

The epidemiology of cryptosporidiosis: application of experimental sub-typing and antibody detection systems to the investigation of water-borne outbreaks

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Abstract. A technique based on the analysis of banding patterns obtained by SDS-PAGE Western-blotting of an oocyst wall antigen obtained from faeces has been evaluated to subtype *Cryptosporidium parvum* Tyzzer, 1912. This technique appears to have sufficient stability to recognise multiple types of this parasite. A similar Western-blotting technique has also been used to assess antibody responses to cryptosporidial antigens in human sera. Two systems were developed: one against three antigens of apparent molecular weights 6, 14 and 17 kDa; the second against oocyst wall antigens of apparent molecular weights 57, 69, 75, 89, 128, 151 and 173 kDa. Antibodies to three antigens of apparent molecular weights 6, 14 and 17 kDa were most successful as diagnostic markers in that they were found in >88% of convalescent phase sera from confirmed cryptosporidiosis patients and were uncommon (7%) in control subjects. Faecal samples from human and animal sporadic cases yielded a wide range of cryptosporidial antigen banding patterns. Samples from patients in a water-borne outbreak in South Devon (England) in 1995 also yielded a wide range of banding patterns including members within individual household family groups. These results are in contrast with those from samples collected from other defined geographical areas, including some from a second water-borne outbreak where much more homogeneous banding patterns were obtained. Sera collected for other purposes from apparently uninfected individuals 9 months after the South Devon 1995 outbreak were examined. Antibodies to the three antigens of molecular weights 6, 14 and 17 kDa were detected in 32-49% of individuals resident in the outbreak water supply area, and in 15-21% of those resident in an adjacent water supply area. The significance of these findings is discussed in relation to data obtained from epidemiological field studies.

During the past decade a number of water-borne outbreaks of cryptosporidiosis have been described, particularly in the USA and UK (Richardson et al. 1991, MacKenzie et al. 1994, Meinhardt et al. 1996, Furtado et al. 1998). Data on subtyping *Cryptosporidium parvum* Tyzzer, 1912 responsible for water-borne outbreaks and assessment of exposure and/or immunity in the general population would be extremely useful in outbreak investigations, but has not so far been widely applied to this infection.

Because it is not yet possible to amplify *C. parvum* by *in vitro* cultivation, there is only limited information on genotypic or phenotypic diversity of this organism, and definitive methods of subtyping for epidemiological purposes are not well developed. Some diversity, however, has been reported between isolates of *C. parvum* using: antigen banding patterns in SDS-PAGE Western-blotting with monoclonal antibodies (Nichols et al. 1991, Nina et al. 1992); isoenzyme electrophoresis (Awad-el-Kariem et al. 1995); whole DNA restriction fragment patterns (Ortega et al. 1991); arbitrary primer PCR (Morgan et al. 1995); restriction

fragment patterns of amplicons from specific DNA sequences (Bonnin et al. 1996, Spano et al. 1997); and DNA sequence data of a specific gene (Peng et al. 1997). The molecular biological and isoenzyme studies suggests two 'lineages' of *C. parvum*, one found in humans, the other predominantly in animals but with a small proportion also in humans. However, these techniques, at least in part, require relatively large quantities of cryptosporidial material, and have not as yet been extensively evaluated.

It has been reported that there is a specific antibody response to *C. parvum* during infection in both humans and animals, and these have been detected in the IgG, IgM, IgA and IgE antibody subclasses (Ungar and Nash 1986, Casemore 1987, Williams and Burden 1987, Hill et al. 1990, Kassa et al. 1991, Peeters et al. 1993, Reperant et al. 1992). In experimentally infected animals (Williams and Burden 1987, Hill et al. 1990, Reperant et al. 1992, Peeters et al. 1993) serum IgG, IgM and IgA occurred after 4-5 days post infection and persists for at least a month. Similar results have been described for human infections (Casemore 1987, Ungar

et al. 1988, Moss et al. 1994, Braz et al. 1996). Epidemiological studies on larger numbers of human subjects have been performed by investigating antibody responses by immunofluorescence (Casemore 1987, Braz et al. 1996), SDS-PAGE Western-blotting (Moss et al. 1994), or ELISA (Kuhls et al. 1994, Newman et al. 1994). These studies provide valuable evidence for additional undiagnosed cases of cryptosporidiosis as well as exposure in unselected individuals. However, these have, at least in part, relied on complex or poorly characterised antigen preparations. Further work is required to investigate their sensitivity and specificity together with the kinetics of the antibody responses.

It was previously reported that a *C. parvum* oocyst wall antigen recognised by a monoclonal antibody MAb-C1 (McLauchlin et al. 1987) exhibited diversity in SDS-PAGE Western-blotting banding patterns of antigens extracted from the faeces of humans and animals (Nichols et al. 1991). We here report a modification of this technique and further evaluated the stability and discrimination of this method for epidemiological typing.

