ADVANCES IN THE STUDY OF *DIENTAMOEBA FRAGILIS*

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ABSTRACT

Dientamoeba fragilis is a protozoan parasite first described in 1918. It resides in the human large intestine. The distribution of this organism is worldwide. *D. fragilis* has been associated with diarrhoea and abdominal pain in some patients. The goal of the research described in this thesis was to develop new methods in the laboratory diagnosis of *D. fragilis* by using immunological techniques, to develop monoclonal antibodies to study the immunological aspects of the organism, to perform antiparasitic drug susceptibility testing to correlate with treatment recommendations, and to carry out a seroprevalence study for a better understanding of the epidemiology of *D. fragilis*.

An indirect fluorescent antibody (IFA) assay was used to examine the presence of *D. fragilis* trophozoites in preserved faecal specimens. Antiserum to *D. fragilis* trophozoites was raised in a rabbit with a dixenic culture from the American Type Culture Collection (ATCC 30948). After absorption with *Klebsiella pneumoniae* and *Bacteroides vulgatus*, the immune rabbit serum was used for IFA examination. A total of 155 clinical samples were tested: 42 with no parasites, 9 with *D. fragilis* and 104 with other parasites. The IFA assay detected *D. fragilis* in 7/9 known positive samples. There were no false-positive IFA readings. The IFA assay appeared to be a promising method because of its specificity and speed in screening.

Monoclonal antibodies (Mabs) against the parasite was produced in mice and characterized. A MAb (8B2) was obtained against an antigen released when *D. fragilis* was frozen and thawed. MAb 8B2 reacted with cultured *D. fragilis* in indirect

immunofluorescence but did not react with clinical *D. fragilis* strains and it did not immunoblot. Another MAb (1H11) was generated against a sodium acetate-acetic acid-formalin fixed antigen. It reacted with *D. fragilis* isolates in patient specimens and identified a *D. fragilis* 39kDa protein.

Susceptibility testing was performed on *D. fragilis* ATCC 30948. *D. fragilis* was cocultured with the bacteria in ATCC medium 1171. The activities of antiparasitic drugs were assessed by counting viable *D. fragilis* trophozoites with a haemacytometer by trypan blue exclusion. The minimal amoebicidal concentrations of the following four drugs were determined: iodoquinol at 128 μg/ml, paromomycin at 16 μg/ml, tetracycline (questionably) at 32 μg/ml, and metronidazole at 32 μg/ml.

In this seroprevalence study sera from 3 symptomatic patients (F 10 y, M 12 y and F 16 y), 12 age and sex-matched controls, and 189 randomly-selected healthy individuals (age range 6 months to 19 years) were tested by an indirect immunofluorescence (IIF) assay for antibodies. All 3 symptomatic patients infected with *D. fragilis* gave positive IIF titres of 80, and all 12 matched controls were also positive (titre ranges from 20 to 160, geometric mean titer (GMT) was 48). Of the 189 healthy children, 172 (91.0%) were positive at a serum dilution of 1 in 10, or higher. The specificity of the IIF assay was confirmed by immunoblotting 20 representative serum samples against *D. fragilis* (3 negative and 17 positive sera). All 17 IIF-positive serum samples identified the 39 kDa band, suggesting that this may be an important immunodominant protein of *D. fragilis*.

These first reports have contributed to the advances in the study of the epidemiology, immunology, laboratory diagnosis and treatment of *D. fragilis*.

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CHAPTER 1

GENERAL INTRODUCTION

The discovery of *Dientamoeba fragilis*

Jepps and Dobell (1918) published an account of the parasite which is still the most comprehensive morphological description. The following review is largely based on their account. The size of *Dientamoeba fragilis* generally ranges between 8 and 10μm. Pseudopodia are thin, hyaline and "leaf-like" and differ from those of *Endolimax nana, Entamoeba histolytica* and *Entamoeba coli*. Motility can be observed by means of advancing pseudopodia. Food vacuoles, apparently containing bacteria, can frequently be seen within the endoplasm, which is finely granular. Cytoplasmic alveolar differentiation can be seen, but only in stained preparations. Patchy staining with iodine, possibly due to glycogen deposits, can also be seen.

Most trophozoites contain two nuclei, but approximately 20% are mononuclear. Nuclear diameter is related to the diameter of the trophozoites, and is usually around 2 μm. Staining is necessary to visualize the nuclei. The actual appearance of the nucleus is variable and dependent upon the staining process used. A clear zone, with several linin threads radiating through it, can be seen between the nucleus and the karyosome, which is about 3/5 of the diameter of the nucleus. The radiating threads are connected with small chromatin granules, otherwise there is no chromatin visible. Using staining procedures which give adequate differentiation, the karyosome appears to be almost homogeneous, but in specimens in which the stain has been extracted, it consists of distinct granules of chromatin embedded in the plastin matrix. The observation that bacteria and yeasts are frequently found in the food vacuoles suggests that they are the main source of nutrition for *D. fragilis*.

The organism is remarkable for its fragility outside the host, hence the specific name "*Dientamoeba fragilis*". Most of the trophozoites in a stool specimen round up and die quickly after stool evacuation. The initial step is formation of a vacuole which becomes enlarged and oval in shape. Some trophozoites form many vacuoles with the appearance of air bubbles. These trophozoites may live for a short time before death. *D. fragilis* has a large central vacuole which can be mistaken for *Blastocystis hominis*.

Jepps and Dobell were never able to discover any stages of *Dientamoeba fragilis* other than the free trophozoites, nor have cysts been described by any subsequent investigators. *D. fragilis* can be recovered from individuals if the stool specimens are watery or loose, or after a saline purge. The organism is rarely found in formed stool specimens. This characteristic makes *D. fragilis* different from other intestinal amoebae in humans, and more closely related to *Trichomonas hominis* (Jepps and Dobell, 1918).

Based on these observations, Jepps and Dobell (1918) thought it necessary to introduce a new generic name for the organism: and accordingly they proposed the name *Dientamoeba* -- a name suggested by the nuclear structure, which is duplicate in comparison with that of other parasitic amoebae of the genus *Entamoeba*. A diagnosis of the new genus and species was then given:

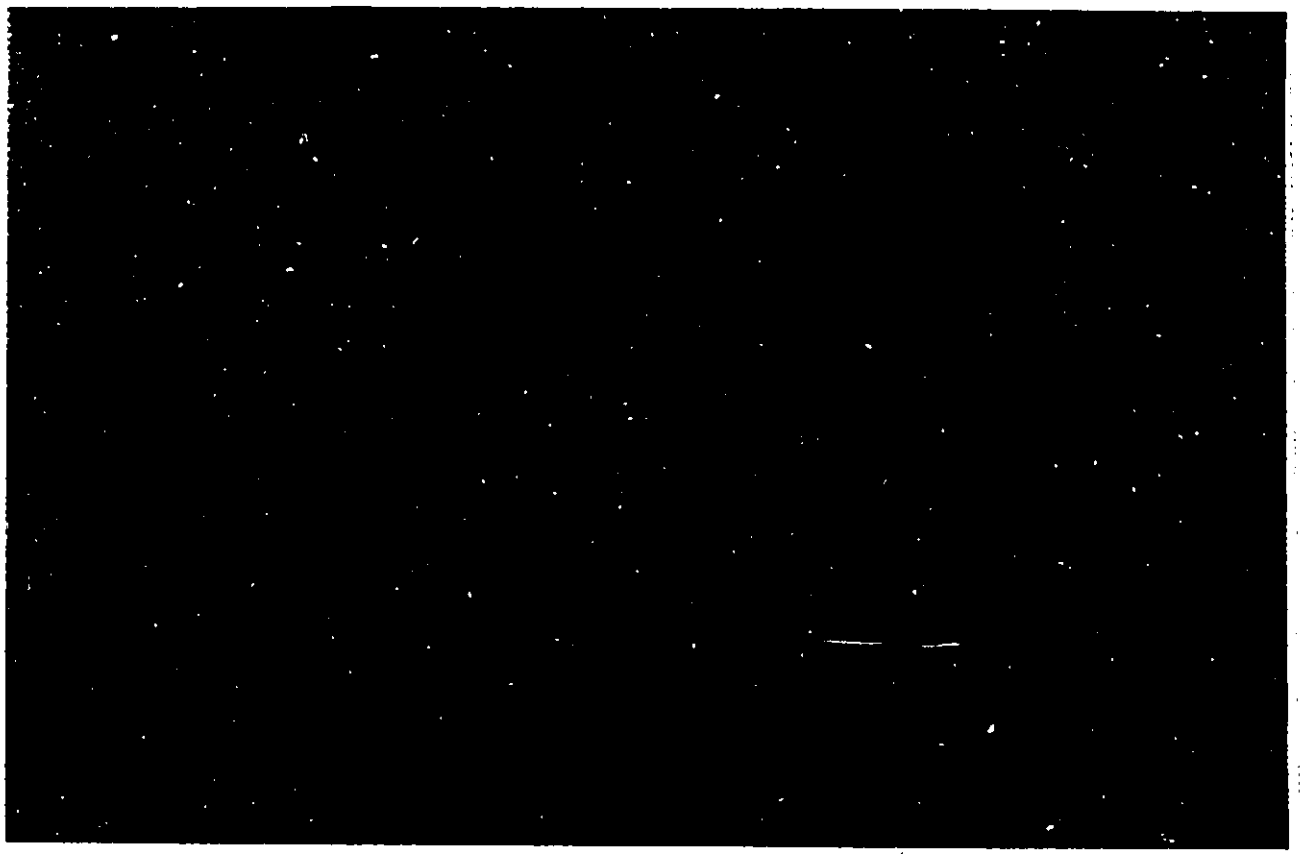
> "*Dientamoeba* nov. gen. -- Small amoeba, with a diameter when rounded of ca. 3.5μ to 12 μ (average ca. 9μ). Cytoplasm differentiated into ectoplasm and endoplasm. Pseudopodia flattened, hyaline, and leaf-like; usually few, with irregularly dentate margins. Progression snail-like. No contractile vacuole. Typically binucleate, both nuclei having the same size and structure. Size of nuclei

ca. 0.8μ -- 2.3μ (average ca. 2 μ). Each nucleus possesses a large central karyosome surrounded by a clear zone containing no peripheral chromatin and limited externally by a very delicate achromatic nuclear membrane. Details of nuclear division unknown; but organism probably divides when binucleate into two uninucleate daughter-individuals, which become binucleate by subsequent division of the nucleus during growth. Cysts unknown. Habitat: intestine (probably colon) of man. Food: small vegetable organisms in intestinal contents.

Only known species *D. fragilis* nov. spec., with characters of genus."

A haematoxylin-stained preparation of the organism is shown in Figure 1.1.

Figure 1.1 ***Dientamoeba fragilis*** **trophozoites (haematoxylin-stained) in a clinical stool specimen.** Both binucleate and uninucleate forms are present.



Dientamoeba fragilis is a flagellate

Since *D. fragilis* was first described (Jepps and Dobell 1918) as a new genus and species of intestinal amoeba from humans a number of different writers have published observations and interpretations concerning this organism. It was regarded (Wenrich 1944) that the most comprehensive paper was that of Dobell (1940), who redescribed the organism, gave an account of nuclear and cell division, recorded observations on cultures and on transmission experiments, and pointed out similarities between *D. fragilis* and *Histomonas meleagridis*, a flagellate that causes "black head" in turkeys.

Dobell (1940) suggested that *D. fragilis* had many of the characteristics of a flagellate. "*Dientamoeba* is indeed, a typical flagellate except for the important circumstance that it possesses no flagella". He cited similarities to *H. meleagridis*, as follows:

1. *Histomonas* grew in all the media that he used for *Dientamoeba*;
2. It ingests red blood corpuscles in cultures, as does the latter;
3. Some of the nuclei contain a group of chromatin granules reminiscent of those of *Dientamoeba*, while others have a "resting" stage like that of the latter species;
4. During division the desmose is a conspicuous feature and may persist until cell division in both species.

To these similarities between *Histomonas* and *Dientamoeba*, Wenrich (1944) added the formation of the peculiar tube-like cytoplasmic differentiation on both organisms, and associated with this the often deeply stainable boundary zone between the ectosarc and the

endosarc. As noted by Wenrich (1944), another flagellate character of *Dientamoeba* is the presence of chromosomal fibres in interphase and prophase nuclei.

In the very limited field of study on the analysis of antigenic relationships of *D. fragilis* with other protozoa, Dwyer (1972a) described a close antigenic relationship was found between *D. fragilis* and *H. meleagridis* by quantitative fluorescent antibody methods. *Trichomonas gallinae* and *D. fragilis* appeared to be less closely related, and still less relationship was noted between *D. fragilis* and *E. histolytica* and *E. invadens* (Dwyer 1972a). Using gel diffusion methods, Dwyer (1972b) reported that *Dientamoeba* antiserum gave many precipitin lines with *Histomonas*, fewer with *Trichomonas*, and fewest with the two species of *Entamoeba*. It was also found by immunoelectrophoresis techniques that *Dientamoeba* shared the most common antigens with *Histomonas*, fewer with *Trichomonas*, and the fewest with *Entamoeba* (Dwyer 1974).

Camp et al (1974) reported light and electron microscopic observations on *D. fragilis* strain A (Bi)1, dealing primarily with the binucleate (arrested telophase) stages. They concluded that their fine-structural investigation lends further support to the earlier observations of Dobell (1940) and Wenrich (1944) that *Dientamoeba* is related quite closely to *Histomonas*. A comparison of the electron micrographs of *H. meleagridis* with those of *D. fragilis* reveals striking similarities between those two species, especially with regard to the periodic filament-Golgi complex assembly, i.e. the parabasal apparatus. As indicated by the light and electron microscopic observations on *Histomonas*, the close relationships between the amoeboflagellate and trichomonads cannot be doubted (Camp et

al 1972). All these kinships are reflected also in the antigenic composition of *Trichomonas*, *Histomonas* and *Dientamoeba* (Dwyer 1972a and 1972b).

The electron microscopic observations by Camp et al (1974) are in complete agreement with the results of Dwyers (1972a and 1972b) based on immunologic comparisons according to which *Dientamoeba* and *Endamoebidae*, as represented by *E. histolytica* and *E. invadens*, are related rather remotely. Camp et al (1974) noted that many basic fine-structural differences exist between *Entamoeba spp.* and *Dientamoeba*:

1. In *Entamoeba* there is no equivalent of the atractophores and of the parabasal apparatus-like organelle, both of which are present consistently in dividing *Dientamoeba*;
2. An extranuclear spindle, typical of trichomonads, and hypermastigotes, and seen in *Dientamoeba*, has not been reported in dividing *Endamoebidae*;
3. The microbody-like cytoplasmic inclusions, very characteristic of trichomonads, have not been found in *Entamoeba*. The "microbodies" of Ludvik and Shipstone (1970), described by these workers in *E. histolytica* as possibly lipid in nature, appear to differ in structure from the microbody-like inclusions of trichomonads and *Dientamoeba*;
4. The ribonucleoprotein bodies described from *E. histolytica* and *E. invadens* as helical fragments and as parallel arrays of helices (typical chromatoid bodies) evidently are absent from *D. fragilis*;
5. The osmiophilic cylindrical bodies (described for *E. histolytica*) have not been

found in *Dientamoeba*.

Based on their observations on the fine structure of *Dientamoeba*, and comparison with those of trichomonads and of *Entamoeba spp*., Camp et al (1974) suggested that the genus *Dientamoeba* to be placed among trichomonas in the family Dientamoebidae Grassé, emend. In a newly revised classification of the protozoa, *Dientamoeba* is placed in Phylum I. Sarcomastigophora, Subphylum I - Mastigophora, Class 2 Zoomastigophorea, Order 7. Trichomonadida (Levine et al 1980). Trichomonadida is represented by the flagellates *Dientamoeba*, *Histomonas*, *Monocercomonas* and *Trichomonas*.

Pathogenicity of *Dientamoeba fragilis*

In their 1918 publication of the first description of *D. fragilis*, Jepps and Dobell gave a detailed account of their investigations on this protozoan parasite. At that time they expressed their opinion that there was no reason to believe that *D. fragilis* was potentially pathogenic. Yet in three of the seven cases they investigated, there were clinical histories of diarrhoea and/or abdominal pain long before the demonstration of the parasite that could not be satisfactorily explained on other grounds. Evidence that *Dientamoeba* might sometimes be a pathogenic agent was presented subsequently in a number of reports (Gittings and Waltz 1927, Hakansson 1936, Wenrich 1937, Sapero 1939, and Hood 1940). In his later detailed account of the life history of *D. fragilis*, Dobell (1940) pointed out the possible relationship of this parasite to the pathogenic flagellate *Histomonas meleagridis*. He therefore did not rule out the possibility of a pathogenic potential of *Dientamoeba*, even though he was still in doubt.

