination, of potable supplies mitigates the risk from bacteria, given their relative susceptibility to disinfection. Infection with these species tends to be more commonly associated with untreated waters, including consumption of well water and exposure to environmental or recreational water. Given the relative ease of isolation of the etiologic agents in clinical specimens, sero-epidemiology is not commonly used. However, epidemiological studies have shown that regular consumption of water from at-risk supplies (e.g. private wells) is associated with increased resistance to illness that masks the risk to the immunologically naive (Raina *et al.* 1999; Strauss *et al.* 2001).

### Selected study reports

The source and transmission routes for most *Campylobacter* infections are not clearly known; infection is widely blamed

on poultry although definitive evidence that poultry is the primary source of infection is generally lacking (Adak *et al.* 1995; Rodrigues *et al.* 2001; Michaud *et al.* 2004; Meldrum *et al.* 2005; Nichols 2005). It has sometimes been associated with the water route, mainly recreational use and consumption of untreated supplies but occasionally resulting from contamination of and/or operational problems with treated municipal supplies (Anon 2000; Said *et al.* 2003; Michaud *et al.* 2004; M. Evans, personal communication). General population sero-surveys for antibodies to *Campylobacter* are problematic for several reasons, including the ubiquity of the infection, complex antigenic structure (including cross-reacting antigens), the difficulty of typing outbreak strains and identifying key genotypic markers, etc. The development of SDS-PAGE purified flagellar antigen has been used in a WB test that seems to offer some benefits but the use of this antigen in an ELISA test leads to some loss of sensitivity (J. Cheesbrough, personal communication). Rural inhabitants regularly exposed to livestock may have increased antibody titres to pathogens such as *E. coli* 0157 and *Campylobacter* and reduced likelihood of symptomatic infection, presumably through recurrent boosting of immunity (Adak *et al.* 1995; Evans *et al.* 2000; Belongia *et al.* 2003). Salmonella has been the cause of waterborne outbreaks in the USA but is much more commonly attributed to food. Waterborne *E. coli* 0157 infection is much less common than either of the preceding two species. In the USA, a survey revealed that 9% of outbreaks from 1982–2002 were waterborne but only 10 (3%; 15% of reported cases) were associated with drinking water. Of these, only three involved municipal supplies, two of which did not use chlorination and the other system had a malfunction (Rangel *et al.* 2005). Sero-diagnostic tests for *E. coli* 0157 have been developed but not widely used for seroprevalence studies, other than in the limited case of studying close contacts of microbiologically confirmed cases. Asymptomatic infected subjects do not appear to produce a serologic response to the 0157 lipopolysaccharide (LPS) antigen used (H. Chart, personal communication). *Helicobacter pylori* infection of the gastric mucosa is very common and may result in chronic gastric inflammation that may lead to ulceration and other disorders. It has been postulated that this infection may be acquired from water, as well as other sources but its ecology is poorly understood (Hopkins *et al.* 1993). The organism has been detected in water in the US but serological studies may be problematic (Khanna *et al.* 1998; Hegarty *et al.* 1999).

### Discussion of bacterial serology

While bacterial infections are associated with transmission by the water route from time to time, this is only a small fraction of infections transmitted from infected food animals or via contaminated food, for example by infected food handlers. The majority of incidents of waterborne transmission tend to be associated with untreated supplies. Regular users of untreated supplies may have reduced risk of IID, due to the effects of acquired immunity. However, such supplies that may appear safe to those consumers represent a particular risk to the previously unexposed such as infants and visitors (Strauss *et al.* 2001).

It is difficult to envisage sero-epidemiologic data providing meaningful information for the risk estimation although undoubtedly it sometimes provides valuable insights in conventional epidemiological outbreak investigation.

## DISCUSSION OF THE ROLE OF SEROPREVALENCE IN RISK ESTIMATION

Immunology is primarily the study of observable phenomena associated with reactions between antibodies and/or cellular immune mechanisms and their homologous antigens (not whole organisms *per se*). These reactions are evidence for exposure to the source of that antigen but not necessarily evidence of disease and recovery; nor do they necessarily denote resistance to re-infection. The presence of high or moderately high levels of IgM and/or IgA especially in the absence of IgG, or weak IgG avidity, is usually interpreted as evidence of recent infection; the presence of IgG alone, and/or of high avidity, is generally taken to denote past infection. Similarly, molecular studies indicate that reactions with particular specific mw antigens may provide similar temporal associations. While the presence of antibody cannot generally be taken to imply that the individual may be resistant to re-infection, or be asymptomatic on re-infection, it may denote

the likely presence of other functional protective mechanisms (e.g. CMI). A factor perhaps not taken sufficiently into account in many studies is the significance of the intensity of an antibody response, whether expressed as titre, size and/or intensity of a WB band, OD value, etc., and the impact that this may have on the level of immunity (resistance). It is generally understood that multiple exposure to an antigen given in a course of vaccine shots is required to produce adequate (high titre) immunity and prolong the effect (prolonged half-life) associated with ongoing protection.