We have also used a similar Western-blotting techniques for the detection of antibodies in human serum samples and have applied these results with those from subtyping to findings from epidemiological field studies.

MATERIALS AND METHODS

Subtyping system using an oocyst wall antigen

Faecal samples

Human and animal faecal samples were collected by hospital, public health and veterinary laboratories and were tested for the presence of cryptosporidial oocysts using conventional techniques (Casemore 1991). Approximate numbers of oocysts detected (high, medium or low) were recorded for each sample. Seven faecal samples containing a serially passaged *C. parvum*, obtained from the faeces of experimentally infected lambs or calves (Blewett 1989) were obtained from the Moredun Institute, Edinburgh and are thereafter referred to as the 'Moredun' isolate.

Human faecal samples in which *C. parvum* oocysts were not detected but from which *Campylobacter*, *Giardia*, *Salmonella* or *Cyclospora* were detected using conventional techniques were also tested. All faecal samples were stored at 4°C without preservative for up to two years.

Samples of human faeces were collected from patients with apparently unrelated confirmed cryptosporidiosis infections from restricted geographical areas, as well as those involved with two outbreaks of cryptosporidiosis where evidence suggested water-borne transmission.

Outbreak 1

An outbreak occurred in 1995 in an area of South Western England which is a popular holiday destination. There had been one or more earlier outbreaks in the same location and between July and November 1992, 204 confirmed cases of

cryptosporidial diarrhoea were recognised (Anon 1994a). Most of the 1992 cases occurred within a single water distribution area and were mainly in children but with a secondary peak in the elderly and adult visitors. A case control study revealed borderline significance for water consumption but did not indicate any other associations. The water catchment area included a river in a livestock farming area, and the water treatment process included 'blending' of the river water into the supply prior to chlorination but without further treatment. Cryptosporidial oocysts were not found in the water supply.

In 1995, 575 confirmed cases occurred during August to September, largely in the same water distribution area (Anon 1995). The age distribution was similar to that in 1992, but with more patients in the intermediate age groups. There was a significant association with water consumption (descriptive cohort study), as well as a highly significant cohort dose response effect (attack rate) which correlated with the home postal address, water distribution area and the proportion of their water derived from a particular treatment works. Although the water treatment had been modified as a result of the earlier outbreak, low levels of oocysts were detected in the water, and a 'boil notice' was in place for 26 days. Cases from the same general area but who did not receive or consume the suspect water were significantly more likely to have a history of other risk exposures such as direct contact with animals. Faecal samples tested in this study were from patients infected during the 1995 outbreak only.

Outbreak 2

During August to December 1994 there were 224 confirmed cases of cryptosporidiosis within a restricted urban area of Southeast England (Anon 1994b). This outbreak showed a higher proportion of both secondary cases and cases amongst adults and juveniles than in outbreak 1. A case control study of the primary cases showed a significant association with increased consumption of tap water from a blended supply which included river water. The level of association was related to the proportion of river water in the blended supply. Further clusters of cases also occurred in adjacent water supply areas which also received water same river and blended with various proportions of water derived from other sources.

Reagents

Single batches of whole ascitic fluid produced 'in house' were used as a source of a mouse monoclonal antibody. Monoclonal antibody MAb-C1 (IgM isotype) against a cryptosporidial oocyst component (McLauchlin et al. 1987), and two further monoclonal antibodies of the IgM isotype (CL2 and CL17) were used which specifically bind to cell surface antigens on the bacterium *Listeria monocytogenes* (McLauchlin et al. 1988) but not to *Cryptosporidium* sp. oocysts. Detection of antibody was achieved using goat anti-mouse whole molecule horseradish peroxidase (HRP) conjugate (Sigma A412).

Preparation of oocyst lysates

Oocyst suspensions were prepared using a single ether extraction by a modification of the method of Casemore

(1991). The resulting particulate material was washed four times in distilled water by centrifugation (1000 g for 10 min at 4°C) and resuspended in distilled water. The number of oocysts present in selected samples was estimated using a haemocytometer. Oocysts were resuspended in an equal volume of SDS-sample buffer (0.125M Tris-HCl pH 6.8, with 4% w/v sodium dodecyl sulphate (SDS), 10% v/v 2-mercaptoethanol, 20% v/v glycerol, and 0.004% w/v bromophenol blue) and placed in a boiling water bath for 3 minutes. Extracts were stored on ice and usually tested on the day of preparation. The protein content was estimated using the method of Bradford (1976) on oocyst lysates prepared as above but without the addition of bromophenol blue.

SDS-PAGE and Western-blotting

Electrophoresis was carried out using the Mighty Small II electrophoresis system (Hoefer) in 80 × 100 × 0.5 mm sized gels with 5 mm tracks. Discontinuous gels with 10% acrylamide separation gel were prepared using standard techniques. Wells were loaded with 10 µl of oocyst lysate (equivalent to approximately 20 µg of protein): prestained molecular weight markers (193-36 kDa, SDS-7B, Sigma) and duplicate Moredun extracts were included on each gel. Electrophoresis at 20 mA constant current per gel was carried out until the marker dye reached the bottom of the separation gel.