Further published reports suggest that the presence of *D. fragilis* may evoke various symptoms (Yang and Scholten, 1977a, Table 1.1) which, in most cases, disappear with the elimination of the parasite with anti-parasitic therapy. Clinically, the commonest syndromes are abnormal stools, abdominal pain, fatigue, loss of appetite, and weight loss. Pathological and microscopic features of *D. fragilis* infection reported include: ingestion of red blood cells (Dobell 1940, Burrows et al 1954, Swerdlow and Burrows 1955), mucosal edema (Kean and Malloch 1966), and fibrosed appendix (Burrows et al 1954). Low-level eosinophilia (Gittings and Waltz 1927, Hood 1940, Spencer et al 1979) has been reported in some patients. The usual site of infection is the large intestine, but infection of the biliary tract (Talis and Stein 1961, Steinitz and Talis 1964, Talis et al 1971) has also been reported. In the latter, the description based on microscopy is consistent with that of *D. fragilis*. Cases have been described in parasitologists, physicians themselves and their families (Hakansson 1936, Wenrich 1944, Burrows and Swerdlow 1956, Desser and Yang 1976). It was reported by Wenrich et al (1936) that more college students were symptomatic when infected with *D. fragilis* than with *Entamoeba histolytica*. Sapero (1939) found that 27.3% of individuals harboring *D. fragilis* had symptoms. In the study by Steinetz et al (1970), 15.1% of subjects infected with *D. fragilis* were symptomatic. In Canada, over 50% of a series of 255 patients were symptomatic in a study reported by Yang and Scholten (1977a).

Gastrointestinal disturbances were seen in children as well as in adult patients infected with *D. fragilis* alone. In a retrospective study of "pure" *D. fragilis* infections, Spencer et al (1979) found gastrointestinal symptoms in 32 (91%) of 35 children. It was

noted that diarrhoea was the most common finding in patients with acute illness, whereas abdominal pain was more common in children with chronic symptoms. Investigations by Ockert (1990) of 387 kindergarten children, with or without diarrhoea, yielded a significantly higher number ($p < 0.001$) of patients infected with *Dientamoeba* in children with diarrhoea than in the asymptomatic group (Table 1.2). Spencer et al (1982) also reported a study in an adult population with similar findings as in their earlier study in children (Spencer et al 1979). Millet et al (1983) described that there were significant differences in the frequencies of symptoms between adults with and without *D. fragilis* infection. Eighty-five percent of 26 infected adults exhibited more than one of the following symptoms: flatus, irritability, and weakness in 54% of the patients; abdominal pain lasting longer than one month in 46%; anorexia in 27%; nausea and vomiting in 19%; constipation in 15%; and diarrhoea in 8%. Ockert (1990) observed that the most frequent symptom was abdominal pain in 21 of 37 adult patients. This was followed by changing stool consistency in 20 patients, diarrhoea in 16, meteorism and mucus in stools in 11, loss of weight in 9, and bloody stools in 5 patients.

Very little knowledge has been gained on the study of pathogenic effects of *D. fragilis* and of the pathologic changes it may cause. Burrows et al (1954) and Swerdlow and Burrows (1955) reported histologic findings obtained by examining the appendices removed from patients infected with *D. fragilis*. In the appendix of 10 of 15 patients *Enterobius vermicularis* was also present. The histopathologic changes observed in the wall of the appendix ranged from suppurative appendicitis to lymphoid hyperplasia and marked fibrosis. The most common change was the increased expansion of the fibrosis

area of the connective tissue in the submucosa, which in most cases displaced lymphoid tissue of the mucosa. To some degree, these changes occurred also in the internal layer of the muscular coat, or bandlike or finger-shaped eversions extended to the mucosa. It was suggested that fibrosis of the appendix may occur with increased age and perhaps also as a result of repeated inflammation. However, the patients examined by Swerdlow and Burrows (1955) were young (4 to 15 years of age). It seemed that *D. fragilis* might have acted as the causal agent of the inflammatory process, over a lengthy period of time. The result of this could be a minimal inflammatory change in the appendix that ultimately led to increased fibrosis of the connective tissue. It was also noted that red cells had been ingested by the organisms.

Shein and Gleb (1983) reported a case of colitis caused by *D. fragilis* diagnosed endoscopically. Numerous punctiform ulcers, about 0.2 cm in diameter, were found in the intestine of the female patient infected with the parasite. The ulcers had yellowish centres and erythematous halos. The nonfriable areas of the mucosa located between the ulcers also showed erythematous changes. Flat ulcerations and acute as well as chronic inflammation could be demonstrated in a biopsy. In the patients examined by Ockert and Schulz (cited in Ockert 1990), there appeared in many instances inflammatory changes of the mucosa of the rectum and sigmoid colon. The areas of inflammation had the form of superficial rounded infiltrations of cells in loosened stoma yielding a picture of low-grade chronic proctitis.

It could not be demonstrated through animal studies that *D. fragilis* could cause ulcers in the colon (Ockert 1990). Lamy (cited in Ockert 1990) asserted that

multiplication of *Dientamoeba* by itself cannot cause disturbance of intestinal functions, but that the parasite may play a role in such a disturbance in concert with suitable intestinal bacterial flora. Such a combination of bacteria and the parasite may lead to the development of an optimal environment for the colonization by and multiplication of *Dientamoeba*. An example of such a situation is the irritability of the colon. Under these conditions *Dientamoeba* could assume the role of a pathogen. There must be taken into account the host-specific standard responses to pathologic stimuli as well as the individual susceptibility of the host as essential factors determining the progression of the disease. It is thus determined if such an infection can cause a shift of balance in the host-parasite relationship and thereby a disease in the host. For example, morphologic damage to the intestine, disturbances in the microbiology of the large intestine, and/or serious primary diseases as well as colonization and multiplication of *D. fragilis* give rise to the appearance of clinical symptoms (Ockert 1990). However, *D. fragilis* is often considered a harmless commensal (Yang and Scholten 1977a), mainly because studies of its prevalence and associated symptoms were uncontrolled and they suffered from selection bias. Randomized, double-blind controlled studies are needed to determine the pathogenic roles of this parasite. It is worthwhile to note that the inclusion in the Medical Letter (1992) of recommendations of treatment for *D. fragilis* infections implies an acceptance of the organism as a pathogen.

Table 1.1 Symptoms in *Dientamoeba fragilis* infections*

Symptoms	% of patients	
	OPH**	Literature
Diarrhoea	58.4	42.5
Abdominal pain	53.7	46.2
Anal pruritus	11.0	2.7
Abnormal stool (bloody, with mucus, loose)	9.8	22.6
Urticaria	6.7	0
Flatulence	5.9	19.9
Fatigue or weakness	5.9	13.4
Eosinophilia	5.1	4.3
Alternating diarrhoea and constipation	3.9	13.4
Nausea or vomiting	3.5	20.4
Weight loss	3.1	10.2
Constipation	2.4	6.5
Belching	2.0	5.4
Tenesmus	1.2	5.9
Anorexia or malaise	1.2	5.4
Others	2.0	18.3
No. of patients	255	186

** Records of Ontario Public Health Laboratory, 1970 to 1974

* Reference: Yang and Scholten (1977a)

Table 1.2 Diarrhoea in *D. fragilis*-positive and -negative preschool children*

Diarrhoea	Number	*D. fragilis*	
		Positive	Negative
Present	168	69	99
Absent	219	54	165

$X^2 = 11.85$, $p < 0.001$

* Reference: Ockert (1990)

Prevalence, epidemiology and transmission of *Dientamoeba fragilis*

If appropriate techniques are used, *D. fragilis* may prove to be ubiquitous. It has been reported in many parts of the U.S. (Burrows and Swerdlow 1956, Kean and Malloch 1966, Bruckner et al 1979, Spencer et al 1979, Millet et al 1983) and in Germany (Ockert 1990). Defined populations such as those of mental institutions, missionaries or Arizona Indians, may have a higher prevalence (Svensson 1928, Brug 1938, Weiner et al 1959, Melvin and Brooke 1962, Mcquay 1967, Ockert 1972a). The use of laxatives increased the recovery rate of the organism (Svensson 1928, Sapero 1939). Different collection techniques and variation in sensitivity of microscopic observations make it difficult to compare on frequency.

One major epidemiological difference between *D. fragilis* and the other protozoa is that *D. fragilis* is no commoner in children from residential homes than from day care centres (Ockert 1990). This contrasts with *G. lamblia* and *E. histolytica*, for both of which residential boarding is a risk factor.

Recovery rates for *D. fragilis* in Canada (Porter 1934, Fantham and Porter 1936, Miller 1939, Saunders 1949) are very variable, and the high incidence reported in Toronto (Scholten and Yang 1975) in comparison with the rest of the country may be a reflection of the intensity of the search for the organism and the use of more sensitive techniques.

Of 64,544 preserved stools submitted by 43,029 individuals over the years from 1970 to 1974, 2,664 samples from 1,791 patients were found to contain *D. fragilis* alone or in combination with other parasites in a study reported by Yang and Scholten (1977a). It was noted by the authors that yearly findings of *D. fragilis*, although fluctuating in absolute

numbers, remained, within limits, constant relative to the number of preserved stools (av. 4.1%). The occurrence of *D. fragilis* in single and multiple infections was compared with a similar analysis of 1,697 *E. histolytica* cases. In contrast to infections by the latter, which occurred most often in association with other faecally transmitted parasites, nearly 60% of the *D. fragilis* cases occurred as single infections only (compared to less than 15% for *E. histolytica*).

In summary, the prevalence of *D. fragilis* differs in several respects from that of other intestinal protozoa. It indicates that the epidemiologic factors responsible for transmission of *Dientamoeba* cannot be equally favourable for transmission of the other protozoan species (Ockert 1990).

Transmission by means of faecally contaminated food or water seems unlikely (Yang and Scholten 1977a) in view of the absence of a cyst stage in *D. fragilis* and because of observations that the parasite cannot survive for any length of time in water (Hakansson 1935 and 1936, Wenrich 1944). Attempts to infect a human volunteer and animals orally also failed (Dobell 1940, Wenrich 1944, Knoll and Howell 1945). Yang and Scholten (1977a) also reported that they could not keep trophozoites alive in simulated gastric juice. This raises the question of how *D. fragilis* is transmitted between individuals.

The possibility of transmission of *D. fragilis* via the egg of an intestinal nematode was first pointed out by Dobell (1940), on the basis of the observations of Graybill and Smith (1920), and also those of Tyzzer (1934), who recognized the vector role of the eggs of the bird nematode *Heterakis gallinarum* in the transmission of *Histomonas meleagridis*, a

flagellate parasite in poultry. Because this species is related to *Dientamoeba*, Dobell assumed a similar relationship of *D. fragilis* with worms, such as *Trichuris*, the whipworm. However, *Dientamoeba* has high prevalence in areas with low prevalence of the whipworm, e.g. in Scandinavia (Svensson 1928). Burrows and Swerdlow (1956) postulated for the first time a connection between *Dientamoeba* and the pinworm, *Enterobius vermicularis*. They examined 1,518 appendices and found that *D. fragilis* and *E. vermicularis* occurred 20 times more frequently than was theoretically expected on the basis of random and independent distribution of these two parasites. Burrows and Swerdlow (1956) also found structures resembling *D. fragilis* in the eggs of pinworms occurring in appendices dually infected with *D. fragilis*, but not in eggs from pinworms in appendices in which *D. fragilis* was absent. Examination of the published photographs revealed structures within the eggs which would be consistent with *D. fragilis*, but the appearance is not definitive. Investigations aimed at a conclusive confirmation of these relationships did not yield positive results. Burrows and Swerdlow (1956) attempted to demonstrate *Dientamoeba* in the eggs, or in the larvae enclosed in pinworms with the following methods:

1. Pinworm eggs from patients positive for *Dientamoeba* were crushed, then inoculated into culture media for *Dientamoeba*.
2. Before being inoculated into the culture medium, pinworm eggs were stimulated to develop in artificial digestive juices.
3. The egg material was inoculated into white mice.

None of these methods yielded cultures of *D. fragilis*. However, when Burrows became accidentally infected with *D. fragilis*, he also found himself infected with *E. vermicularis*, although several stools were negative for both parasites before (Burrows and Swerdlow 1956). One investigator (Ockert 1972b) having examined his own stools and found them negative for *D. fragilis* trophozoites, voluntarily infected himself with acid washed pinworm ova for a convincing clean preparation free from any possible surface contamination with *D. fragilis*, and was subsequently able to demonstrate *D. fragilis* in his stools. Additional studies by Ockert (cited in 1990) included a total of 37 isolated attempts from 11 patients (children 3 to 6 years of age) who harboured simultaneously *Enterobius* and *Dientamoeba*. Eggs obtained from worms were allowed to develop in artificial digestive juices, and then inoculated to culture medium for *D. fragilis*. Growth of *Dientamoeba* could not be demonstrated, despite repeated passages (up to seven).

Yang and Scholten (1977a) attempted to determine if there was an association between infections of *D. fragilis* and a nematode with observed and statistically expected numbers of infections involving *D. fragilis* and each of four common nematodes. Statistically expected numbers were calculated as follows:

> "Expected number of samples (or patients) containing both parasite A and parasite B equals the product of the relative prevalence of A and of B. multiplied by total number of samples (or patients). i.e. E = pA x pB x N."

Observed and statistically expected numbers of infections involving *D. fragilis* and *Ascaris lumbricoides*, hookworms, *Trichuris trichiura*, and *E. vermicularis* are presented

in Table 1.3.

Table 1.3 Observed and expected frequencies of infections involving *D. fragilis* (p=0.0416) and each of four nematodes, regardless of other species of parasites present (N = 43,029)

Nematode species	P	Observed	Expected	Ratio (O/E)
Ascaris lumbricoides	0.0159	22	28.5	0.8
Hookworm	0.0274	31	49.0	0.6
Trichuris trichiura	0.0775	144	138.7	1.0
Enterobius vermicularis	0.0013	20	2.3	8.7

Reference: Yang and Scholten (1977a)

The frequency with which *D. fragilis* is associated with pinworm infection is nine times the statistical expectation, whereas there is no such association between *D. fragilis* and other helminths (Yang and Scholten 1977a). The circumstances described above have been advanced to support the concept that the pinworm may serve as a vector for *D. fragilis*. However, whether the pinworm can function as an agent in the transmission of *D. fragilis* from host to host has yet to be proven.

Laboratory diagnosis of *Dientamoeba fragilis* infections

As pointed out by Wenrich early in 1944, the frequent absence of *Dientamoeba* from lists of parasites in survey reports or its mention with a very low percentage of incidence might indicate either that the methods employed were unsuitable for its recognition, or that the investigators were not sufficiently familiar with the organism to identify it. For the diagnosis of *Dientamoeba*, the following are needed (Wenrich 1944):

1. Sufficient experience in its recognition;
2. soft or fluid faeces;
3. examination while the faeces are still fresh; and
4. recognition of the organisms in fresh smears and/or on properly fixed and stained slides.

Wenrich (1944) also mentioned that cultures might reveal positives missed by direct examination, but it was stressed that the most reliable method was to make properly fixed (in Schaudinn's fluid) and stained slides (stained with iron-haematoxylin).

As described by Wenrich (1936), the nuclei of *D. fragilis* trophozoites (typically with two nuclei) are made up of 4 to 6 granules. The laboratory diagnosis of *D. fragilis*

infections relies on the demonstration of these trophozoites with the typical nuclear structure. Because these trophozoites are fragile it is mandatory that stool specimens must be collected and processed properly, preferably with multiple specimens collected over several days, for a reliable diagnosis (Yang and Scholten 1977a). The authors also stressed that "fresh" specimens should be examined promptly, or alternatively, the specimens should be collected in appropriate fixatives to preserve the trophozoites. Acceptable fixatives for the preservation of *D. fragilis* include polyvinyl alcohol fixative (Goldman and Brooke 1953), modified Schaudinn's fixative (Scholten 1972), or sodium acetate-acetic acid-formalin fixative (Yang and Scholten 1977b). Yang and Scholten (1977a) did not find merthiolate-iodine-formalin (Sapero and Lawless 1953) acceptable because of its suboptimal staining for *D. fragilis*. It is recommended that a permanent stain should be performed on smears made from fresh or fixed stool specimens for the identification of *D. fragilis* trophozoites. This is based on the fact that concentration procedures (either flotation or sedimentation methods) will not recover trophozoites, and that simple wet preparations do not reveal adequate details of the organism for proper identification (Yang and Scholten 1977a). Haematoxylin (Scholten and Yang 1974) was the stain preferred by Yang and Scholten (1977a). They also found that trichrome (Wheatley 1951, Alger 1966) and celestine blue B (Yang and Scholten 1976) were acceptable stains for the identification of *D. fragilis* trophozoites.

It may be difficult to identify *D. fragilis* under some circumstances. This happens when the majority of the trophozoites have only one nucleus, or when the granules of the nucleus do not appear as distinct entities, or when these granules are obscured by stain

deposits. Under these circumstances *D. fragilis* may be mistaken as *Endolimax nana* trophozoites (Yang and Scholten 1977a). Conversely, *E. nana* trophozoites may have an "atypical" nuclear structure (Yarinsky and Burrows 1966), or they may have two nuclei, or they may be colonized by *Nucleophaga* (a hyperparasite). As a result of these complications, *E. nana* may be misidentified as *D. fragilis* trophozoites. Additionally, *Pentatrichomonas hominis* and *Blastocystis hominis* may mimic *D. fragilis* in morphology, and the microscopist can be confused by these organisms in the identification of *D. fragilis* (Yang and Scholten 1977a).