Various studies cited above showed seroprevalence against a number of the agents tested tends to be high in young infants (<6 months) reflecting maternal antibody levels; levels then dip and subsequently increase with age, reaching a plateau at a variable age. The rate at which this occurs generally reflects hygiene levels in the communities studied. This is consistent with the reciprocal age-related decline in frequency of symptomatic cases resulting from recurrent exposure and is an argument in favour of assuming that antibody is linked to or correlated with immunity.

An additional consideration in the risk equation is that of herd immunity, in which high rates of individual immunity act as an inhibitor of secondary (person-to-person) spread. Such secondary spread may amplify the impact of waterborne transmission in low- or non-endemic settings and will often result in a fall in mean age of cases as the outbreak progresses (Casemore 1995; Meinhardt *et al.* 1996; Harrison *et al.* 2002). More importantly perhaps, the absence of detectable antibody can generally be interpreted as indicating likely susceptibility. This is important in populations generally protected from waterborne infection by the provision of good quality, safe drinking water. Penetration of pathogens through exceptional challenge and/or breakdown in treatment will likely result in a much increased attack rate in such communities. This is increasingly likely with the climatic instability and increase in exceptional weather associated with global change (Rose *et al.* 2001). Such communities can be identified by the evidence of seroprevalence studies and improved protective measures then initiated where indicated. Where there are high levels indicating frequent exposure, the greatest concern is for immunocompromised persons, especially those in whom that condition has not been diagnosed. Again, seroprevalence studies can help identify these at-risk communities.

Public water suppliers are required to provide potable water that does not represent a risk to public health. However, investigations of outbreaks of waterborne disease affecting such supplies have usually revealed treatment deficiencies or operational failings. Poor quality source water may result in frequent, usually low level, penetration that may enhance seroprevalence and result in lower attack rates except in young children, visitors and the immunocompromised. Improved water treatment in areas with a challenged supply may lead to a fall in seroprevalence. When source waters are of high quality and in the absence of other exposures, seroprevalence is likely to be low or very low. Breakdown in treatment is then likely to result in a high attack rate in the exposed population. Small local and private supplies, including those in many rural areas, many of them untreated, are often inherently unable to meet public health quality requirements. Seroprevalence studies may indicate the frequency of transmission resulting in sub-clinical infection in the immune and the potential risk to others.

## SUMMARY AND CONCLUSIONS

This review of published serologic and seroprevalence studies on a wide range of waterborne pathogens concentrates on information relevant to estimating risk of waterborne infection. A number of conclusions can be drawn concerning the organisms and the antibody detection methods that are of importance.

In assessing the importance of different potentially waterborne pathogens in relation to seroprevalence, it needs to be recognized that:

- Most species have multiple sources and routes of transmission. Nonetheless, antibody from infection acquired by other routes (e.g. person-to-person, food) will also impact susceptibility when transmission occurs via water.
- Many have multiple types or sub-types that make it difficult to be sure from serologic findings of the precise organism or strain of organism involved.
- Some organisms (e.g. vegetative bacteria) are readily controlled in drinking water by simple disinfection. Priority has therefore been given in this report to *Cryptosporidium* and *Giardia*, both of which are frequently waterborne and

relatively resistant to disinfection. The most extensive literature available relates to *Cryptosporidium*. The relevant features of papers on seroprevalence for *Cryptosporidium* in North America are shown in Table 1. Findings are highly variable in place and time, between different population groups, and by different methods.

- The immune response is complex and interpretation of laboratory findings requires caution and considerable expertise.
- A serological response indicates that the subject has been infected with the organism concerned or an antigenically related species. It does not necessarily imply that the subject has suffered acute clinical (symptomatic) infection.
- Primary infection usually results in clinical (symptomatic) infection and a typical pattern of antibody responses that can be used to estimate the likely period of infection. Such kinetic studies may be based on the different antibody classes, seroconversion in time series samples, and/or the precise antigen for which there is a measurable response. In *Cryptosporidium* infection, there has been debate about the pattern of the kinetic response, but this may reflect the relative sensitivity of the methodologies used.
- Some infections, for example those occurring non-invasively in the gut (e.g. *Giardia*), may produce little or no measurable antibody response. Conversely, invasive infection is usually associated with a measurable antibody response.
- Continuing chronic infection may result in persistence of IgM, which is often associated with recent primary infection.
- After primary infection, antibody declines and usually returns to baseline generally in less than a year. However, subsequent reinfection with the same or antigenically related species may boost the level and may result in a different pattern of antibody response.
- Reinfection may occur in the presence of antibody. Such infection may or may not be symptomatic but will produce a boost to the antibody response. Although reinfection has been demonstrated in antibody-positive subjects, symptoms are usually less severe.
- Antigenically related organisms may also boost antibodies produced against the earlier infecting organism (anamnestic response). Subjects may not be protected against the effects of infection with an antigenically related but distinct species or sub-species. Whether this is true for *Cryptosporidium* species is not definitively known.
- Antibodies are variably protective but may indicate likelihood of the presence of other mechanisms (e.g. CMI) more closely associated with protection (resistance) in some infections such as *Cryptosporidium*.
- High rates of antibody in a population reflect frequency of exposure but not necessarily of overt disease. Such antibody prevalence may produce indirect protection to individuals from person-to-person (secondary) transmission through the mechanism of herd immunity. In the event of contamination of drinking water, the impact (attack rate) will likely be lower than in a similar event in areas of low seroprevalence.
- In the case of organisms with multiple routes of transmission in communities where there is high prevalence of antibody, the immunocompromised, visitors and young children may act as sentinels of the high endemicity and are at particular risk.
- Seroprevalence rates are generally higher in areas supplied by surface water sources, compared with groundwater. However, incidence (disease) rates may be lower than expected and this may be due to natural immunization of the resident population to symptomatic infection. Not all strains present will necessarily be pathogenic or virulent in humans but may be capable of provoking or boosting immunity.
- The multiple routes of transmissions (food, water, person-to-person, etc.) of many of the pathogens described above make it difficult to estimate the proportion due to water. The only positive indication of source or route is in well-defined outbreaks that are investigated epidemiologically using case-control studies. In some of the studies described, there is a clear increase in prevalence of infection (symptomatic and asymptomatic) in areas supplied with water from surface water supplies compared with groundwater sources. It is reasonable to ascribe the excess, but not the total contribution, to water, if other potentially confounding risks can be excluded.
- Antibody resulting from infection acquired from other sources and routes (generally more common) will