Antigens from SDS-PAGE gels were transferred onto 0.45 µm nitro-cellulose membrane (BioRad) by Western blotting as described previously (Bjerrum and Schafer-Nielsen 1986) in a Trans Blot Semi-dry Transfer System (Bio-Rad) at 15 Volts for 25 minutes. Relative mobilities (Rf) for each molecular weight marker were calculated and if one or more exceeded a 90% confidence limits in a linear regression analysis, data from the blot was not considered and the gel run was repeated.

Western blots were immersed for 1 h in approximately 25 ml of blocking buffer (5% w/v dried skimmed milk, 1% w/v bovine serum albumin, 0.05% v/v Tween-20 in Dulbecco A phosphate buffered saline (PBS), Unipath). The membrane was then incubated in 10 ml of MAb-C1 diluted 1 : 500 in blocking buffer for one hour, washed in 100 ml PBS-T (PBS with 0.05% v/v Tween-20) for 5 min three times and incubated for 1 h in 10 ml of anti-mouse-HRP conjugate diluted 1 : 250 in blocking buffer and washed as above. Bound HRP was detected using the enhanced chemiluminescence ECL™ system (Amersham) and photographic film (Hyperfilm-ECL™, Amersham) according to the manufacturers instructions. Sequential photographs were taken with exposure times varying between 1 and 180 seconds. All incubations were carried out at room temperature.

The migrations of all bands from the sample origin were measured and entered into a spreadsheet and a composite picture of bands identified on all exposures generated using the QuattroPro graph plotter (QuattroPro, Borland International). From the composite picture, gel bands were entered into the GelCompar gel analysis program (Applied Maths, BVBA) using a Genius ColorPage-I flatbed scanner with iPhoto Deluxe software (U-Lead Systems Inc.). The characteristic pattern of seven bands of apparent molecular weights 173, 151, 128, 89, 75, 69 and 57 kDa (Fig. 1)

obtained from the two Moredun extracts were used to normalise each gel run. Composite data from all gels were analysed by calculation of similarities between banding patterns using the Ward algorithm (Ward 1963) and Dice band matching coefficient (Dice 1945). Cluster analysis was carried out on the basis of the Ward and Dice similarities.

Antibody detection in serum

Serum samples and reagents

Human serum samples were obtained during the convalescent-phase following confirmed cryptosporidial infection and from apparently healthy individuals where infection had not been diagnosed and which had been collected for other diagnostic purposes. Ethical approval was obtained for the testing of all sera.

Bound human antibodies were detected using goat anti human polyvalent HRP conjugate (Sigma A8400) and MAb-C1 was detected using anti-mouse HRP conjugates as described previously.

Antigen preparation

Antigen was prepared from oocysts of the Moredun strain of *C. parvum* using two extraction methods. Method 1 (for antigens of >50kDa) comprised of the purification of oocysts from faeces using acid flocculation, a single salt flotation, and ether extraction (Casemore 1991) followed by suspension in SDS sample buffer as described previously. Method 2 (for antigens of <50kDa) consisted of treating an oocyst suspension as above but extracting twice with ether followed by treatment with Sanichlor Super-X disinfection solution (1 tablet dissolved in 2 litres of distilled water to give 2500 PPM available chlorine) for 10 min. The oocysts were washed three times in distilled water, resuspended in an equal volume SDS-sample buffer and freeze thawed (-70 to 100°C) three times. Oocyst numbers were estimated using a haemocytometer.

SDS-PAGE and Western-blotting

SDS-PAGE electrophoresis was carried out as described above but using 10% or 20% resolving SDS-PAGE gels for antigen prepared by methods 1 and 2, respectively. Just prior to electrophoresis, the antigen suspensions (equivalent to approximately 10⁶ oocysts) were heated for 5 min at 100°C and approximately 100 µl of the lysate applied to a single well extending over the entire width of the SDS-PAGE gel together with prestained molecular weight markers as a single track. Molecular weight markers of 193-36 kDa (SDS7B, Sigma) or 49-6.6 kDa (C 3187, Sigma) were used with antigen prepared by method 1 and 2, respectively. Electrotransfer and immuno-blotting using a PR 150 Deca-Probe manifold (Hoefer Scientific Instruments) with the ECL detection system was performed as described before (Patel et al. 1997). Blots were stripped to remove bound antibodies and retested up to three times (Patel et al. 1997).

To identify high molecular weight antigens prepared using method 1 which coincidentally react with both human sera and MAb-C1, immunoblotting was carried out in two stages. Blots were first tested with human sera (using the Deca-Probe) followed by anti-human conjugate, stripped of bound antibodies and retested with MAb-C1 followed by the anti-mouse conjugate. Antigens coincidentally reacting with both

human sera and MAb-C1 were identified by superimposing the two photographic images on a lightbox as described previously (Patel et al. 1997).