Ockert (1990) believed that the great variation in the recovery rates of *D. fragilis* reported by different centres was method-dependent. Microscopic examination of wet mounts prepared directly from stool specimens is a common practice in laboratories because it is a simple procedure. However, this procedure is reliable only when fresh specimens are examined without delay. Under optimal conditions, *D. fragilis* trophozoites are usually seen in wet preparations as rounded structures with a size range of 5 to 15 μm. *D. fragilis* trophozoites sometimes exhibit actively-advancing pseudopodia and they may resemble *Entamoeba hartmanni* or *Endolimax nana* (Ockert 1990). The pseudopodia of *D. fragilis* are distinctly different from other intestinal amoebae by their serrated presentation, and, together with its leaf-shaped lobopodia, the organism offers characteristic features for its identification in wet preparations. Phase contrast or darkfield microscopy may also help to observe movement of pseudopodia (Ockert 1990). Unfortunately, the usefulness of wet preparations for identification is very limited because

D. fragilis trophozoites will deteriorate rapidly in morphology. Once outside the body for more than an hour, the trophozoites may no longer be recognized despite the fact that they are still viable and culturable (Hakansson 1937, Dobell 1940).

Ockert (1990) also felt that *Dientamoeba* can be positively identified in permanently stained smears made from fresh stool specimens. The procedure must be carried out promptly as older specimens (a few hours old) will not exhibit the characteristic morphology of the organism. Ockert (1990) also prefers the haematoxylin staining method. An adequate volume of glacial acetic acid in fixatives is recommended for proper nuclear fixation and staining. Attention to the following is advised by Ockert (1990) for simple staining methods:

"1. Fixation of thin (20 μm) faecal smears.

2. Sufficiently large amounts of a fixative or a mixture of stain and fixative.

3. Rapid placement of the faecal smear into the stain-fixative mixture or rapid overlay of the smears with this mixture.

4. Complete rinsing out of the mixture.

5. Employment of very pure solutions of reagents."

Ockert (1990) stated that culture is the most reliable method to identify *D. fragilis* infections. In a study carried out in the Halle District in Germany, the rapid staining method identified *D. fragilis* in 0.6% of 3,670 children. The same procedure demonstrated that 3% of another 576 children were infected by the organism. Surprisingly, 35% of the same population were found infected by a combination of staining and culture procedures. Similar results were obtained in a further study involving

1,066 individuals. In this further study, the identification rate by stained smears was 1.97%, versus a rate of 39.31% by the combination of staining and culture (Ockert 1990).

It has been suggested that the diphasic medium of Dobell and Laidlaw (1926) is appropriate to recover *D. fragilis* trophozoites. Ockert (1990) modified the medium and used coagulated human serum in the solid phase. A liquid medium could also be used to satisfactorily recover the protozoan. The formulation of this liquid medium includes addition of 5 to 10% bovine serum to the Ringer's solution. To meet with the growth requirement for *D. fragilis*, rice starch granules are also added to either the diphasic or liquid medium.

Primary cultures may be somewhat restricted to yield optimal growth of *D. fragilis* (Ockert 1990). One way to compensate for this limitation is to perform further subcultures. It was reported by Ockert (1990) that primary cultures yielded 40% of positives, whereas a first subculture would increase the yield to 80%, and a second subculture to 100%.

Recently Sawangjaroen et al (1993) reported the first survey in Australia to use faecal culture to detect *D. fragilis* in patients with diarrhoea of 3 different protozoal culture media evaluated in a case of known infection. Modified Boeck and Drbohlav's medium was the most suitable. The organism could be grown from faeces stored up to 24 hours at room temperature, but for only 10 hours at 4°C. Culture was then used, in combination with microscopy of smears fixed with polyvinyl alcohol and trichrome-stained, to examine single stool specimens from 260 consecutive patients with diarrhoea in the city of

Brisbane. *D. fragilis* was detected in 4 (1.5%) specimens, only 2 of which were positive by microscopy. The authors noted that they were successful in reliably growing *D. fragilis* only by the modified Boeck and Drbohlav's medium. However, failure of trophozoites to grow does not invariably mean that a particular medium is unsuitable; strain variation in the protozoan species, or changes in the accompanying bacterial flora, may influence the adaptation of protozoa to the culture medium (Robinson 1968). Robinson medium (1968) was also found to be suitable for the cultivation of *D. fragilis* (Gonzalez-Ruiz 1994).

Although culture appears to be an appealing method for the recovery of *D. fragilis*, the demand on fresh specimens to be delivered to the laboratory within a short time is a great drawback on the requirements of the culture technique. Extreme weather conditions in places such as Canada may further hamper the viability of the trophozoites in transportation and therefore affecting their recovery. These limitations will make culture an impractical method for the demonstration of *Dientamoeba* in many laboratories.

To date the only available practical method of laboratory diagnosis of *D. fragilis* is microscopic examination of stained smears of preserved faecal specimens for the characteristic trophozoites (Scholten and Yang 1974, Grendon et al 1991). Of 15,807 paired preserved (modified Schaudinn's fluid, haematoxylin-stained smears) and unpreserved (formalin-ether concentrates) stool samples, 564 specimens were found to contain *D. fragilis* in the report by Scholten and Yang (1974). One hundred percent of *D. fragilis* was identified with the preserved samples, while 0% was found in the unpreserved samples (Scholten and Yang 1974). Grendon et al (1991) carried out a survey of State

Public Health Laboratories for their study on *D. fragilis* detection methods and prevalence. They also found that failure to use recommended stool fixation and permanent staining techniques almost precludes identification of *D. fragilis*. In order to increase the probability of detecting *D. fragilis* in stool specimens, the authors suggested that all stools should be submitted fixed in polyvinyl alcohol fixative, sodium acetate-acetate acid-formalin fixative, or Schaudinn's fixative. Furthermore, all specimens, regardless of consistency, should be permanently stained prior to microscopic examination (Grendon et al 1991).

To assess the need for routinely submitting three stool samples per patient for recovery of enteric parasites, Senay and MacPherson (1989) reviewed the records of their parasitology laboratory for 1985-87 to determine the number of parasites that would not have been detected if only one or two samples had been submitted. A total of 16% of all stool samples were positive. For each sample that was positive for a parasite (index sample) a search was done for other stool samples, positive or negative, received from the same patient within 6 days of reception of the index sample. They identified 676 sets of two (276) or three (400) samples of which at least one was positive. A total of 93% of the enteric parasites were detected in the first sample in the two-sample sets. Among the three-sample sets 90% of the parasites were detected in the first sample, 8% in the second and 2% in the third. The authors recommended waiting for the result from the first stool sample rather than routinely submitting three samples for recovery of enteric parasites (Senay and MacPherson 1989).

It is reckoned that diagnostic parasitology, especially protozoology, is perhaps one of

the most difficult of all routine laboratory investigations (Elsdon-Dew 1971). Because competence to accurately diagnose parasitic infections is not easily obtained, as has been emphasized by the American Academy of Pediatrics (1974) and the World Health Organization (1969), physicians should perhaps be more concerned about the competence of the laboratory to which they submit samples and be better informed of the techniques used by the laboratory before accepting positive or negative reports at face value. It may well be that many cases of abdominal distress of hitherto unknown etiology are, in fact, due to *D. fragilis* (Yang and Scholten 1977a).

Treatment of *Dientamoeba fragilis* infections

In the earlier days carbarsone (Hankasson 1936) was used to treat patients infected with *D. fragilis*. The use of emetine, carbarsone and tetracycline was also mentioned by Kean and Malloch (1966) in the treatment of *D. fragilis* infections.

The current drug of choice (The Medical Letter 1992) to treat *D. fragilis* infections is iodoquinol, paromomycin or tetracycline. Information on treatment is given in Table 1.4.

Table 1.4 Drugs for treatment of *Dientamoeba fragilis* infections

	Drug	Adult Dosage*	Pediatric Dosage*
Drug of choice	Iodoquinol[1]	650 mg tid x 20d	40 mg/kg/d in 3 doses x 20d
OR			
	Paromomycin	25-30 mg/kg/d in 3 doses x 7d	25-30 mg/kg/d in 3 doses x 7d
OR	Tetracycline[7]	500 mg qid x 10d	40 mg/kg/d in 4 doses x 10d (max. 2 grams/d)[13]

* The letter d stands for day.

1. Dosage and duration of administration should not be exceeded because of possibility of causing optic neuritis; maximum dosage is 2 grams/day.
7. An approved drug, but considered investigational for this condition by the U.S. Food and Drug Administration
13. Not recommended for children less than eight years old.

Reference: The Medical Letter (1992)

In a retrospective study of "pure" *D. fragilis* infections, Spencer et al (1979) found gastrointestinal symptoms in 32 (91%) of 35 children. It was noted that diarrhoea was the most common finding in patients with acute illness, whereas abdominal pain was more common in children with chronic symptoms. Eighteen of the 35 children were treated with the following: iodoquinol, 30 to 40 mg/kg of body weight/day for 21 days, was given to 12 children; metronidazole, 250 mg three times a day for one week, was given to five children; and tetracycline hydrochloride, 250 mg twice a day for five days, was given to one child. Twelve children completed the course of therapy and returned for a follow-up examination. Two of these children were initially asymptomatic and tests of their post treatment stools were negative for parasites. The remaining ten symptomatic children no longer had symptoms after therapy. Documented reinfection with gastrointestinal symptoms occurred in two children, both of whom were re-treated; both were asymptomatic when a follow-up examination was performed. No organisms were observed in stools tested for ova and parasites collected later from eight of these children (Spencer et al 1979). Similar findings were also reported by the same group of investigators for an adult population (Spencer et al 1982).

Millet et al (1983) described the clinical findings and treatment of 26 adults with *D. fragilis* alone or with a commensal. Gastrointestinal symptoms were observed in 22 (85%) of infected subjects. Iodoquinol 650 mg three times a day for 20 days eliminated the parasite in 10 (83%) of the 12 treated, although three subjects required a second course of therapy.

Iodoquinol is a halogenated 8-hydroxy quinoline. It is practically insoluble in

water and sparingly soluble in alcohol. Iodoquinol contains approximately 64% iodine (AHFS Drug Information 1994). Iodoquinol is referred to as a luminal or contact amoebicide because it acts primarily in the intestinal lumen. The precise mechanism is unknown.

Iodoquinol is amoebicidal against protozoa, especially *Entamoeba histolytica*. The drug is believed to act against both the trophozoites and encysted forms of the parasite; however, elimination of the cyst form probably results from destruction of the trophozoites (AHFS Drug Information 1994).

Some clinicians consider iodoquinol a drug of choice for the treatment of *Blastocystis hominis* and *D. fragilis* infections. Iodoquinol also has been used for the treatment of balantidiasis, and *Giardia lamblia* infection (AHFS Drug Information 1994).

Paromomycin is an aminoglycoside antibiotic obtained from *Streptomyces rimosus* var. *paromomycinus*. The drug is structurally related to neomycin, streptomycin, and kanamycin. Paromomycin sulphate has solubilities of greater than 1 g/ml in water and less than 0.1 mg/ml is alcohol at 25°C (AHFS Drug Information 1994).

Paromomycin sulphate is also considered a luminal or contact amoebicide. Unlike tetracyclines, paromomycin is a direct-acting amoebicide, and is effective either in the presence or absence of bacteria. Similar to other aminoglycosides, paromomycin is bactericidal and appears to inhibit protein synthesis in susceptible bacteria at the 30S segment of the ribosome (AHFS Drug Information 1994).

Paromomycin sulphate has a broad spectrum of activity, including activity against protozoa, bacteria and cestodes. It has been used with some success and is considered an

effective alternative to iodoquinol for the treatment of *D. fragilis* infections. Additionally, because paromomycin is poorly absorbed from the gastrointestinal tract, the drug may be useful in pregnant women with giardiasis (AHFS Drug Information 1994).

Tetracyclines are antibiotics and semisynthetic antibiotic derivatives obtained from cultures of *Streptomyces*. All commercially available tetracyclines contain the tetracycline nucleus. Tetracycline bases are amphoteric and very slightly soluble in water while tetracycline salts are generally sparingly soluble to freely soluble in water (AHFS Drug Information 1994).

Tetracyclines are usually bacteriostatic in action, but may be bactericidal in high concentrations or against highly susceptible organisms. Tetracyclines appear to inhibit protein synthesis in susceptible organisms mainly by reversibly binding to 30S ribosomal subunits, thereby inhibiting binding of aminoacyl transfer-RNA to those ribosomes. In addition, tetracyclines appear to reversibly bind to 50S ribosomal subunits. There is preliminary evidence that tetracyclines also alter cytoplasmic membranes of susceptible organisms resulting in leakage of nucleotides and other intracellular components from the cell. At high concentrations, tetracyclines also inhibit mammalian protein synthesis (AHFS Drug Information 1994).

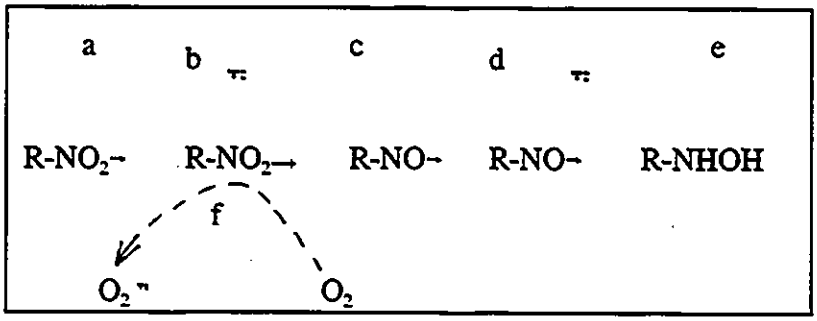
Tetracyclines have a broad spectrum of activity and are active against most *Rickettsia*, *Chlamydia*, *Mycoplasma*, spirochaetes, many gram-positive and gram negative bacteria and some protozoan parasites (AHFS Drug Information 1994). The use of tetracyclines is contraindicated in pregnancy and in young children.

Metronidazole is a synthetic, nitroimidazole-derivative antibacterial and

antiprotozoal agent. It is commercially available as the base and the hydrochloride salt. Metronidazole hydrochloride is very soluble in water and soluble in alcohol (AHFS Drug Information 1994).

Metronidazole is bactericidal, amoebicidal and trichomonacidal in action and selectively kills anaerobic microorganisms. The antimicrobial activity of reduced metronidazole is proposed to result from the reactivity and dismutation of short-lived intermediates formed as the nitro group of the drug is reduced in single electron steps to a hydroxyamine (Johnson 1993, Figure 1.2).

Figure 1.2 **Activation of metronidazole (a) in single electron steps to yield nitro-free radical (b) nitroso (c) nitroso-free radical (d) and ultimately, hydroxyamine (e).** Under aerobic conditions, re-oxidation (f) of the nitro-free radical produces the inactive form of the drug and a superoxide anion.



Reference: Johnson (1993)

$R\text{-}NO_2$ radical can be re-oxidized to inactive $R\text{-}NO_2$ in the presence of O_2. Thus O_2 may be sufficient to protect cells from the drug.

The reduction product appears to be responsible for the cytotoxic and antimicrobial effects of the drug which include disruption of DNA and inhibition of nucleic acid synthesis. Metronidazole is equally effective against dividing and nondividing cells (AHFS Drug Information 1994).

Metronidazole is referred to as both a luminal or contact amoebicide and a tissue amoebicide because the drug is active against trophozoites in the intestinal lumen, intestinal wall, and at extra intestinal sites such as the liver and lungs following oral administration. However, because metronidazole is well absorbed from the gastrointestinal tract, the drug usually has greater activity systemically than locally following oral administration (AHFS Drug Information 1994).

It is worthy to note at this point that although iodoquinol, paromomycin, tetracycline and metronidazole have been described to be successful in the treatment of *D. fragilis* infections, experimental data such as minimal inhibitory concentrations of the drugs on this organism cannot be found in the literature.

Difficulties encountered in the study of *Dientamoeba fragilis*

The difficulties encountered in the study of *D. fragilis* can be summed up by the following quotation: "To the protozoologist --if not to the physician--*D. fragilis* is now, perhaps, the most interesting of all the intestinal amoebae of man: for we know less about it than about any of the others, and its life history and activities are still mysterious. Ever since I first saw this curious organism in 1917, I have been intrigued by its peculiarities

and have taken every opportunity of studying it further: yet after more than 20 years of work and cogitation, I am still baffled....." Clifford Dobell,1940.

Twenty-five years later, a second quotation appeared: "Nothing has changed." B.H. Kean, 1965.

Over the last 28 years (from 1965 to 1993) little has been achieved in the study of *D. fragilis*, as reflected by the lack of information whenever a literature search is done on this topic. While tremendous amount of knowledge is being accumulated on the study of other intestinal protozoan parasites such as *Entamoeba histolytica, Giardia lamblia, Cryptosporidium parvum* and, most recently, *Microsporidium spp.*, there is no research whatsoever on *D. fragilis*.