contribute to moderating the impact of exposure derived from water.

- Antibody studies are valuable in the investigation of waterborne and other outbreaks as they are a more sensitive indicator of exposure rates than detection or isolation of the putative causal organism. This is especially true of the detection of parasites by stool microscopy, which is very insensitive (excretion of parasites is often intermittent; PCR detection can be used but is expensive and technically more demanding).
- Seroprevalence found in outbreak investigations can be used as an upper boundary for estimation of endemic prevalence.
- Absence of antibody indicates likely susceptibility to infection. In the event of widespread exposure through contamination of the water supply in a population with a low rate of seroprevalence, the attack rate is likely to be high. There is often a marked increase in primary cases amongst adults, followed by increased secondary transmission, mainly through children.
- Whether or not antibody *per se* is protective is not necessarily of importance if it acts as a correlate for immunity (resistance). Although subjects with antibody can often be reinfected (naturally and experimentally), and sometimes have symptoms, these are often reduced in severity. In addition, the age-related increase in seroprevalence correlates well with the reciprocal decline in incidence of acute clinical (symptomatic) infection.

Antibody measurement can be achieved by many different methods, some more suitable than others for epidemiologic purposes. Each method may have specific benefits or shortcomings. The results obtained from various studies using different methods or variations of methods cannot be compared in absolute terms but can be useful for illustrating general trends in relative frequency (prevalence). All tests used need to be rigorously controlled (QA/QC), including the use of known positive and negative samples. The main methods for use are western blot and ELISA, as follows:

- Large format western blot is both sensitive and specific. It provides a "gold standard" for identifying antibody positive and negative control samples and assessing equivocal results. However, it is cumbersome and expensive, which makes it unsuitable for epidemiological surveys.
- Minigel format western blot is economic and convenient to use. It has provided much useful epidemiologic information in relation to waterborne infection. It has a reduced sensitivity to a key antigen (27 kDa) and the significance of this needs to be investigated. Other workers have reported poorer results than those who developed the method and the robustness of the method for transfer needs to be established.
- The ELISA test is relatively cheap and easy to use. It is open to criticism if crude antigens are used but the use of clearly defined antigens in the CDC second-generation test makes an improved epidemiologic tool for estimating seroprevalence in different populations. However, false-positive and false-negative results have been reported and this needs to be further investigated. Although it has been demonstrated that it is robust and capable of transfer to other laboratories, the more widespread and economic availability of the antigens is crucial.

Certain caveats apply to methods using well-defined antigens:

- Two *Cryptosporidium* antigens are used in the current tests as important marker antigens but others have been identified and these need to be further investigated.
- The evaluation of such antigens has been with sera from adults. Their performance for sera from children needs to be evaluated.
- Some tests have been done to exclude cross-reacting antigens (e.g. *Toxoplasma*) but others may need to be tested. It is known, for example, that *Eimeria* may cross-react (Ortega-Mora *et al.* 1992), but this is thought to be of little significance in humans.
- The current tests have been developed with, and use, a particular strain of bovine-derived *C. parvum* and not *C. hominis* and or any of the other species known to infect humans. The relative sensitivity and specificity of these antigens in tests for antibodies in those who have been infected with species other than *C. parvum* is not currently clear and needs to be further evaluated.
- The significance of the intensity of an antibody response, whether expressed as titre, size and/or intensity of a WB

band, OD value, etc., and the impact that this may have on the assessment of the level of immunity (resistance) needs to be further investigated.

The current information is of value in assessing the approximate seroprevalence in certain populations of North America but it is unclear if these are sufficiently representative or robust enough to be used yet in the calculation of a national risk estimate.

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## DISCLAIMER

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