RESULTS

Polymorphisms of an oocyst wall antigen

Sample quantification

Satisfactory electrophoresis without ‘overloading’ could be achieved with up to 20 µg of total protein per gel lane. To obtain maximal cryptosporidial antigen concentration per track, samples were adjusted to this protein concentration and this was used in all subsequent experiments. Since the actual amount of cryptosporidial antigen present in the immunoblots, however, varied between samples and with the total protein concentration, dilutions of purified oocysts were added to faeces to simulate a range of 3×10^6 to 10^3 oocysts/g of faeces (equivalent to between high and low numbers of oocysts in the original sample). Following extraction and retesting by immunoblotting, a relationship between the dilution and strength of signal was detected: those with the highest numbers of oocysts gave the strongest signal. Examination of the photographic images showed that some bands caused over-exposure of the film before fainter bands became visible. However, identical or very similar banding patterns were obtained despite decreasing numbers of oocysts (and differences in antigen concentration) by analysis of the sequential series of photographic images obtained with increasing exposure. For example, the typical banding pattern of the Moredun extract (which comprised seven bands, Fig. 1) was obtained in all dilutions of the extract except for that equivalent to initial levels of 1×10^3 oocysts where one of the bands was not detected.

Initially, faecal samples with high numbers of oocysts only were tested. On extending the analysis to samples with low numbers of oocysts, no or only faint bands were usually detected. Therefore, all subsequent data refers to antigen banding patterns from samples originally containing high and medium numbers of oocysts.

The number of bands detected varied from 1 to 15 per sample, and judging by the strength of signal obtained, the number of bands present was unrelated to the concentration of antigen, i.e. single or small numbers of bands did not reflect low antigen concentration or small numbers of oocysts present in the original sample.

Specificity of band detection by MAb-C1

Western blots were prepared with oocyst lysates from 14 different faecal samples (including the Moredun isolate) and also seven similarly treated faecal

samples containing either *Campylobacter*, *Giardia*, *Salmonella* or *Cyclospora* but where *C. parvum* had not been detected. Blots were tested using anti-mouse HRP conjugate alone, plus either the anti-*L. monocytogenes* monoclonal antibodies CL2, or CL17, or MAb-C1 followed by the anti-mouse HRP conjugate. No antigen bands were detected on blots tested using either the anti-mouse conjugate alone, or the anti-*L. monocytogenes* monoclonal antibodies. Bands were only observed in those samples where *C. parvum* had been detected using MAb-C1, and were not observed when testing the seven *Cryptosporidium* ‘negative’ faecal samples.

Stability of banding patterns

Extracts of the Moredun isolate were prepared from the faeces of 5 different experimentally infected animals tested on > 50 occasions. Characteristic banding patterns were obtained when tested over a > 2 year period despite the occasional loss of some of the antigen bands (Fig. 1).

Extracts from 11 human faecal samples were prepared in duplicate on different occasions and tested on different SDS-PAGE gels together with samples from three patients where faecal samples were collected on two different occasions. The 28 samples were assessed for stability of banding patterns and there was a high similarity between the two extracts prepared from the same patient’s sample or extract. Differences in banding patterns obtained from extracts of faeces stored at 4°C for up to 2 years were not observed.

To further assess the stability of banding patterns, six sample of whole faeces containing high numbers of oocysts (one sheep faeces containing the Moredun strain and five human samples) were left at room temperature and re-extracted after one and four weeks.

Table 1. Grouping of 227 cryptosporidial oocyst lysates on the basis of banding patterns of an oocyst wall antigen by SDS-PAGE Western blotting on the basis of cluster analysis using a Ward and Dice similarity matrix.

Group Number	Number of samples			Notes
	Total	Outbreak 1	Outbreak 2	
1	60	0	0	All samples Moredun isolate from experimentally infected animals
2	11	0	0	All samples from a single region
3	42	7	19	
4	9	9	0	
5	25	15	3	
6	57	30	2	
7	9	0	0	All samples from a single region (different from those in Group 2)
8	14	3	0	8 samples from a single region (same region as those in group 7)

Table 2. Detection of specific antibodies in human sera against 10 cryptosporidial antigens by SDS-PAGE Western blotting