Apart from being a fragile trophozoite organism with no known cyst form, another factor contributing to the difficulty in the study of *D. fragilis* is the lack of an axenic culture. To date *D. fragilis* can only be propagated in the presence of bacteria in culture, and most certainly the presence of bacteria in a mixed culture will cause unnecessary complications in any experimental design. *D. fragilis* has been alleged to be pathogenic in some patients, but its pathogenic mechanism is not known. Laboratory diagnosis of *D. fragilis* infections is limited to microscopic examination of stained smears prepared from preserved faecal specimens. Non-morphologic or immunodiagnostic techniques have not yet been developed. The antigens of *D. fragilis* have not been thoroughly studied. Monoclonal antibodies have not been developed to study the immunodominant proteins of this organism. Modern diagnostic techniques such as DNA probes and the polymerase

chain reaction have yet to be developed for *D. fragilis*. While treatment information is available, there are no experimental data on susceptibility testing to substantiate the treatment recommendation. As for the transmission of *D. fragilis*, circumstantial evidence and statistical analysis point to the link of the protozoan with the pinworm, but the hypothesis has yet to be proven. Additionally, seroprevalence of many intestinal protozoan parasites (such as *E. histolytica, G. lamblia* and *C. parvum*) has been studied, but the seroprevalence of *D. fragilis* is unknown.

Statement of Objectives

A dixenic culture of *Dientamoeba fragilis* is available at the American Type Culture Collection (ATCC 30948). It is intended that this culture will be obtained for the following studies:

1. The dixenic culture will be characterized and attempts will be made to axenize this culture.

   However, there is no guarantee that the culture can be axenized. Therefore other experiments will be carried out with the dixenic culture for ongoing research.
2. Polyclonal antibodies to *D. fragilis* will be produced from rabbits. Indirect immunofluorescence techniques will be developed to identify *D. fragilis* trophozoites in clinical specimens.
3. Attempts will be made to develop and characterize monoclonal antibodies for the study of *D. fragilis* antigens.
4. Activities of the anti-parasitic drugs used in the treatment of *D. fragilis* infections will be determined using *D. fragilis* ATCC 30948 as a test strain.

5. Seroprevalence of *D. fragilis* antibodies in symptomatic patients and healthy controls will be determined by an indirect immunofluorescence technique.

None of the proposed studies mentioned above has been reported in the literature.

CHAPTER 2

CHARACTERIZATION OF *DIENTAMOEBA FRAGILIS* DIXENIC CULTURE (ATCC 30948) AND ATTEMPTS TO AXENIZE THIS CULTURE

INTRODUCTION

A few definitions of culture systems based on Dougherty's classification (Dougherty 1959) are given below:

Axenic: Cultures in which the parasite is grown in the absence of all other metabolizing cells.

Monoxenic: Parasite is grown in the presence of a single known metabolizing microbial associate or cell.

Dixenic: Two known associates are present in culture.

Polyxenic: Several known associates are present in culture.

Xenic: Several associates are present, none of which are known. Also referred to as conventional or crude cultures.

The advantage of working with an axenic culture (if available) is obvious. An axenic culture is a pure culture of the parasite and there are no unnecessary complications caused by the presence of the metabolizing microbial associate(s).

A dixenic culture of *Dientamoeba fragilis* is available from the American Type Culture Collection (ATCC 30948). It was deposited there by L.S. Diamond (designated strain Bi/pa) in 1981. This strain originated from an adult human female in Evanston, Illinois. It was initially isolated by W. Balamuth in 1948. When deposited at the ATCC this *D. fragilis* culture was stated to be dixenic with *Clostridium perfringens* and *Klebsiella pneumoniae* (ATCC catalogue of Protists 1985).

MATERIALS AND METHODS

D fragilis strain and maintenance of culture.

A dixenic culture of *D. fragilis* (ATCC 30948) was acquired from the American Type Culture Collection (ATCC). It was maintained in TYGM-9 medium (ATCC medium 1171). The medium was dispensed aseptically to screw-cap tubes (16 by 125 mm) in 8 ml amounts. Immediately before use, 0.15 ml of rice starch solution was added aseptically to each tube. The rice starch solution was prepared by heat sterilizing 0.5 g of rice starch (BDH Inc., Toronto, Ontario) at 150°C for 2 h and adding 9.5 ml of sterile phosphate-buffered saline (PBS, FA buffer [pH 7.2]; Difco Laboratories, Detroit, MI) prior to use. After 3 to 4 days of incubation at 35°C and thorough mixing, *D. fragilis* was subcultured with the bacteria in the culture to two new tubes in 0.5- and 1.0-ml volumes. The maximum yield of *D. fragilis* trophozoites was estimated to be between 10^5 and 10^6 cells per ml, with a haemacytometer used for counting

D. fragilis was also preserved in liquid nitrogen using 2.75% of dimethyl sulphoxide in the freezing mixture (Dwyer and Honigberg, 1971).

Bacteria in *D. fragilis* dixenic culture.

Isolation. The *D. fragilis* dixenic culture was gently mixed and the culture fluid was inoculated to a 5% sheep blood Columbia agar plate and a MacConkey agar plate for aerobic incubation as well as a 5% sheep blood Columbia agar plate and a phenylethanol blood agar for anaerobic incubation. All inoculated plates were incubated at 35°C and examined after 24 and 48 hours of incubation.

Identification. Bacteria were identified by their growth requirements and characteristics,

gram-stain morphology, oxidase and catalase tests, and biochemical reactions with commercial systems. The identity of the *Klebsiella pneumoniae* was confirmed with the Microbact System (Disposable Products Pty. Ltd. Adelaide, South Australia). The anaerobic organism was identified by the AN-IDENT System (API Laboratory Products Ltd. St. Laurent, Quebec).

Antimicrobial susceptibility testing. Antimicrobial agents were used to eliminate the bacteria in the *D. fragilis* dixenic culture in an attempt to axenize the *D. fragilis* culture. Minimal inhibitory concentrations (MIC) of antimicrobial agents against the *K. pneumoniae* isolate identified in the dixenic culture were determined by the Vitek automated system (bioMerieux Canada, Inc., St. Laurent, Quebec). Because there is no approved standard for disc diffusion tests for anaerobic organisms, zone sizes of inhibition for aerobic organisms, published by the National Committee of Clinical Laboratory Standards (NCCLS 1993), were used only to indicate whether the antimicrobial agents were active on the anaerobes for axenization experiments. Mueller-Hinton agar supplemented with 5% defibrinated sheep blood was used as the test medium.

Attempts to axenize *D. fragilis* ATCC 30948

Several attempts with different approaches were carried out to axenize *D. fragilis* ATCC 30948. Axenization experiments were performed with ATCC medium 1171 (described under "Maintenance of *D. fragilis* Culture" earlier in this section) with the following additional supplements described under separate experiments. Imipenem (100μg/ml) and piperacillin (200 μg/ml) were included in the final preparation of medium to eliminate the bacteria in the *D. fragilis* culture. (Please refer to results of antimicrobial

susceptibility testing on bacteria). The dixenic *D. fragilis* culture was inoculated to two tubes containing 8 ml of supplemented medium (described below) in 0.5 and 1.0 ml volumes and incubated at 35°C.

Axenization experiment 1. Culture tubes were incubated anaerobically.

Axenization experiment 2. Culture medium was supplemented with sodium thioglycollate (0.5 g/L in final medium).

Axenization experiment 3. Culture medium was supplemented with 1% (v/v) of Isovitalex (BBL Microbiology Systems, Cockeysville, MD).

Axenization experiment 4. Culture medium was supplemented with Vitamin K (1 mg/L in final medium).

Axenization experiment 5. Culture medium was supplemented with Isovitalex (1% v/v) and Vitamin K (1 mg/L).

Axenization experiment 6. Culture medium was supplemented with 20% (v/v) bovine serum.

Axenization experiment 7. Culture medium was supplemented with 20% (v/v) human serum.

Axenization experiment 8. Culture medium was supplemented with 50% (v/v) bacterial pre-conditioned medium (prepared by growing the bacteria in ATCC medium 1171 and then filtering the culture supernatant).

Axenization experiment 9. Culture medium was supplemented with 0.1 ml of heat-killed (70°C for 1 hour) bacteria.

Axenization experiment 10. Culture medium supplemented with *Crithidium sp.* strain

ATCC 50083 (Diamond et al 1978).

Axenization experiment 11. ATCC hybricare medium supplemented with 15% (v/v) bovine serum and 5% (v/v) conditioned medium (Sigma Chemical Company, St. Louis, MO).

RESULTS

Maintenance of *D. fragilis* culture

The dixenic culture of *D. fragilis* ATCC 30948 was successfully maintained in ATCC medium 1171. Growth of *D. fragilis* usually peaked in three days, after which the number of live trophozoites declined. The maximum density of *D. fragilis* trophozoites on the third day was estimated to be between 10^5 and 10^6 cells per ml, with a haemacytometer used for counting. Growth of *D. fragilis* was maintained by subculturing every 3 to 4 days.

Bacteria in *D. fragilis* dixenic culture

Bacterial isolation and identification. The biochemical reactions and identification by the Microbact System are presented in Table 2.1. The organism was identified as *K. pneumoniae*, as listed in the ATCC catalogue.

We were unable to isolate *Clostridium perfringens* on the anaerobic culture plates. Instead, anaerobic, small, gram-negative bacilli were isolated. These anaerobic organisms consistently produced two different colony sizes which were designated B_1 (small colonies) and B_2 (large colonies). They were both identified as *Bacteroides sp.* by the AN-IDENT system with only minor differences in biochemical reactions (Table 2.2). These anaerobic organisms were referred to the Laboratory Centre for Disease Control, Ottawa, Ontario,

where they were confirmed as *Bacteroides vulgatus*. The discrepancy in bacterial identification was discussed with the staff at ATCC, who indicated that they would further investigate this dixenic culture (Nerad 1993).

Antimicrobial Susceptibility Testing. MIC values of antimicrobial agents on *K. pneumoniae* were determined by the Vitek Automated System (bioMerieux). Susceptibility testing results are shown in Table 2.3. Notably, *K. pneumoniae* was susceptible to both imipenem and piperacillin, the two antimicrobial agents used in the axenization experiments to eliminate the bacteria.

Results of disc diffusion tests on B_1 and B_2 variants of *Bacteroides vulgatus* are shown in Table 2.4. NCCLS guidelines for aerobic organisms were used to indicate if the antimicrobial agents exerted activities on the anaerobes, and the zone sizes of inhibition were by no means used as standards to indicate susceptibility. Both variants were "susceptible" to imipenem, while piperacillin was "active" on variant B_1 and less active on variant B_2. In the axenization experiments, imipenem (100 μg/ml) and piperacillin (200 μg/ml) were used as the antimicrobial agents to eliminate the bacteria in the *D. fragilis* culture.

Attempts to axenize *D. fragilis* ATCC 30948

All of 11 axenization experiments had the same outcome. The combination of imipenem and piperacillin effectively eliminated the bacteria from the *D. fragilis* dixenic culture, so that no viable bacteria could be recovered after the second or the third passage. Unfortunately, when the bacteria were eliminated, so was D. *fragilis* and we were unable to obtain an axenic culture of *D. fragilis* despite many attempts with different approaches.

DISCUSSION

A dixenic culture of *D. fragilis* was acquired from ATCC. It was successfully maintained in ATCC medium 1171.

The two bacterial species listed by ATCC for this dixenic culture were *Klebsiella pneumoniae* and *Clostridium perfringens*. We identified *K. pneumoniae* and *Bacteroides vulgatus* with two different colonial variants (B_1 and B_2) in the culture tube we received from ATCC.

It is believed that one of the major obstacles hindering the progress in the study of *D. fragilis* is the absence of an axenic culture. We attempted to axenize *D. fragilis* by different approaches, including the use of anaerobic conditions (Zierdt 1991), supplements of additional nutrients and vitamins (Diamond 1968), increasing the concentration of bovine serum (Diamond et al 1978), preconditioned medium with bacterial growth, heat-killed bacteria, and using *Crithidium sp.* strain ATCC 50083 (Diamond 1968). Unfortunately, none of these approaches has been successful. When the bacteria in the *D. fragilis* culture were eliminated by the antimicrobial agents, *D. fragilis* trophozoites also disappeared from the culture.

Because we were unable to obtain an axenic culture, we had no option but to proceed with our study using the dixenic culture.

Table 2.1 Identification of aerobic bacteria in *D. fragilis* ATCC 30948 dixenic culture by the Microbact System.

Biochemical reactions in Microbact 24 E	
Lysine	-
Ornithine	-
H_2S	-
Glucose	+
Mannitol	+
Xylose	+
ONPG	+
Indole	-
Urease	+
V.P.	+
Citrate	+
TDA	-
Gelatin	-
Malonate	+
Inositol	+
Sorbitol	+
Rhamnose	+
Sucrose	+
Lactose	+
Arabinose	+
Adonitol	-
Raffinose	+
Salicin	-
Arginine	-

Identification: *Klebsiella pneumoniae*

Table 2.2 Identification of anaerobic bacteria in *D. fragilis* ATCC 30948 dixenic culture by the AN-IDENT System.

Biochemical reactions in AN-IDENT System

Test	B_1(small colonies)	B_2(large colonies)
Indole production	-	-
N-acetyl-glucosaminidase	+	+
α-Glucosidase	+	+
α-Arabinosidase	-	-
ß-Glucosidase	-	+
α-Fucosidase	+	+
Phosphatase	+	+
α-Galactosidase	-	+
ß-Galactosidase	-	-
Indoxyl-acetate	+	+
Arginine utilization	-	-
Leucine aminopeptidase	+	+
Proline aminopeptidase	-	-
Pyroglutamic acid arylamidase	-	-
Tyrosine aminopeptidase	-	-
Arginine aminopeptidase	+	+
Alanine aminopeptidase	+	+
Histidine aminopeptidase	+	+
Phenylalanine aminopeptidase	+	+
Glycine aminopeptidase	+	-
Catalase	-	+

Identification: *Bacteroides sp.*

Confirmation by reference laboratory: *Bacteroides vulgatus*

Table 2.3 **Susceptibility testing results of *Klebsiella pneumoniae* with the Vitek automated system.**

Antimicrobial Agent	MIC (μg/ml)	Interpretation
Ampicillin	≥32	R*
Cefazolin	≤8	S**
Cefotaxime	≤4	S
Cefoxitin	≤2	S
Ciprofloxacin	≤0.5	S
Gentamicin	≤0.5	S
Imipenem	≤4	S
Piperacillin	≤8	S
Ticarcillin/clavulanic acid	≤16	S
Tobramycin	≤1	S
Trimethoprim-Sulphamethoxazole	≤10	S

* R = Resistant
**S = Susceptible

Table 2.4 **Susceptibility testing results of *Bacteroides vulgatus* (variants B_1 and B_2) with disc diffusion tests.**

Antimicrobial Agent	Zone size (mm)		Interpretation	
	B_1	B_2	B_1	B_2
Ampicillin	0	0	R*	R
Cefoperazone	19	0	I**	R
Ceftazidime	0	0	R	R
Ciprofloxacillin	10	18	R	I
Clindamycin	26	24	S***	S
Imipenem	34	34	S	S
Piperacillin	26	15	S	R
Ticarcillin	26	0	S	R

*R = Resistant
**I = Intermediate
***S = Susceptible

Because there is no approved standard for disc diffusion tests for anaerobes, the interpretation of R, I or S was used only as indicators for activity of antimicrobial agents for their selective use in axenization experiments.

CHAPTER 3

APPLICATION OF INDIRECT IMMUNOFLUORESCENCE FOR THE DETECTION OF *DIENTAMOEBA FRAGILIS* TROPHOZOITES IN FAECAL SPECIMENS.

The study described in this chapter has been published in The Journal of Clinical Microbiology 31:1710-1714 (1993).

INTRODUCTION

Dientamoeba fragilis is a protozoan parasite of the human large intestine. The organism was originally seen by Wenyon in 1909, but was not recognized at that time as a new species. It was then described and named by Jepps and Dobell in 1918. Since then it has been found in most parts of the world where careful surveys have been taken with an estimated incidence of 1.4 to 53% (Burrows and Swerdlow 1956, Kean and Malloch 1966, Talis and Lengy 1971, Yang and Scholten 1977a, Bruckner et al 1979, Spencer et al 1979, Millet et al 1983).

Although *D. fragilis* is often considered a harmless commensal, a number of reports have been published suggesting that infection by the parasite may evoke various symptoms, which, in most cases, disappear with the elimination of the parasite. Abnormal stools, diarrhoea, abdominal pain, fatigue, loss of appetite and weight loss were among the symptoms experienced (Yang and Scholten 1977a). Fibrosis of the appendix (Swerdlow and Burrows 1955), phagocytosis of red blood cells (Burrows and Swerdlow 1956), low grade eosinophilia (Spencer et al 1979), occurrence in biliary tracts (Talis et al 1971), and colitis (Shein and Gleb 1983) have also been reported. Wenrich et al (1936) found a higher incidence of gastrointestinal disorders among college students harbouring *D. fragilis* than among those infected by *Entamoeba histolytica*. Sapero (1939) reported that 27.3% of cases with *D. fragilis* infection presented symptoms, and Steinitz et al (1970) reported symptoms in 15.1% of infected persons. An incidence of 25% symptomatic cases was reported by Yang and Scholten (1977a) in Canada.

Spencer et al (1979) conducted a retrospective study on 35 children in whom *D.*

fragilis was the only parasite found in the gastrointestinal tract. Gastrointestinal symptoms were present in 32 (91%) of these children. Diarrhea was the most common finding in patients with acute illness, whereas abdominal pain was more common in children with chronic symptoms. Therapy with iodoquinol or metronidazole was effective. Symptoms were eliminated or diminished on follow-up evaluation after treatment. From this association between therapy and symptomatic relief, the authors stressed that *D. fragilis* should be considered pathogenic in those children with gastrointestinal symptoms. Similar findings were also reported in the adult population by the same group of authors (Spencer et al 1982).

D. fragilis is classified as a flagellate (Levine et al 1980) but with no demonstrable flagella. It does not have a cyst stage. The only available method in laboratory diagnosis is microscopic examination of stained smears of preserved fecal specimens for its characteristic trophozoites (Scholten and Yang 1974, Grendon et al 1991). The objective of this study was to determine the usefulness of indirect fluorescent antibody (IFA) assay for the identification of *D. fragilis*.

MATERIALS AND METHODS

***D. fragilis* strain and maintenance of culture.** Please refer to Chapter 2 (page 45).

Bacteria in *D. fragilis* culture. Please refer to Chapter 2 (page 45).

Preparation of *D. fragilis* antigen. Twelve tubes of 3 to 4 day old *D. fragilis* culture were centrifuged at 600 xg for eight minutes to deposit *D. fragilis* trophozoites. They were washed three times to remove bacteria in the supernatant with warm (35°C) FA

buffer containing piperacillin (200 μg/ml) and imipenem (100 μg/ml). These two antibiotics were active against the two bacterial species and were included in the preparation to protect the rabbit for immunization. After the final washing the *D. fragilis* trophozoites were counted. The suspension prepared following this procedure contained 3.4×10^6 trophozoites per ml. It was frozen and thawed once (frozen at -20°C and then thawed at 4°C), to disintegrate the trophozoites. After microscopic examination to confirm the disintegration of trophozoites, the antigen preparation was kept at -20°C until use.

Production of antiserum. A New Zealand white rabbit of 2 kg in weight were used. Equal volumes of antigen preparation and complete Freund's adjuvant (Difco) were mixed. A 0.5 ml amount of the mixture was injected intramuscularly into the biceps femoris muscle. One month after the initial vaccination a booster (0.5 ml mixture of equal parts of antigen preparation and incomplete Freund's adjuvant, [Difco]) was given. The rabbit was bled two weeks after the booster.

Absorption of antiserum. The two bacterial isolates present in the culture (*K. pneumoniae* and *B. vulgatus*) were grown in TYGM-9 medium overnight at 35°C. They were washed three times in FA buffer by centrifugation at 1,000 xg for 10 min. Equal volumes of rabbit antiserum and bacterial suspension (to which 0.15 ml of rice starch suspension had been added after the final washing) were mixed to react for 1 h, at 35°C, with gentle mixing every 10 minutes. At the end of 1 h, the mixture was placed in 1.5 ml Eppendorf tubes, and spun in an Eppendorf micro centrifuge at 10,000 xg for 3 minutes to pellet the bacteria and starch particles. The supernatant was removed, filter sterilized

with a 0.22 μm Millipore filter unit, and stored at -70°C. Because residual antibody at low titres towards the bacteria was detected by IFA, the antiserum was absorbed a second time to ensure specificity for *D. fragilis*.

Specimens. All faecal specimens for parasitological examination were collected in sodium acetate-acetic acid-formalin (SAF) fixative (Yang and Scholten, 1977b). Specimens collected for this study included those received at the Children's Hospital of Eastern Ontario, the Ottawa Civic Hospital, Queensway-Carleton Hospital, and the Ottawa Public Health Laboratory. Because many samples were known positives stored at different laboratories, the findings reported here would not reflect the true prevalence of the parasites in our region.

Concentration and staining of specimens. Specimens collected in SAF fixative were strained through gauze into 15 ml centrifuge tubes, centrifuged at 600 xg for 2 minutes, and the deposit washed twice in saline. For permanent staining a drop of Mayer's albumin was placed on a glass slide, and mixed with a drop of the sediment. The mixture was then spread with an applicator stick using a "dabbing" motion to produce a smear of varying thickness. After drying at room temperature, the smear was stained with a modified iron haematoxylin/Kinyoun stain (Palmer 1991). To continue with the concentration procedure, 8 ml of 10% formalin were added to the remaining sediment. This was followed with 4 ml of ethyl acetate (Young et al 1979), and shaking for 1 minute. After centrifugation, the plug of debris in the ethyl acetate layer was removed, and the supernatant was decanted. The sediment was then examined microscopically for ova and parasites. The wet preparation of the concentrates was examined with a 10x objective

covering overlapping fields under a 22 x 22 mm cover slip. Confirmation of findings was made with a 25x or 40x objective. This examination procedure averaged about five minutes per preparation. The haematoxylin/Kinyoun stained slide was mounted with a 22 x 40 mm cover slip and examined with a 50x oil-immersion objective for protozoan parasites, covering at least two-thirds of the mounted area. This procedure took approximately 15 minutes per slide.

IFA. The pre-immunization rabbit serum (diluted 1 in 20 in FA buffer) and immune absorbed rabbit serum were tested by IFA. Smears of samples known to be abundant in *D. fragilis* trophozoites and smears made from *D. fragilis* dixenic culture (preserved in SAF fixative) were used to assess the immunoassay. Teflon coated welled-slides of 6mm diameter (Cel-Line Associates Inc., Newfield, NJ) were used. Two-fold serial doubling dilutions of rabbit serum were made in FA buffer and applied to the smears for 1 h at 35°C in a moist chamber. After three washings in FA buffer (5 minutes each) fluorescein-labelled sheep anti-rabbit immunoglobulin (Wellcome Diagnostics, Dartford, UK) diluted 1 in 40 in FA buffer was applied for 1 h. After washing slides were mounted with buffered glycerol (Gull Laboratories, Inc., Salt Lake City, UT) and were examined with a 25x objective on a Leitz epifluorescence microscope with a 490nm exciter filter, a 510nm dichromatic beam splitter, and a 520nm barrier filter. The pre-immunization rabbit serum yielded a negative IFA reading (Fig. 3.1A). The immune absorbed rabbit serum gave a strong fluorescence (4+) with *D. fragilis* (Fig. 3.1B) up to a dilution of 1 in 256. A dilution of 1 in 128 of the immune absorbed rabbit serum was chosen for the detection of *D. fragilis* in clinical samples. The entire well was examined with a 25x

objective. Each examination was accomplished in less than one minute.

RESULTS

Routine examination of ova and parasites. A total of 155 clinical samples were included in this study. Concentration procedures and a modified iron haematoxylin/Kinyoun stain were carried out to examine for helminths and protozoal parasites. Parasites were not found in 42 specimens. *D. fragilis* was present in 9 samples, while other parasites were found in the remaining 104 examinations. In total four species of helminths (*Enterobius vermicularis, Hymenolepis nana, Strongyloides stercoralis* and *Trichuris trichiura*) and 11 species of protozoa (*Blastocystis hominis, Chilomastix mesnili, Cryptosporidium sp., D. fragilis, Entamoeba coli, E. hartmanni, E. histolytica, Endolimax nana, Giardia lamblia, Iodamoeba buetschlii* and *Retortamonas intestinalis*) were found (Table 3.1). Infection by a single parasite was identified in 67 specimens, while mixed infections were observed in 46 samples. Of the nine specimens positive for *D. fragilis*, 8 were "pure" infections. *E. nana* and *E. hartmanni* were also identified with *D. fragilis* in the remaining specimen.

IFA. IFA was performed on all of 155 clinical samples. Immunofluorescence was read without previous knowledge of the results of routine parasitological examination. *D. fragilis* was identified in seven specimens by IFA in less than one minute of examination time per specimen. Reaction was strong as demonstrated by bright (3+ to 4+) fluorescence (Fig. 3.1C). There were two specimens of doubtful reading by IFA. On cross reference with routine parasitology results it was found that there were only very

scanty amounts (less than 5 trophozoites) of *D. fragilis* on the stained smears. None of the helminths or protozoa (Table 3.1) cross-reacted with the immune absorbed rabbit serum. There was also no false positive IFA reading with the 42 specimens in which no parasites could be found.

DISCUSSION

Since its first description in 1918 *D. fragilis* has been a frequent finding in most laboratories where careful parasitological examination is performed. The organism does not have a cyst stage, and the trophozoites disintegrate rapidly in unpreserved faecal specimens. To date, the only reliable method in laboratory diagnosis is microscopic examination of stained smears of preserved stools. Laboratories which do not employ this procedure would almost certainly miss the diagnosis (Scholten and Yang 1974, Grendon et al 1991).

While examination of stained smears enables the identification of *D. fragilis* and most other parasites, the procedure is lengthy and the microscopic examination is time-consuming because it takes 15 minutes or longer. Fluorescence microscopic methods have been developed for other parasites such as *Giardia lamblia* (Wilson and Schantz 1991) and *Cryptosporidium sp* (Garcia et al 1987). These methods are very useful as screening procedures because of their sensitivity and speed. Dwyer studied the antigenic relationship of *D. fragilis* and other protozoa by immunofluorescence (1972a) and gel diffusion (1972b). He found that *D. fragilis* was least related to *Entamoeba spp.*, more related to *Trichomonas* and most related to *Histomonas meleagridis*. However, the data suggested that cross-reactivity was minimal among species. The indication was that the antigenicity

of *D. fragilis* was specific and cross reactivity with other parasites should not occur with clinical samples.

Axenic cultures of *Entamoeba spp.*, *Giardia lamblia* and *Trichomonas spp.* are readily available from ATCC. This is not the case for *D. fragilis*. It is unfortunate because its availability would simplify the procedure to obtain a specific antiserum and absorption procedures would be obviated. The unavailability of an axenic culture would probably also explain why so little progress has been made in the study of *D. fragilis*. However, in the absence of an axenic culture, we have managed to produce an absorbed antiserum suitable to carry out our immunofluorescence study on the identification of *D. fragilis* trophozoites in preserved faecal specimens.

In the IFA study described here, *D. fragilis* was identified in 7 out of 9 specimens which were positive by routine microscopic examination. The two positive specimens missed by the IFA assay contained only very scanty numbers (less than 5 trophozoites) of *D. fragilis*. The failure of IFA on these two specimens is not surprising when we consider that the surface area of the 6 mm diameter circle on the slide for IFA examination is 28 mm^2, compared to a 22 mm x 40 mm (880 mm^2) cover-slipped area for routine microscopic examination. If the entire surface area of the permanently-stained smears is read, then the area examined is 31 times that for IFA examination. Theoretically there should be at least 31 *D. fragilis* trophozoites on the smear for routine microscopic examination before the smear for IFA examination would be read as a positive. Even if only one-third of the cover-slipped area is examined, the surface area covered by routine microscopic examination would still be 10 times more than that examined by the IFA

assay. We believe that if a larger amount of sample was spread on an increased surface area for examination, the sensitivity of the IFA assay would be improved.

The IFA assay was highly specific for *D. fragilis* in the present study. None of the four species of helminths or the other 10 species of protozoa (Table 3.1) cross-reacted with *D. fragilis* trophozoites. There was also no false positive IFA reading with any of the specimens in which no parasites could be found.

It has been more than 70 years since *D. fragilis* was first described. Over these years there has been little if any advancement in the laboratory diagnosis of this parasite. Published reports (Burrows and Swerdlow 1956, Kean and Malloch 1966, Talis and Lengy 1971, Yang and Scholten 1977a, Bruckner et al 1979, Spencer et al 1979, Millet et al 1983) showed that the prevalence of *D. fragilis* equalled or surpassed that of *G. lamblia*. In our laboratory *D. fragilis* is one of the most frequently found parasites perennially. Clinical data also suggested that the pathogenicity of *D. fragilis* resembled that of *G. lamblia* (Turner 1985). Yet, despite many recent advances in the study of *G. lamblia*, little knowledge has been gained about *D. fragilis*, including the field of laboratory diagnosis. One of the main reasons for the lack of progress is probably due to the absence of an axenic culture. With a dixenic culture the presence of the two bacteria undoubtedly would cause undesirable complications in any type of experimental design. In the present study, we demonstrated that it is possible to raise an antiserum to *D. fragilis* in a mixed bacterial culture and specificity can be obtained by absorption. This specific antiserum can then be employed to identify *D. fragilis* trophozoites in preserved faecal specimens. To the best of our knowledge this is the first study that has used immunological techniques

to identify *D. fragilis* in clinical samples, and the results of this preliminary study are very encouraging. The IFA assay might prove to be a useful screening method. The time of the IFA procedure could be reduced if a direct immunofluorescence technique were developed. There have been recent reports (Rosoff et al 1989, Stibbs 1989) describing the successful use of enzyme-linked immunosorbent assay (ELISA) techniques to identify *G. lamblia* antigens in stool specimens. It is one of our future objectives to develop an ELISA or a simpler method to identify *D. fragilis* antigens, preferably in both fresh and preserved faecal samples. As indicated in the study on stool diagnosis of giardiasis (Rosoff et al 1989), an enzyme immunoassay could detect *Giardia* infection in at least 30% more cases than microscopic examination. It is possible that this would also hold true for *D. fragilis*, and a similar antigen detection test might help to improve the underdiagnosis of this infection. Also the availability of a simple test would permit epidemiological studies and clarify the pathogenic role of this organism.

Table 3.1 Helminths and protozoa recovered in 113 preserved faecal specimens.

Helminths	No. of occurrences*
Enterobius vermicularis	2
Hymenolepis nana	5
Strongyloides stercoralis	1
Trichuris trichiura	8
Protozoa	
Blastocystis hominis	25
Chilomastix mesnili	3
Cryptosporidium sp.	6
Dientamoeba fragilis	9
Entamoeba coli	32
Entamoeba hartmanni	12
Entamoeba histolytica	3
Endolimax nana	37
Giardia lamblia	24
Iodamoeba buetschlii	4
Retortamonas intestinalis	1

* a single parasite was identified in 67 specimens, while mixed infections were observed in 46 samples.

Figure 3.1 ***D. fragilis*** **trophozoites grown in culture and visualized by indirect fluorescent antibody staining with (A) rabbit pre-immunization serum (no fluorescence), and (B) immune rabbit serum (strong fluorescence).** (C) *D. fragilis* trophozoites in clinical stool specimen showing fluorescence with immune rabbit serum. Final magnification, x2000.