Category of human subject	No. of subjects	Numbers of subjects with antibodies against cryptosporidial antigens (% of subjects in each category)									
		173	151	128	89	75	69	57	17	14	6
Molecular weight in kDa		173	151	128	89	75	69	57	17	14	6
Confirmed cryptosporidiosis cases (convalescent phase)	9	1 (11%)	2 (22%)	3 (33%)	3 (33%)	3 (33%)	3 (33%)	7 (77%)	8 (89%)	8 (89%)	8 (89%)
Control subjects (blood donors, pregnancy tests, antinatal screens etc.)	58	0* –	0* –	3* (14%)	2* (9%)	3* (14%)	6* (27%)	10* (45%)	4 (7%)	4 (7%)	2 (3%)
Not identified as infected, resident in the water supply area+	37	0 –	8 (22%)	4 (18%)	6 (27%)	7 (32%)	3 (8%)	6 (16%)	18 (49%)	16 (43%)	12 (32%)
Not identified as infected, resident in the adjacent water supply area+	34	0 –	2 (6%)	2 (6%)	4 (12%)	3 (9%)	11 (32%)	7 (21%)	7 (21%)	7 (21%)	5 (15%)

* 22 samples only tested

+ Sera from individuals not identified as infected and collected in the 9 month period after water-borne cryptosporidiosis Outbreak 2

Banding patterns of these extracts showed only minor differences, although disappearance of some higher molecular weight bands occurred in some samples after four weeks.

Intensity of staining of individual bands

Much inter- and intra-sample variation in the intensity of staining of individual antigen bands (and presumably antigen concentration) was noted and analysis of either all or only intensely staining bands was carried out. Results from 118 samples were analysed in a cluster analysis using Ward and Dice similarities which included 22 duplicate extractions and Moredun isolate controls as described in the previous section. Analysis was initially carried out considering all bands detected, and secondly with only the strongly staining bands (those which were detected within the first ten seconds of exposure to film). The similarity between the replicate extracts was compared under both conditions.

When all bands were considered, all replicates (including extracts from the Moredun isolate) grouped together into single clusters (see analysis, next section). However, when considering only the strongly staining bands, the replicates and Moredun isolate controls were not all segregated together into individual groups. Subsequent analysis was therefore carried out using data considering all bands.

Segregation of banding patterns into discrete groups

Results from 167 samples (10 from animals, 157 from humans) and 60 Moredun isolate controls were analysed using Ward and Dice similarities. Considering an arbitrary similarity of 80%, the banding patterns were segregated into 8 groups (Table 1). Using this method of analysis, all of the patterns obtained from the Moredun controls, duplicate extracts, sequential samples, and stability samples after 0 and 2 weeks storage at room temperature segregated together into their appropriate groupings.

Epidemiological relationships of samples

There was a marked segregation in the patterns obtained dependent on the origins of some of the isolates. For example, all of the Moredun isolates segregated into a single group (Group 1), and almost all samples found in Groups 2, 7 and 8 were obtained from apparently unrelated sporadic cases of human infections collected from three separate geographical locations (Fig. 2).

On analysis of faeces collected from patients involved with the two water-borne cryptosporidiosis outbreaks, samples from outbreak 2 (Fig. 2) showed a high degree of homogeneity and were almost all confined to Group 3 (Table 1). However, those samples collected from individuals involved with outbreak 1 showed very diverse banding patterns (Fig. 1). Furthermore, samples obtained from patients infected during outbreak 1 from 2 or 3 members of seven single household family groups showed considerable intra-family variation in banding patterns in six out of the seven groups.

Antibodies in human sera

Sera were collected from 9 confirmed cryptosporidiosis patients (convalescent phase) together with 58 individuals investigated for other purposes (blood donors, pregnancy tests, antinatal screens etc.). Sera were also tested from individuals not identified as having cryptosporidiosis and resident in either the water supply area (37 individuals) or the adjacent water supply area (34 individuals) of outbreak 1 during the 9 months after the 1995 outbreak. The results for the detection of specific antibodies to the 10 cryptosporidial antigens are shown in Table 2.

The antigens prepared by method 2 (6, 14 and 17 kDa) were most successful as diagnostic markers and were significantly different between controls and infected individuals ($p < 0.001$, Fisher's exact test), although there was also a significant difference between the same groups with the results obtained from the 69,

Fig. 1. SDS-PAGE Western blotting banding patterns of a *Cryptosporidium parvum* oocyst wall antigen isolated from the faeces of 74 patients involved with water-borne outbreak 1 of cryptosporidiosis. Banding patterns were clustered on the basis of a Ward and Dice similarity matrix. Samples marked M are derived from the Moredun isolate.

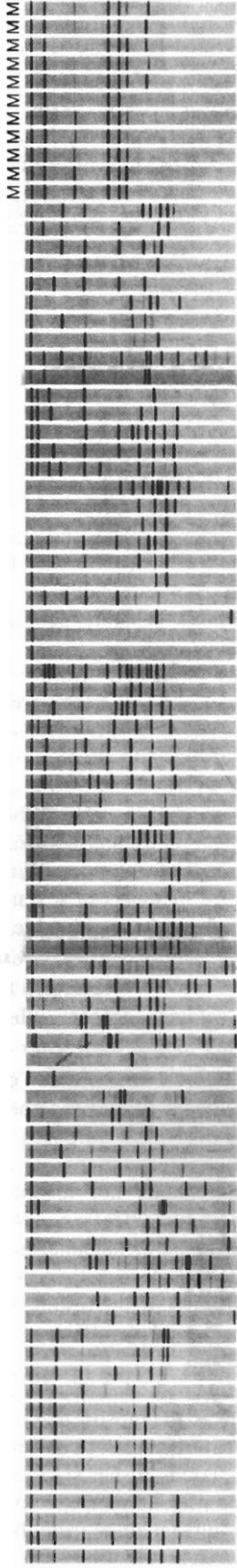
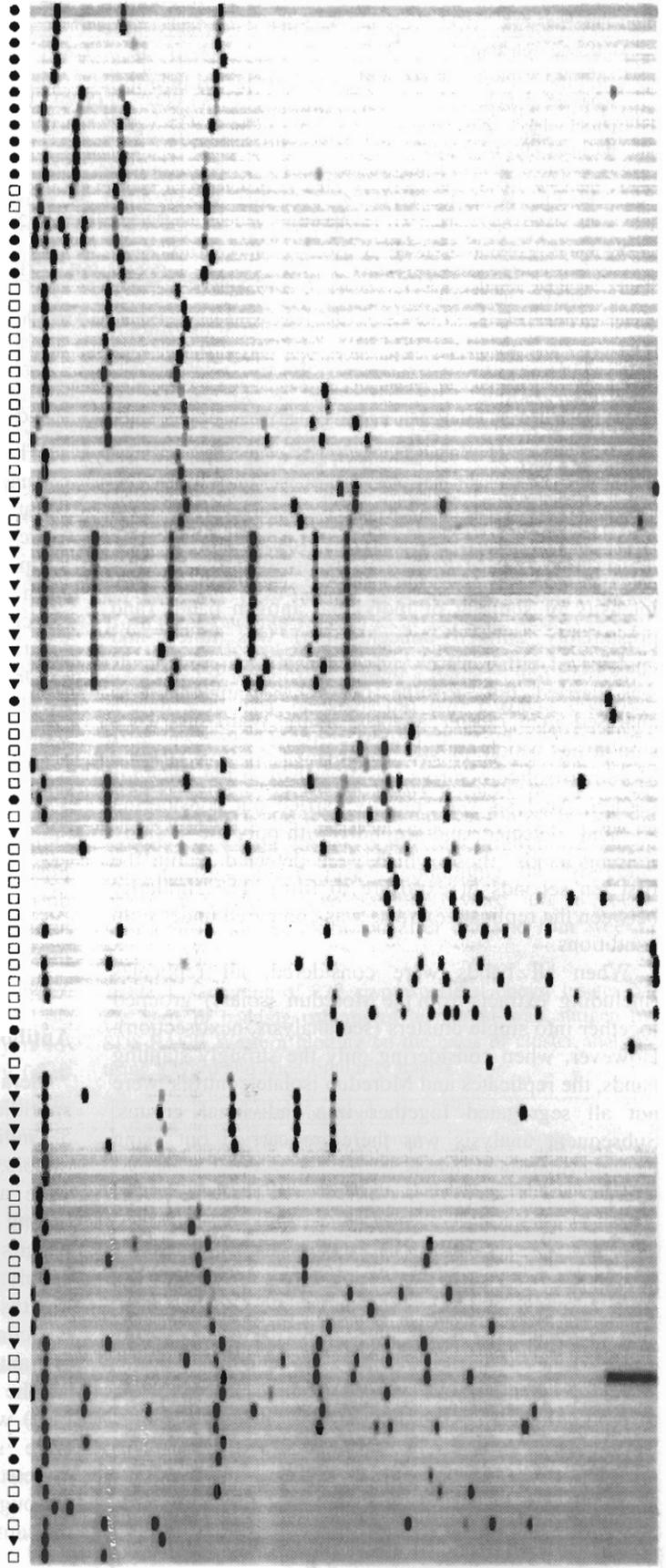


Fig. 2. SDS-PAGE Western blotting banding patterns of a *Cryptosporidium parvum* oocyst wall antigen isolated from the faeces of 97 patients. Banding patterns were clustered on the basis of a Ward and Dice similarity matrix.

- ▼ Apparently unrelated cases, Region A
- Apparently unrelated cases, Region B
- patients involved with water-borne outbreak 2



75 and 151 kDa antigens ($p < 0.05$ Fisher's exact test). Antibodies to the 6, 14 and 17 kDa antigens were significantly more common (χ^2 test, $p < 0.001$) in individuals resident in the water supply area in the 9 month period following outbreak 1, but not in residents from adjacent area (χ^2 test, $p = 0.1$) as compared to the control group.