Figure 3.1 (A) rabbit pre-immunization serum (no fluorescence)

Figure 3.1 (B) immune rabbit serum (strong fluorescence)

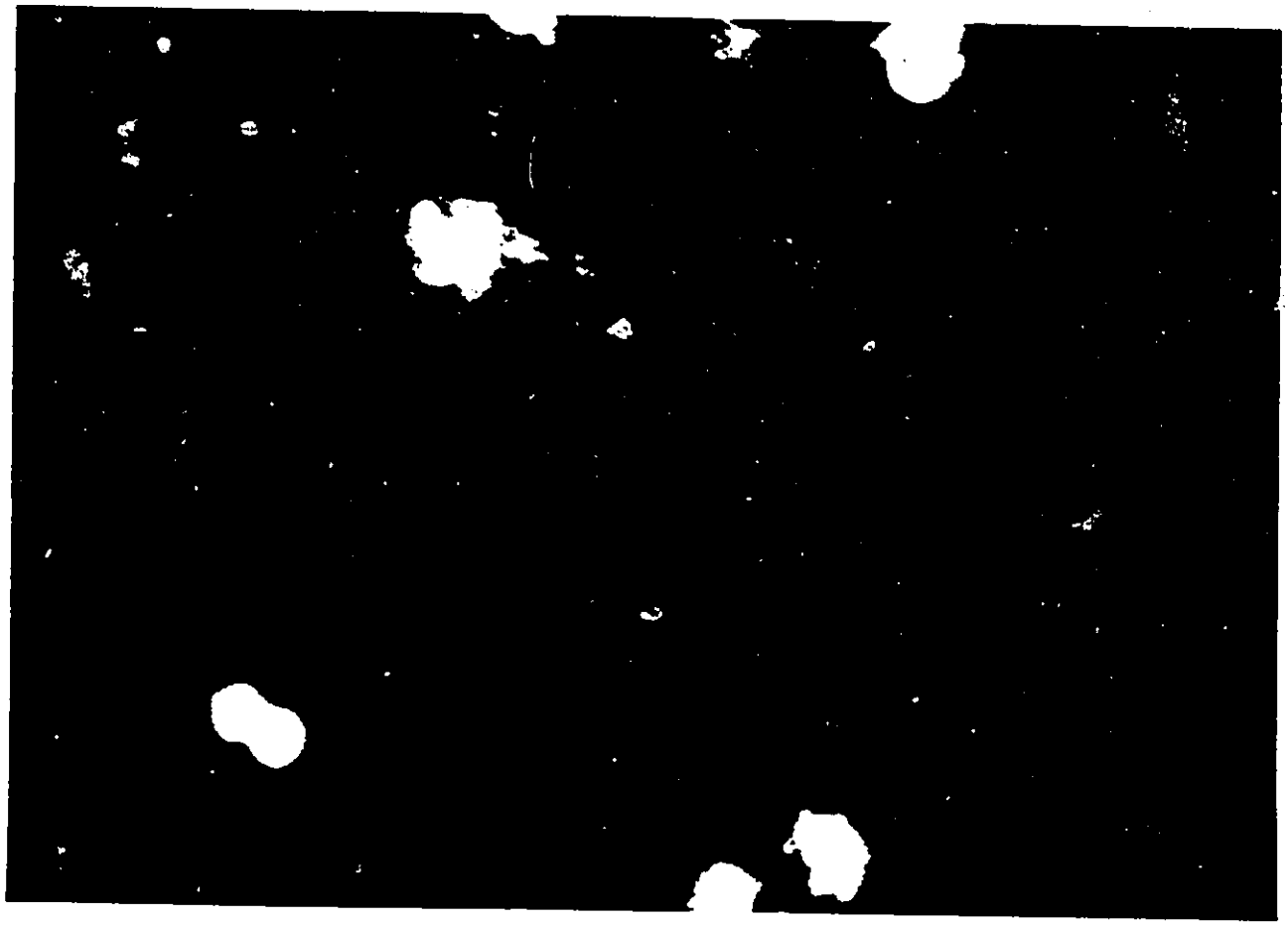


Figure 3.1 (C) *D. fragilis* trophozoites in clinical stool specimen showing fluorescence with immune rabbit serum.

CHAPTER 4

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES IN THE STUDY OF *DIENTAMOEBA FRAGILIS*

A manuscript based on this chapter is being prepared for publication.

INTRODUCTION

Köhler and Milstein (1975) developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. In this technique an antibody-secreting cell, isolated from an immunized animal, is fused with a myeloma cell, a type of B-cell tumour. These hybrid cells (or hybridomas) can be maintained in vitro and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by hybridomas are known as monoclonal antibodies (MAbs) (Harlow and Lane 1988). A diagrammatic explanation of the production of MAbs is presented in Figure 4.1 (Diamond et al 1981).

MAbs have at least three distinct advantages over polyclonal antibodies: specificity of binding, homogeneity, and ability to be produced in unlimited quantities (Harlow and Lane 1988). The production of MAbs allows the isolation of reagents with a unique, chosen specificity. Because all of the antibodies produced by descendants of one hybridoma cell are identical, MAbs are powerful reagents for testing for the presence of a desired epitope. Hybridoma cell lines also provide an unlimited supply of antibodies (Harlow and Lane 1988). In addition, one unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single-cell cloned prior to use, MAbs can be produced after immunizations with complex mixtures of antigens (Harlow and Lane 1988), as in the case of the dixenic ATCC *Dientamoeba fragilis* culture.

In spite of being known for more than 75 years, a worldwide distribution and an alleged pathogenicity, there has been little progress in the study of *D. fragilis* . Apart

from the reports by Dwyer (1972a, 1972b, 1974), there are virtually no data on the antigenic and immunologic aspects of this organism. We have described the application of indirect immunofluorescence (IIF) to detection of *D. fragilis* in fecal specimens (Chan et al, 1993). Here we report the development and characterization of monoclonal antibodies (MAbs) in the study of *D. fragilis*.

MATERIALS AND METHODS

***D. fragilis* strain and maintenance of culture.** Please refer to Chapter 2 (page 45).

Bacteria in *D. fragilis* culture. Please refer to Chapter 2 (page 45).

Preparation of *D. fragilis* antigen for mouse inoculation.

Two antigenic preparations were made to inoculate mice in two separate studies. In the first study, twelve tubes of 3- to 4-day-old *D. fragilis* cultures were centrifuged at 600 x g for 8 min to deposit the *D. fragilis* trophozoites. To remove the bacteria in the supernatant, the tubes were washed three times with warm (prewarmed to 35°C) FA buffer containing piperacillin (200 μg/ml) and imipenem (100 μg/ml). These two antibiotics were active against the two bacterial species and were included in the preparation to protect the mouse for immunization (Chan et al, 1993). After the final washing, the number of *D. fragilis* trophozoites was counted. The antigen prepared by this procedure contained 3.4 x 10^6 trophozoites per ml. It was frozen and thawed once (frozen at -20°C and then thawed at 4°C) to disintegrate the trophozoites. After microscopic examination to confirm the disintegration of the trophozoites, the antigen preparation was kept at -20°C until use.

In the second study, the *D. fragilis* culture was fixed in sodium acetate-acetic acid-

formalin (SAF) (Yang and Scholten 1977b) for one hour. The fixed trophozoites were then washed three times with FA buffer for mouse inoculation.

Production of MAbs

MAbs to each antigen preparation were produced according to the method described by Harlow and Lane (1988) and procedures established at the Animal Care Service at the University of Ottawa. BALB/c mice were immunized intraperitoneally with an equal volume of antigen preparation emulsified with complete Freund's adjuvant (Difco Laboratories). A volume of 0.2 ml of the mixture was inoculated into each mouse. Mice were given two booster injections intraperitoneally (0.2 ml) with equal volumes of antigen preparation and incomplete Freund's adjuvants (Difco Laboratories) at 2-week intervals. They were test bled for antibodies to *D. fragilis* by an indirect immunofluorescence (IIF) assay (described below). A final booster of 0.1 ml antigen preparation without adjuvant was given one week later. Four days after the final booster, the spleen of the mouse was removed and the spleen cells were fused with P3X63-Ag8.653 non-secreting mouse myeloma cells (ATCC CRL1580) by using 50% polyethylene glycol (Sigma Chemical Company, St. Louis, MO). Hybridomas secreting MAbs against *D. fragilis* antigen were screened by IIF and cloned twice by limiting dilution using ATCC hybricare medium supplemented with 10% fetal calf serum and 10% conditioned medium (Sigma), and selection by 1% hypoxanthine aminopterin and thymidine (Sigma). Ascites were produced by pristane-primed mice. MAbs were isotyped by ELISA with a commercial kit (Southern Biotechnology Associates Inc., Birmingham, AL). The specificity of MAbs was determined by negative IIF readings against *K pneumoniae* and the two variants of *B.*

vulgatus in the *D. fragilis* dixenic culture.

Preparation of *D. fragilis* antigen slides

Tissue culture flasks (25 cm^2; Corning Glass Works, Corning, N.Y.) containing 8 ml of culture medium and 0.15 ml of rich starch suspension were inoculated with 0.5 ml of the *D. fragilis* dixenic culture. These inoculated flasks were laid on their flat sides (allowing larger areas to be covered by the culture medium) and incubated for 3 days at 35°C. Many *D. fragilis* trophozoites became attached to the surface of the flasks. The culture fluids were decanted and the flasks were rinsed gently 5 to 7 times with warm (pre-warmed to 35°C) FA buffer to wash off the bacteria. After rinsing, the attached trophozoites were washed off the flask surface by squirting FA buffer with a sterile Pasteur pipet. The harvested trophozoites with minimal bacterial contamination were collected in 16 x 100 mm tubes and centrifuged at 100xg for 5 min. The deposit containing the trophozoites was washed one more time with warm FA buffer before fixation in SAF. The fixed trophozoites were then washed three times with FA buffer, counted with a haemacytometer, and diluted to 3 x 10^6 trophozoites per ml. A 10-μl amount was spread on teflon-coated welled slides of 6mm diameter (Cel-Line Associates Inc., Newfield, N.J.) and air-dried. After fixing with methanol, these antigen slides were kept at 4°C prior to an IIF assay to detect MAbs to *D. fragilis*.

Indirect immunofluorescence assay to screen for MAbs

Supernatants of hybridoma cell cultures were screened for the presence of MAbs to *D. fragilis*. For each assay 25 μl of culture supernatant were applied to the *D. fragilis* antigen slides for 1 h at 35°C in a moist chamber. After three washings in FA buffer (5

min each) a blocking solution of 3% fetal calf serum in FA buffer was applied for 30 min. This was followed by three washings in FA buffer, and 25 μl of fluorescein-labelled goat anti-mouse immunoglobulins (Sigma) diluted 1 in 40 in FA buffer with Evans blue (dilution of 1 in 20,000 in buffer) were applied for 1 h. After three washings, the air-dried slides were mounted with FA mounting fluid (Difco) and were examined with a 25x objective on a Leitz epifluorescence microscope with a 490-nm exciter filter, a 510-nm dichromatic beam splitter, and a 520-nm barrier filter.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

D. fragilis culture was prepared as described under "Preparation of *D. fragilis* antigen slides" (page 75). Trophozoites washed with FA buffer were resuspended in 0.5 ml PBS - 0.05% Tween 20 instead of sodium acetate-acetic acid-formalin fixative. The bacteria in the *D. fragilis* dixenic culture were also propagated for comparative study in SDS-PAGE and immunoblotting. *K. pneumoniae* was cultured aerobically on 5% sheep blood agar and incubated at 35°C for 24 hours. B1 and B2 variants of *B. vulgatus* were grown on 5% sheep blood agar and incubated anaerobically at 35°C for 48 hours. These bacteria were suspended and washed 3 times in FA buffer at 10,000xg for 3 minutes in an Eppendorf centrifuge. After the final wash, the bacteria were suspended in 0.5 ml PBS-0.05% Tween 20. *D. fragilis* and bacterial preparations were sonicated 6 times for 10 seconds each time in ice cold water. They were then frozen overnight at -20°C. After thawing they were centrifuged at 12,000xg for 6 minutes. Protein concentrations were determined for the supernatants with the Kodak Ektachem™ analyzer (Eastman Kodak

Company, Rochester, N.Y.). The method utilizes reflectance spectrophotometry and a copper azo-dye complex. The supernatants were mixed with sample buffer (0.5M Tris-HCl [pH 6.8], 10% glycerol, 10% SDS, 5% ß-mercaptoethanol, and 0.05% bromophenol blue) and then boiled for 5 minutes. Three different concentrations of *D. fragilis* protein preparations (75 μg, 50 μg and 25 μg), and 5 μg of each bacterial protein preparation were loaded per gel track for electrophoresis. A 10-μl sample of a boiled prestained solution of molecular mass standards with the following molecular masses (in kilodaltons): phosphorylase b, 106; bovine serum albumin, 80; ovalbumin, 49.5; carbonic anhydrase, 32.5; soybean trypsin inhibitor, 27.5; lysozyme, 18.5 (Bio-Rad Laboratories Inc., Mississauga, Ontario, Canada) was loaded in one track of each gel. SDS-PAGE was performed according to procedures of Laemmli (1970), and Harlow and Lane (1988) with 15% separating gel in a mini-Protean II cell apparatus (Bio-Rad) at 100 V for 2 hours. Electrophoresed proteins were transferred to nitrocellulose (BA-S NC supported nitrocellulose, poresize 0.45 μm, Schleicher and Schuell, distributed by Mandel Scientific Co. Ltd., Guelph, Ontario, Canada) as described by Towbin et al (1979), at 100 V for 1 hour. Replica gels were stained with Coomassie brilliant blue. Nitrocellulose was blocked with 3% gelatin and 1% bovine serum albumin in Tris-buffered saline (TBS), (60 minutes at 35°C), washed (once for 10 minutes in 0.05% Tween 20-Tris buffered saline [TTBS]), and probed with mouse ascites (1/50 dilution, overnight at 35°C) containing MAbs. Normal mouse serum, diluted 1 in 50, was used as a negative control. After 3 washings (10 minutes each) with TTBS, the membrane was exposed to a 1/500 dilution of peroxidase-conjugated goat anti-mouse IgG (Sigma) in TBS-1% gelatin (2 hours at

35°C). The membrane was washed 3 times in TTBS, and the protein bands were developed in TBS-5% 4-chloro-1-naphol (Pierce, Rockford,IL)-0.015% H_2O_2 for 30 minutes.

RESULTS

Production of MAbs

Two MAbs (8B2 and 1H11) to *D. fragilis* were identified by the IIF assay (Fig. 4.2). MAb 8B2 was produced from frozen and thawed antigen and MAb 1 H11 from *D. fragilis* trophozoites fixed in SAF. The specificities of these two MAbs to *D. fragilis* were confirmed by negative IIF assay with *K. pneumoniae*, and both B1 and B2 variants of *B. vulgatus*, as antigens.

SDS-PAGE

Results of SDS-PAGE are presented in Figure 4.3. Protein bands of *D. fragilis* were readily visualized on gels loaded with 75 μg and 50 μg of protein respectively, but the bands were hardly visible on the gel track loaded with 25 μg of protein. Bacterial protein bands were readily visible, and they were distinctly different in molecular sizes from those of *D. fragilis* (Fig. 4.3). In order to have a better view of the *D. fragilis* protein bands, the gel track loaded with 75 μg of *D. fragilis* protein was magnified with that of the molecular mass standards (Fig. 4.4). At least 6 discernable *D. fragilis* protein bands (with molecular masses 65,39,30,29,28 and 27 kDa) were observed.

Characterization of MAb 8B2 and MAb 1H11

Both MAb 8B2 and MAb 1H11 were isotyped as IgG1, with kappa light chains.MAb 8B2 did not give any band on immunoblotting with *D. fragilis* protein

antigens, while MAb 1H11 identified a 39 kDa protein on immunoblot (Fig. 4.5). Normal mouse serum (used as a negative control) did not yield any band on immunoblotting with *D. fragilis*, B1 and B2 variants of *B. vulgatus* or *K. pneumoniae* proteins (Fig. 4.6). Both MAbs were positive in IIF assay to the *D. fragilis* ATCC culture on repeated tests. MAb 8B2 did not react with patient strains of *D. fragilis*, but MAb 1H11 gave positive IIF results on *D. fragilis* trophozoites from clinical specimens (Fig. 4.7). The characteristics of these two MAbs are summarized in Table 4.1.

DISCUSSION

Our previous study (Chan et al 1993) showed that *D. fragilis* trophozoites can be readily identified in faecal specimens by an IIF assay and there were no cross-reactions with other protozoa or helminths commonly found in clinical samples. The results of that study indicated that immunological techniques could be useful in the laboratory diagnosis of *D. fragilis* infections, but the specific immunogenic proteins of *D. fragilis* have not been elucidated.

In this study we have identified at least 6 discernable *D. fragilis* protein bands of molecular masses 65,39,30,29,28 and 27 kDa on SDS-PAGE. While the immunological significance of these proteins needs to be further studied, one particular protein of 39 kDa was recognized by MAb 1H11.

This is the first report on the development and characterization of MAbs to *D. fragilis*. Two MAbs of IgG1 isotype with kappa light chains were produced from two different methods of antigenic preparation. Because MAb 8B2 failed to immunoblot we suspect that it probably recognizes a heat-labile antigen of *D. fragilis*. In any case the

failure of MAb 8B2 to react with *D. fragilis* trophozoites in patient specimens would limit its use in clinical application. On the other hand, MAb 1H11 identified a 39 kDa protein on immunoblot, and it showed some promise in its possible clinical application by giving a positive IIF test with *D. fragilis* trophozoites identified in clinical specimens.

MAbs have proven records of success lately in the laboratory diagnosis and differentiation of pathogenicity of other human intestinal protozoan parasites. Garcia et al (1987) reported successful fluorescence detection of *Cryptosporidium* oocysts in human faecal specimens by using MAbs. Stibbs (1989) described a MAb-based enzyme immunoassay to identify *Giardia lamblia* antigen in human stools. Garcia et al (1992) also reported an evaluation of a new MAb combination reagent for direct fluorescence detection of *Giardia* cysts and *Cryptosporidium* oocysts in human faecal specimens. Recently a MAb for distinction of invasive and noninvasive clinical isolates of *E. histolytica* was described by Gonzalez-Ruiz et al (1992). While the usefulness of MAbs to study the immunologic proteins of *D. fragilis*, or to help to establish a laboratory diagnosis of infections due to this parasite has yet to be determined, this preliminary report shows encouraging results that MAbs to *D. fragilis* could be developed and characterized for these purposes.