DISCUSSION

The usefulness of a subtyping system applied to an infectious agent for epidemiological purposes depends upon: the reproducibility of the system to recognise specific characters; the stability of these characters within populations; the numbers of characters recognised (the discrimination); and the ease with which the test can be carried out and applied to adequate numbers of appropriate samples. Since there are difficulties in culturing *C. parvum*, there is therefore limited information on both its phenotypic and genetic diversity which can be usefully utilised for subtyping. In addition, the diversity within *C. parvum* populations is not at present known, and this includes diversity within: an individual oocyst; an individual host; different hosts, or groups of hosts such as human infections involved with a common-source outbreak, or infected animals within a flock or herd. Hence, *C. parvum* presents additional problems to many other culturable and indeed non-culturable infectious agents in both the tools (methodology and materials) available for study, as well as a lack of information on the basic biology of this parasite.

We here applied a simple SDS-PAGE Western-blotting system to study the diversity of an oocyst wall antigen. Our preliminary analysis using samples collected sequentially from individual patients as well as those tested in replicate suggests that this system has sufficient stability to reproducibly recognise at least eight different groupings or 'types' of *C. parvum*. Characterising the same isolates by more than one method has not been previously reported and we will shortly be comparing the results outlined here with those obtained by the application of genetic markers described elsewhere (Bonnin et al. 1996, Peng et al. 1997, Spanno et al. 1997) to the material already tested here. We will also be attempting to analyse the molecular basis of the polymorphisms recognised by MAb-C1 (which are at present not known), although these may not be easily understood by a genetic approach if the diversity is non-protein in nature.

To obtain sufficient antigen to perform the SDS-PAGE analysis, relatively high numbers of oocysts ($>10^4$) are required, and since only faecal samples with relatively large numbers of oocysts were tested here it is not known what bias may have been introduced in the 'types' of *C. parvum* recognised. Clearly this method will be difficult to apply to material from humans or

animals excreting low numbers of oocysts, and is probably not applicable to testing environmental samples such as water. However, the data produced from the techniques described here may provide valuable information on aspects of the epidemiology of cryptosporidiosis (see later discussion) and indicate future directions for the application of methods which can characterise low numbers of *C. parvum*, or indeed individual oocysts.

Data presented here using the Moredun strain showed a high degree of stability in the banding patterns obtained despite passage through both different animals infected over a number of years, and different host species. Preliminary experiments with an oocyst suspension passaged through calves and which had been isolated from a patient infected during water-borne outbreak 1 showed a much higher degree of variations in banding patterns than that showed by the Moredun strain (McLauchlin et al., unpublished data). This observation may represent changes in a single type of the parasite (although on the basis of the results presented here we believe that this is unlikely), or be due to co-infection of the original (or the secondary) host by more than one type of *C. parvum*. This latter explanation together with the possibility of genetic recombinations between types of *C. parvum* will be the subject of future investigations.

It is of note that the pattern obtained using the Moredun isolate differed from all those obtained from material isolated from humans and a small number animal infections (Table 1). This strain has been shown to have a very low infective dose in lambs (Blewett et al. 1993). Subsequent analysis of further samples from humans did not reveal a similar pattern, but this pattern was recognised in samples obtained from other (apparently unrelated) animals.

It has been shown by isoenzyme analysis (Awad-el-Kariem et al. 1995), by analysis of two independent genetic loci (Bonnin et al. 1996, Spanno et al. 1997), by DNA sequencing of a specific gene (Peng et al. 1997), and by using random polymorphic DNA analysis (Morgan et al. 1995) that there are two 'lineages' of *C. parvum*, one of which is primarily involved with infected animals (but also some humans), the other with humans alone. Although these observation should be interpreted with caution, the data presented here that a single animal isolate differed from all those obtained from humans (although animal isolates were recognised which clustered together with banding patterns obtained from humans) further indicates the poor understanding at present of the distribution (and probably classification) of *C. parvum* together with the complexity of the potential reservoirs of disease and multiple potential routes of transmission (Meinhardt et al. 1996). Problems of applying the 'simple' concept of zoonotic transmission for this disease have been commented on elsewhere (Meinhardt et al. 1996).

However, a more complete understanding of the epidemiology of this disease is likely to become apparent when methods become available to track *C. parvum* strains (ideally within populations of other cryptosporidia) within environmental (water and possibly food) samples as well as in a wide variety of hosts.

The preliminary results presented here using a SDS-PAGE Western blotting technique to detect anti-cryptosporidial antibodies suggests that this is a promising method to study the exposure to this parasite and gave similar results with low molecular weight antigens (method 2) to those described elsewhere (Reperant et al. 1992, Moss et al. 1994). The use of the enhanced chemiluminescence detection system, however, has a number of advantages over conventional colorimetric systems, including the ability to take multiple exposures during a single series of reactions and to 'strip' and retest individual Western blots easily. The technique described here detects whole immunoglobulin responses, and we will shortly be refining the technique to characterise the individual subclass-specific responses to both the high and low molecular weight antigens. It is not clear if the differences in the molecular weight of the 6, 14 and 17 kDa antigens to those described elsewhere (Moss et al. 1994) is *C. parvum* strain dependent, or varies for other reasons such as the extraction technique used.

Sera from cryptosporidiosis patients showed strong reactions to antigens of >50 kDa, and these, although present, have not been analysed extensively in previous reports using Western-blotting (Moss et al. 1994). The retest and image overlay technique (Patel et al. 1997) allows simple analysis of these antigens and we will explore further the retesting of blots with antibodies other than MAb-C1 (i.e. polyvalent rabbit sera or convalescent patient sera) to further characterise these antibody responses to other high molecular weight antigens.

The analysis of *C. parvum* types, together with specific antibody responses by SDS-PAGE Western blotting has already presented novel observations concerned with the epidemiology of cryptosporidiosis. For example, it is not clear why *C. parvum* causing human infection within defined geographical areas should be of very restricted types (so called outbreak strains). The presentation of restricted patterns may represent previously unrecognised common source water-borne outbreaks of restricted type (see later discussion), outbreaks originating from a common non-water-borne route (such as food) or outbreaks where there was predominantly person to person transmission within susceptible groups. The latter possibility is supported by the observation that all of the patients within Group 2 (Table 1) were from an urban region with a high proportion of AIDS patients. This was not, however, the situation for the patients within Groups 7

and 8 (Table 1) which were collected largely amongst apparently healthy individuals from the same predominantly rural region, albeit each group largely occurred in different years. In contrast, waterborne outbreaks, particularly following heavy rain, might be expected to involve multiple sources and hence multiple strains of *C. parvum*. Such characteristics might also produce infections in individuals with more than one strain and these two observations are consistent with the results obtained from patients involved with outbreak 1.

Casemore (1995) categorised infective water-borne challenges into three groups dependent on the level of herd immunity:

1) Low herd immunity levels: highest probability that oocysts will be ingested by susceptible persons; high secondary (person to person) transmission rates probable, especially amongst children.

2) Moderate herd immunity: lower probability of ingestion of infective dose by susceptible persons; some secondary cases likely but this is limited by immune individuals creating a secondary (indirect) barrier to transmission.

3) High herd immunity: low probability of ingestion of infective dose by a susceptible person; situation commonly marked by relatively high rates of asymptomatic infection and low rates of secondary transmission. Water-borne transmission may occur but outbreaks are unlikely. Visitors may act as "sentinels", succumbing to symptomatic infection (e.g. travellers diarrhoea).

Field epidemiological studies suggest that the water-borne outbreaks 1 and 2 correspond well to the above descriptions for moderately high and low herd immunity. Epidemiological data suggested that outbreak 1 occurred in a distribution area with prolonged or recurrent contamination of the water supply and hence there was high exposure for residents in the supply area. There was a relatively small proportion of infected individuals in the adult and juvenile age range, except in those who were visitors to the area. Evidence for the high exposure is also supported by the results that apparently uninfected individuals had a significantly higher likelihood of having serum antibodies to the 6, 14 and 17 kDa cryptosporidial antigens (Table 2) after the 1995 outbreak than control subjects. Further work, however, is needed to confirm these preliminary results on a larger series of serum samples and particularly in ensuring that the control groups are adequately matched. We have also obtained serum samples taken from similar individuals collected prior to the 1995 outbreak and predict that these will also show a similarly significantly higher proportion of specific antibodies to those in the control groups. The occurrence of diverse *C. parvum* types both within the outbreak overall and within family groups is also consistent with the epidemiological data that the

prolonged blending of the water supply with contaminated river water is likely to give rise to infections with multiple types. The observation that there was little secondary spread (because of the raised level of herd immunity likely from multiple previous exposures) is also suggested by the diversity of *C. parvum* from within most of the family groups. In contrast, epidemiological data suggested that outbreak 2 showed characteristics associated with low herd immunity in that an initial primary transient exposure through drinking water occurred, followed by a higher proportion of secondary (person to person) spread than in outbreak 1. Outbreak 2 was also of a less prolonged duration and more cases occurred among the adult and juvenile aged groups. Data showing a considerably more homogeneous range of types of *C. parvum* involved with this outbreak (Table 2, Fig. 2) which is also consistent with the hypothesis of a transient common source of infection.

The application of subtyping techniques to *C. parvum* will be instrumental in the elucidation of the overall cycles of transmission for this parasite. If, however, common source outbreaks are usually due to multiple types of *C. parvum*, epidemiological typing may be of limited value in establishing likely routes of

transmission. This remains to be established by the further molecular characterisation of the *C. parvum* responsible for these and other common source outbreaks.

It is not clear what is the clinical significance of the variation of *C. parvum* described here. Evidence has been presented elsewhere that there is variation in infectivity between different *C. parvum* isolates (Pozio et al. 1992, Peng et al. 1997) and that this may be related to the genotype of the parasite. We hope that the further application of the approaches described here will result in a better understanding of the epidemiology of cryptosporidiosis and hence more rational approaches to the control of this disease.

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