Table 4.1 Characterization of Monoclonal Antibodies 8B2 and 1H 11

MAb designation:	8B2	1H11
Produced from:	frozen and thawed antigen	SAF fixed antigen
Immunoglobulin class	IgG1	IgG1
Light chain	kappa	kappa
Protein antigen of *D. fragilis* identified on immunoblot	did not immunoblot	39 kDa
Indirect immunofluorescence with *D. fragilis* culture	yes	yes
Indirect immunofluorescence with patient *D. fragilis* trophozoites	no	yes

Figure 4.1 Production of monoclonal antibodies

Figure 4.1. Production of Monoclonal Antibodies

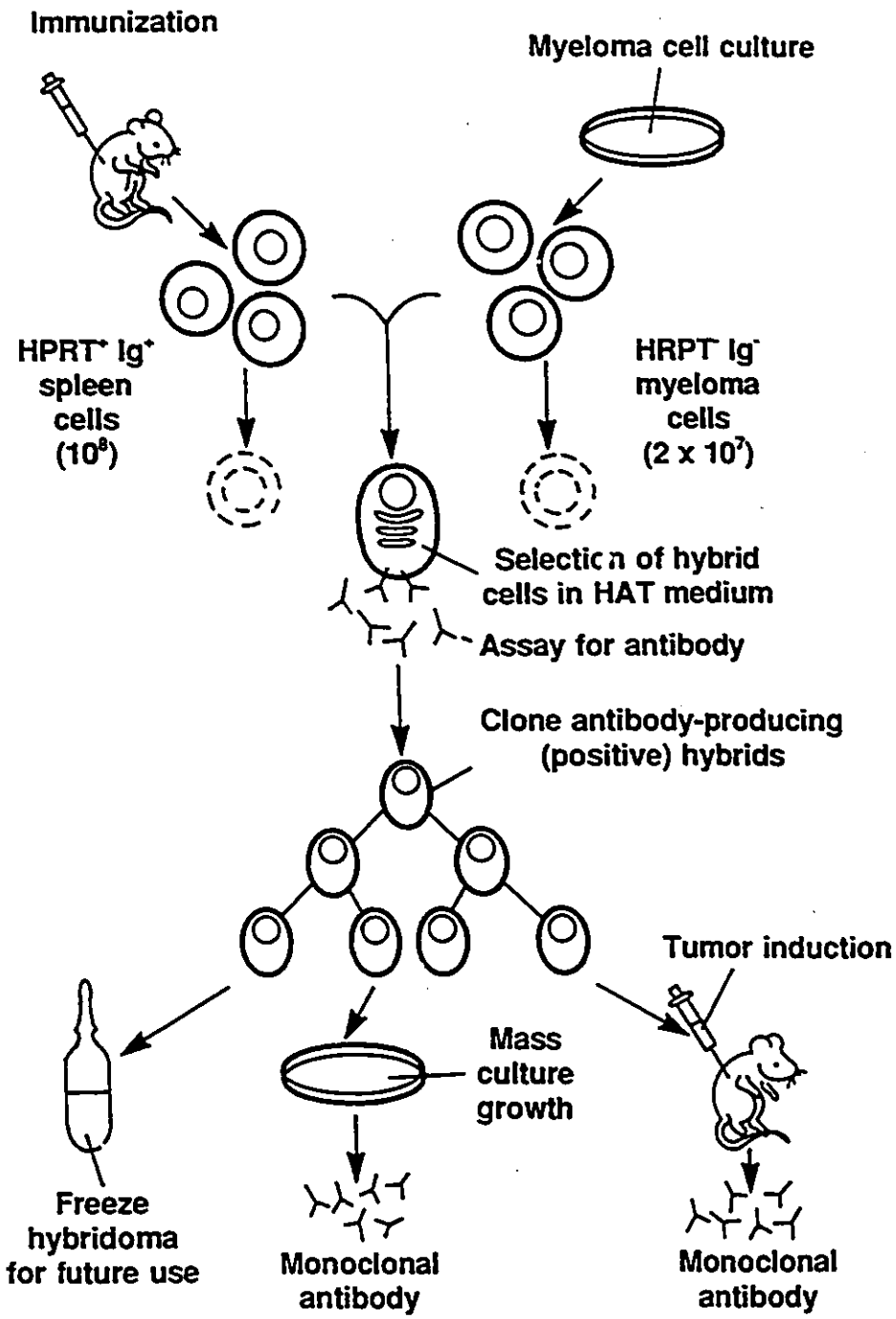


Figure 4.1. Formation of hybridomas between mouse cells and myeloma cells. Mouse myeloma cells that do not produce their own immunoglobulins and lack hypoxanthine phosphoribosyl transferase (HPRT) are fused to splenocytes from an immunized mouse with polyethylene glycol. The hybrid cells are selected in hypoxanthine-aminopterin-thymidine (HAT) medium. Unfused myeloma cells are killed by HAT and unfused splenocytes die out. The hydridomas are cloned, and antibody is produced in tissue culture or by ascites formation. Reference: Diamond et al (1981)

Figure 4.2 *Dientamoeba fragilis* culture visualized by indirect immunofluorescence staining with normal mouse serum (no fluorescence) (A), and positive fluorescence with MAb 8B2 (B) and MAb 1H11 (C).

Figure 4.2 (A) Normal mouse serum (no fluorescence)

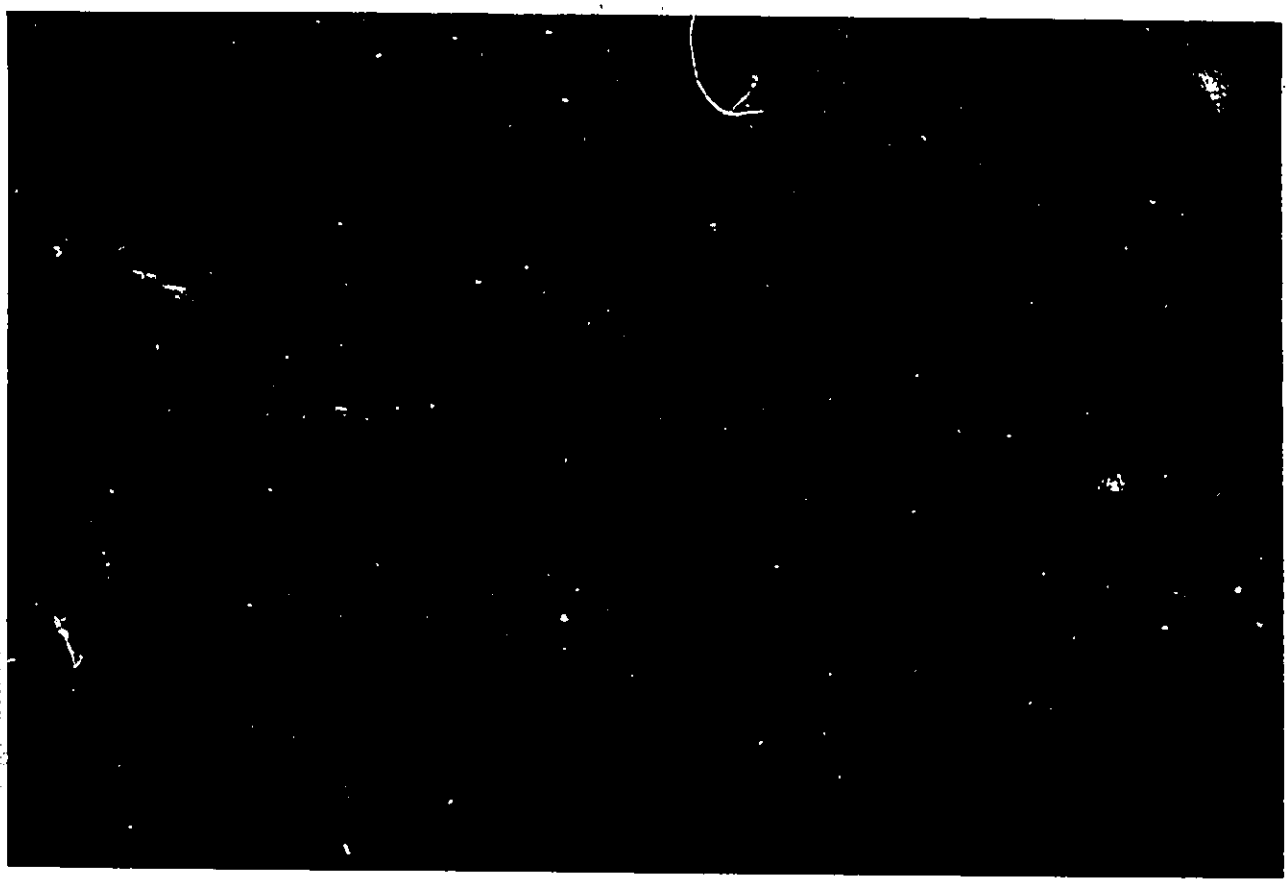


Figure 4.2 (B) MAb 8B2 (positive fluorescence)

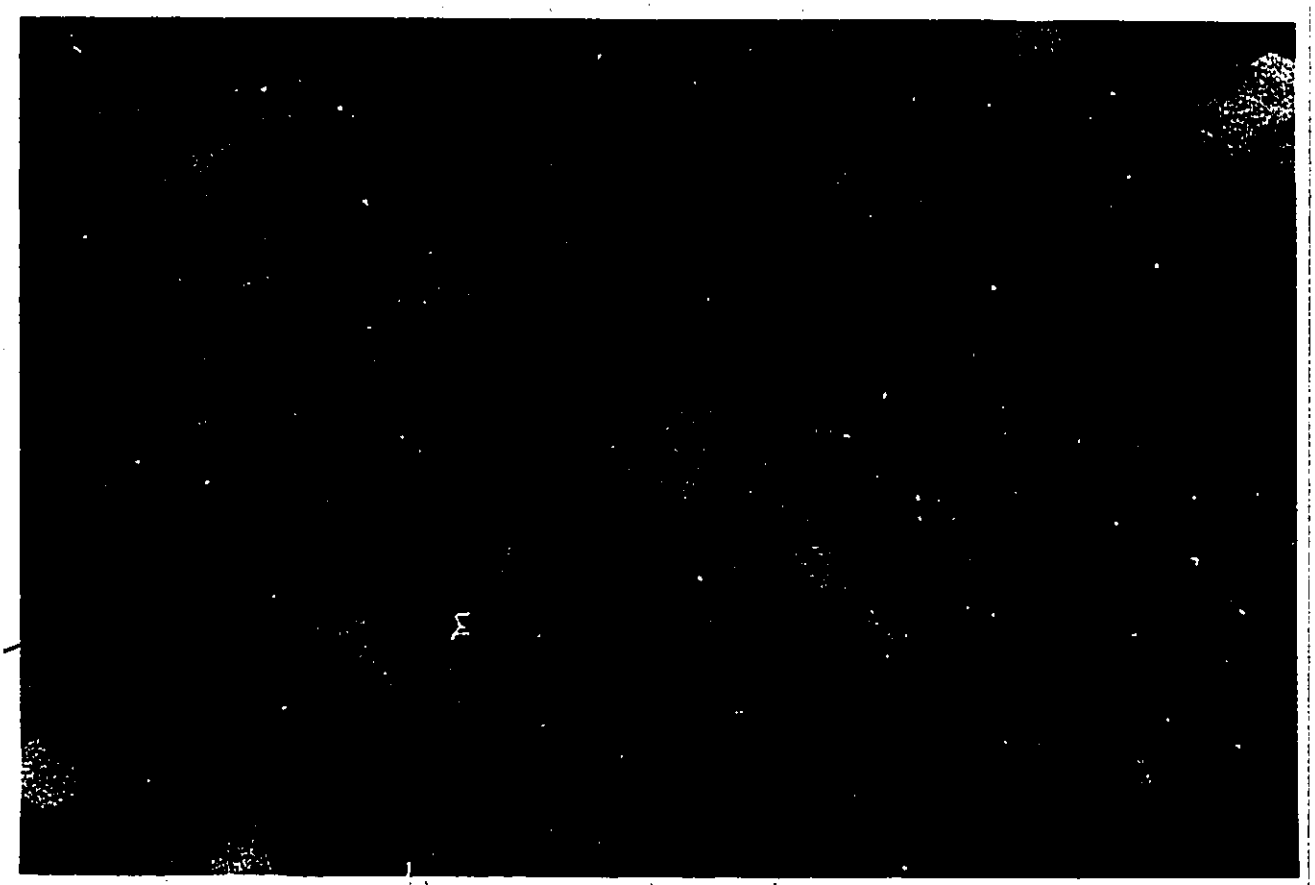


Figure 4.2 (C) MAb 1H11 (positive fluorescence)

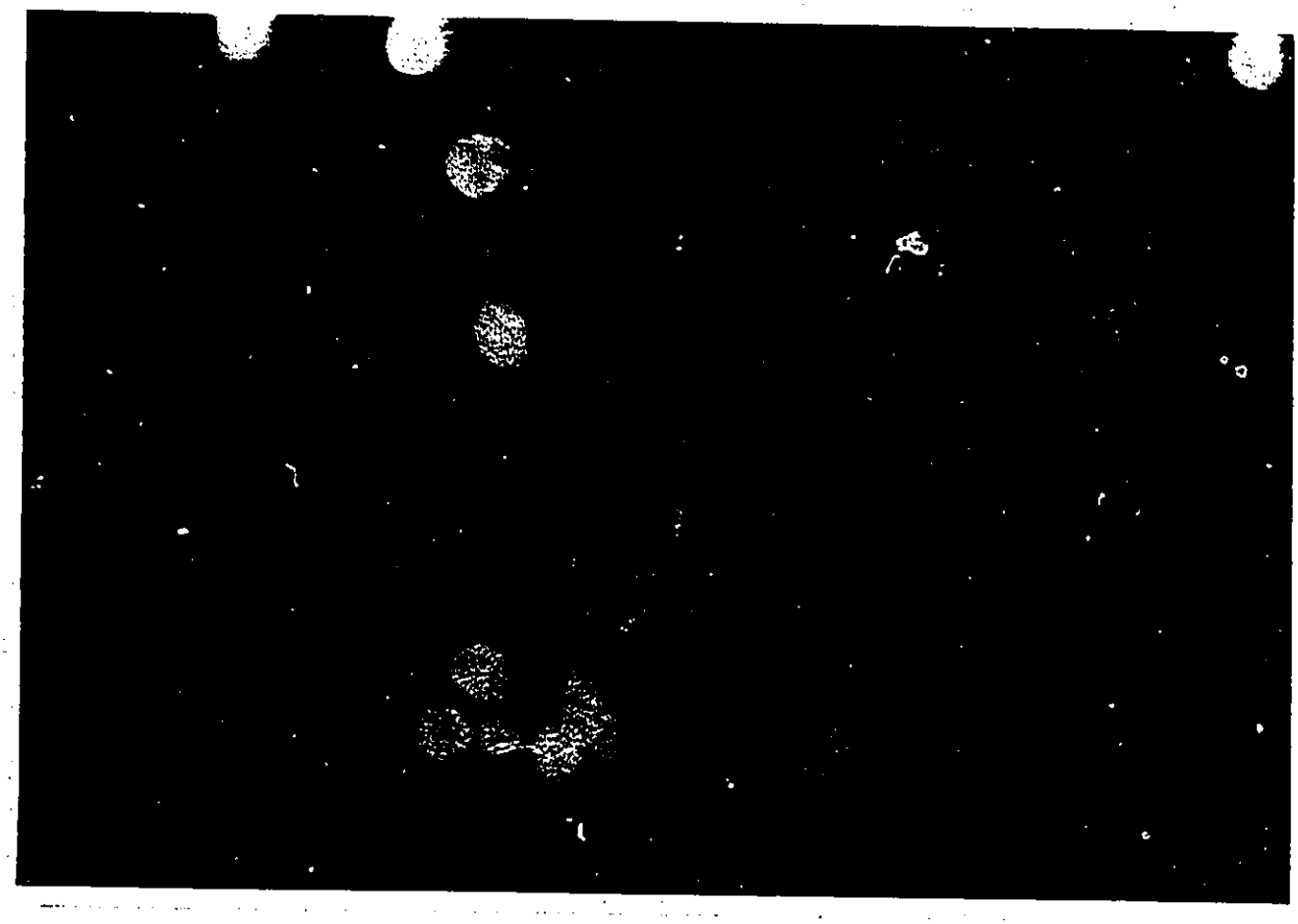
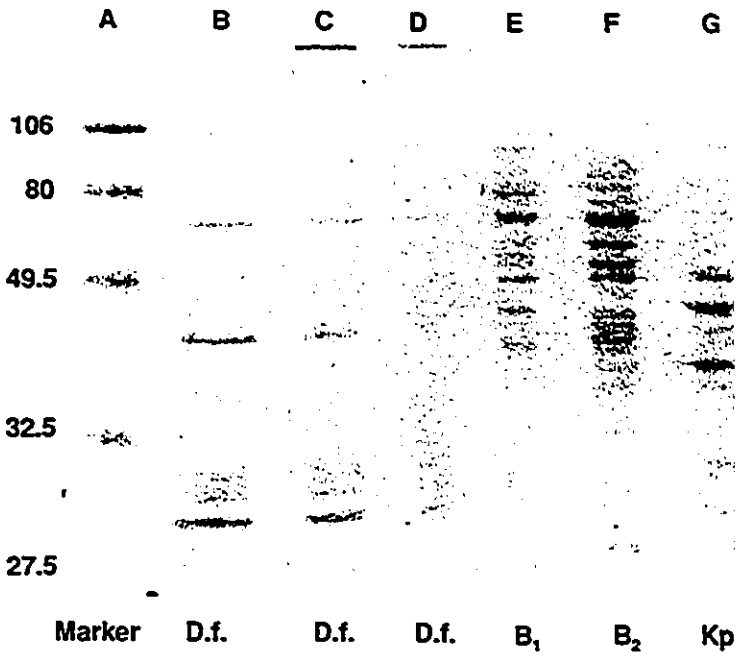
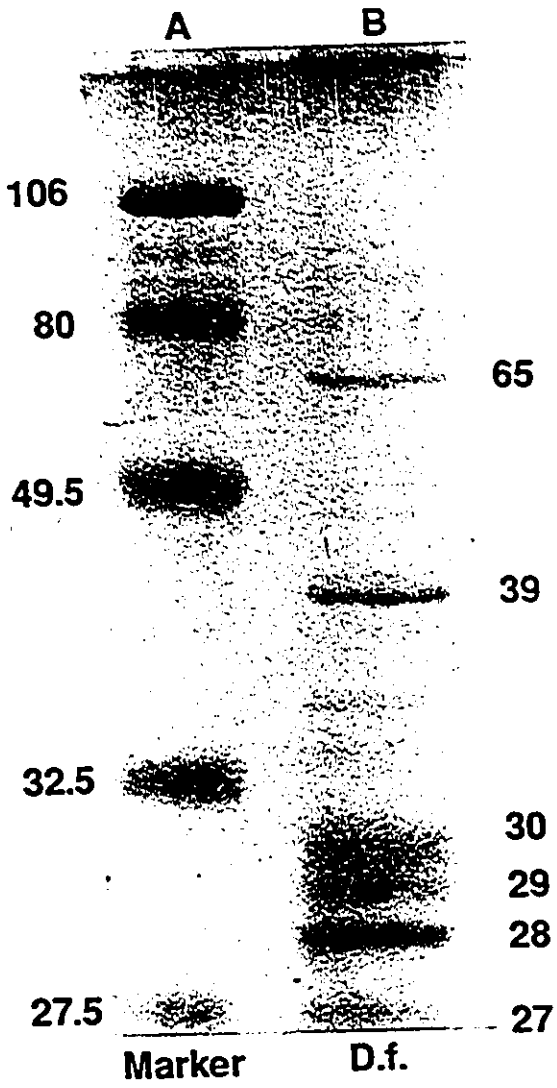


Figure 4.3 SDS-PAGE of *Dientamoeba fragilis* and the bacteria in the ATCC dixenic culture. Lane A: Molecular size marker in kilodaltons, Lanes B, C and D: *D. fragilis* with 75 μg, 50 μg and 25 μg of protein respectively, Lane E: *Bacteroides vulgatus* variant B1, Lane F: *B. vulgatus* variant B2, Lane G: *Klebsiella pneumoniae*. Protein bands of *D. fragilis* are distinctly different in molecular sizes from those of the bacteria.



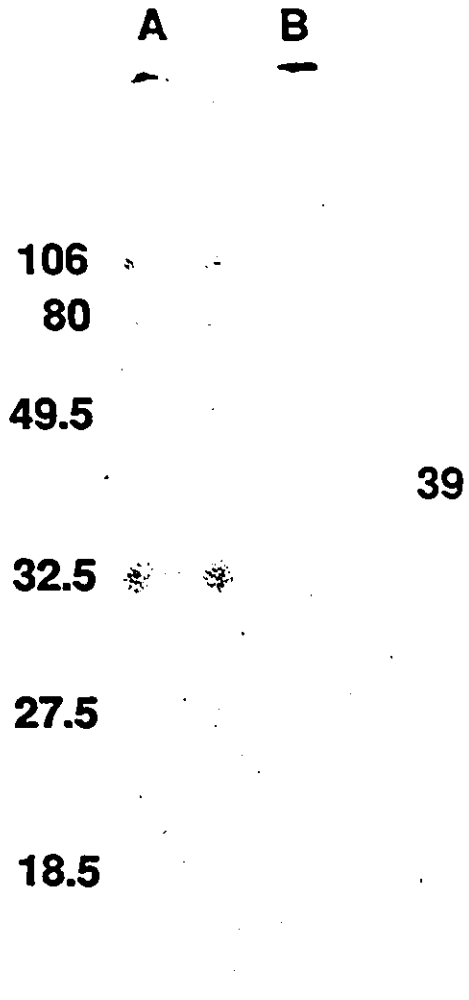
SDS - PAGE of D. fragilis Bacteroides vulgatus (variants 1 and 2) and Klebsiella pneumoniae

Figure 4.4 **SDS-PAGE of *Dientamoeba fragilis* with 75 μg of protein.** Lane A: molecular size marker in kilodaltons, Lane B: *D. fragilis* with at least 6 discernable bands of molecular masses 65,39,30,29,28 and 27 kDa.



SDS - PAGE of D. fragilis

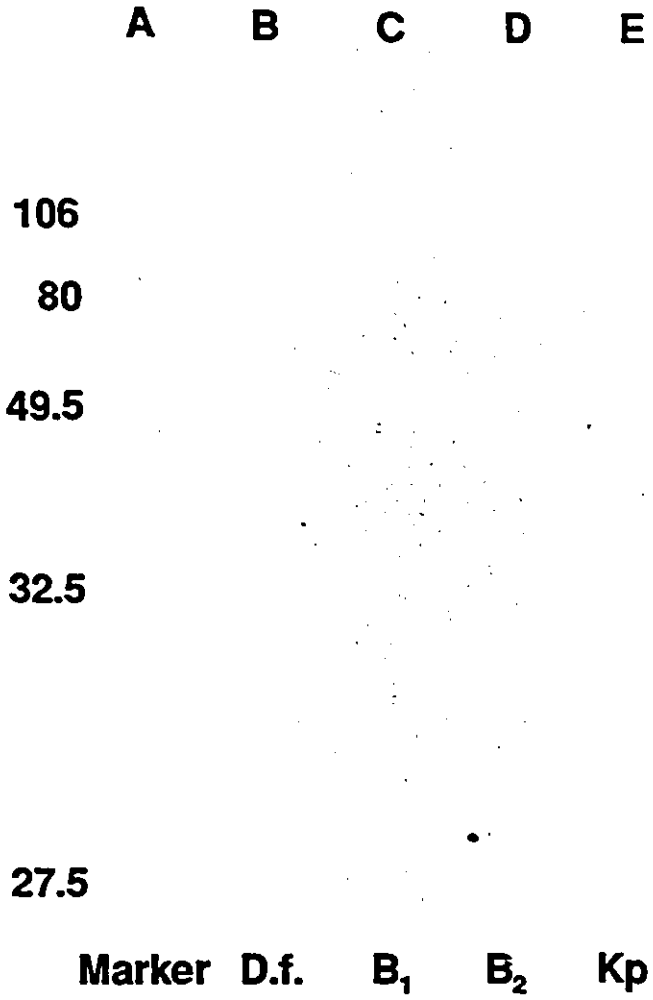
Figure 4.5 **Immunoblot of *Dientamoeba fragilis* using MAb 1H11 (mouse ascites fluids, diluted 1 in 50).** Lane A: molecular size marker in kilodaltons, Lane B: MAb 1H11 identifying a 39 kDa *D. fragilis* protein.



Marker D.f.

Immunoblot Using
MAb IHII

Figure 4.6 **Immunoblot using normal mouse serum diluted 1 in 50, as a negative control.** Lane A: Molecular size marker in kilodaltons, no band was observed for Lane B (*Dientamoeba fragilis*), Lane C (*Bacteroides vulgatus* variant B1), Lane D (*B. vulgatus* variant B2), or Lane E (*Klebsiella pneumoniae*).



Immunoblot Using Normal Mouse Serum

Figure 4.7 **MAb 1H11 giving a positive indirect immunofluorescence reading on *Dientamoeba fragilis* trophozoites from a clinical specimen.**

CHAPTER 5

SUSCEPTIBILITY TESTING OF *DIENTAMOEBA FRAGILIS* ATCC 30948 WITH IODOQUINOL, PAROMOMYCIN, TETRACYCLINE, AND METRONIDAZOLE.

The study described in this chapter has been published in Antimicrobial Agents and Chemotherapy 38: 1157-1160 (1994).

INTRODUCTION

Carbarsone (Hankasson 1936) was the first drug used to treat patients infected with *Dientamoeba fragilis*. Subsequently the use of emetine, carbarsone and tetracycline was mentioned by Kean and Malloch (1966) in the treatment of *D. fragilis* infections. Spencer et al (1979) found that treatment with iodoquinol or metronidazole was effective for their patients. Millet et al (1983) also reported successful treatment of *D. fragilis* infections with iodoquinol. The current drug of choice to treat *D. fragilis* infections is iodoquinol, or paromomycin, or tetracycline (The Medical Letter 1992).

The objective of this study was to determine the *in vitro* susceptibility of *D. fragilis* to iodoquinol, paromomycin, tetracycline and metronidazole.

MATERIALS AND METHODS

A dixenic culture of *D. fragilis* (ATCC 30948) was acquired from the American Type Culture Collection (ATCC). The two bacteria listed by ATCC in this dixenic culture are *Klebsiella pneumoniae* and *Clostridium perfringens*. While the presence of *K. pneumoniae* was evident and confirmed, we were unable to isolate *C. perfringens* from the culture we received. Instead, a *Bacteroides sp.* was isolated. The organism was identified as *Bacteroides vulgatus* by the Laboratory Centre for Disease Control, Ottawa, Ontario, Canada. (Please refer to Chapter 2). The bacterial species that were cocultured helped to maintain *D. fragilis* on continuous passage in TYGM-9 medium (ATCC medium 1171).

The following antiparasitic compounds were kindly provided for our study: iodoquinol (Searle Canada Inc., Oakville, Ontario, Canada), paromomycin (Parke-Davis,

Ann Arbor, MI), tetracycline (Pfizer Canada Inc., Arnprior, Ontario, Canada), and metronidazole (Abbot Laboratories Ltd., Montreal, Quebec, Canada). Stock solutions (10,000 μg/ml) were made in sterile distilled water (except for iodoquinol, which was prepared as a suspension in 10% ethanol because of its poor solubility in water), further diluted and added to tubes of rice starch supplemented culture medium adjusted to contain a volume of 8 ml. We determined in a separate experiment that the amount of ethanol used in the drug dilutions did not have deleterious effects on *D. fragilis*. Serial doubling dilutions of each drug were prepared in these tubes of medium to cover a range of 256 to 1 μg/ml. A tube of supplemented medium with no drug was included as a growth control. The *D. fragilis* inoculum was adjusted to 10^5 trophozoites in 0.1 ml by low speed centrifugation (100 x g for 10 minutes) and counting with a haemacytometer. After seeding with 0.1 ml of inoculum containing *D. fragilis* trophozoites and the two bacteria, all inoculated tubes were incubated at 35°C. The number of live trophozoites in each tube was determined by trypan blue exclusion by taking the average of three counts on a daily basis. Growth of *D. fragilis* in the control tube peaked to 7.7 x 10^5 trophozoites per ml in three days (Table 5.1), after which the number of live trophozoites declined. The daily counting was therefore continued for 3 days for the drug-containing tubes of medium.

RESULTS

Table 5.1 shows the activities of the four drugs on *D. fragilis*. It was a common finding for all four drugs that the number of viable *D. fragilis* trophozoites decreased as the concentration of the drugs increased. Viable trophozoites also disappeared abruptly at a particular concentration of each drug. At and above this amoebicidal concentration,

viable *D. fragilis* trophozoites were not found. This "all or none" phenomenon was probably due to the inability of the fragile trophozoites to repair and revive themselves in the presence of the antiparasitic drugs and the damage became irreversible. From a practical point of view, it was also difficult to find very small numbers of viable trophozoites microscopically with the small volumes used in a haemacytometer for counting. The minimal amoebicidal concentrations of the following drugs were determined: iodoquinol at 128 μg/ml, paromomycin at 16 μg/ml, tetracycline at 32 μg/ml, and metronidazole at 32 μg/ml (Table 5.1).

Because these antiparasitic drugs could also be antibacterial, we performed agar dilution testing on *K. pneumoniae* and *B. vulgatus*, the two supporting bacteria in the *D. fragilis* culture. Serial doubling dilutions of the four drugs were prepared in Mueller Hinton agar (Becton Dickinson Microbiology Systems, Cockeysville, MD) with 5% defibrinated sheep blood. On the basis of the amoebicidal results obtained with *D. fragilis*, the following ranges of serial doubling dilutions of the four drugs were prepared in the agar dilution test: 128 to 1 μg/ml for iodoquinol, 16 to 1 μg/ml for paromomycin, 32 to 1 μg/ml for tetracycline, 32 to 1 μg/ml for metronidazole.

While the *K. pneumoniae* strain in the dixenic culture appeared to be uniform in colonial morphology, *B. vulgatus* presented consistently with two different colony sizes which we designated B1 (small colonies) and B2 (large colonies). The biochemical reactions of variants B1 and B2 were similar. (Please refer to Chapter 2). We included here susceptibility studies on both the B1 and B2 variants of *B. vulgatus*.

Fresh (24 h) cultures of *K. pneumoniae* as well as the B1 and B2 variants of *B.*

vulgatus were suspended in 5 ml of Mueller-Hinton broth (Oxoid Limited, Basingstoke, England) to match a 0.5 McFarland turbidity standard (approximately 10^8 CFU/ml). These bacterial suspensions were further diluted 1 in 10 to give a concentration of 10^7 CFU/ml. A 1-μl volume of this diluted suspension was inoculated to each plate of the drug dilution series. A control plate with no drugs was included as a growth control. All the inoculated plates were incubated anaerobically at 35°C for 48 h.

The MICs of the four drugs for the bacteria are given in Table 5.2. There was no inhibition *of K. pneumoniae* or *B. vulgatus* (both B1 and B2 variants) by iodoquinol (highest concentration tested, 128 μg/ml) and paromomycin (highest concentration tested, 16 μg/ml). The *K. pneumoniae* strain was highly susceptible to tetracycline, (MIC, $\leq$ 1 μg/ml). There was a twofold difference in the tetracycline MIC for the two *B vulgatus* variants: 16 μg/ml for variant B1 and 32 μg/ml for variant B2. Metronidazole up to a concentration of 32 μg/ml did not exhibit any inhibitory activity against *K. pneumoniae*, while both *B. vulgatus* variants were inhibited by metronidazole at a concentration of $\leq$ 1 μg/ml.

By comparing the activities of the four drugs against *D. fragilis* in the dixenic culture and the bacterial isolates, we were led to the following conclusions. Because of the lack of antibacterial activity by iodoquinol and paromomycin under the test conditions of the present study, the amoebicidal activity exerted against *D. fragilis* should be directly related to these two drugs. On the other hand, the activity of tetracycline against *D. fragilis* was difficult to assess. *The K. pneumoniae* strain was highly susceptible to

tetracycline (≤1 μg/ml), and both variants of *B. vulgatus* were inhibited by tetracycline at a concentration of 32 μg/ml, the same concentration at which depletion of live *D. fragilis* trophozoites was observed. The inhibition of growth of *D. fragilis* by tetracycline might be attributed to this concentration of the drug or it may be a result of the depletion of the bacteria which supported the growth of the trophozoites. Metronidazole was highly active against both *B. vulgatus* variants (MIC, ≤ 1 μg/ml), but it had no activity against *K. pneumoniae*. This can be expected because the cytotoxic selectivity of metronidazole results from the necessity of the drug to be reduced to an active cytotoxic form, and this does not occur in aerobic cells (Johnson 1993). It appears that under the action of metronidazole the growth of *D. fragilis* was sustained by *K. pneumoniae* as a monoxenic culture and that the MIC of 32 μg/ml (versus ≤ 1 μg/ml for *B. vulgatus*) was a "true" MIC for *D. fragilis*.

DISCUSSION

To summarize, we performed antiparasitic drug susceptibility testing on the ATCC dixenic culture of *D. fragilis*. With the exception of tetracycline, which exhibited inhibition against both *K. pneumoniae* and *B. vulgatus* therefore making the results of the study on *D. fragilis* inconclusive, all other drugs (iodoquinol, paromomycin, and metronidazole) yielded results which could indicate that they were inhibitory against *D. fragilis* because of their limited or total lack of activity against the supporting bacteria. However, we do not know the complexities and influences of the bacteria in the dixenic culture. It is possible that subinhibitory concentrations of antimicrobial agents could affect

the bacterial populations in more subtle ways, such as causing the release of toxins or growth factors that might have an adverse effect on the viability of the parasite. We tried to avoid performing susceptibility testing on *D. fragilis* in a mixed bacterial culture through axenization with imipenem and piperacillin to eliminate the bacteria (Chan et al 1993). Unfortunately, attempts to axenize *D. fragilis* by this and other means, including increasing the concentration of bovine serum to 15% (Diamond et al 1978), using a *Crithidium sp.* strain ATCC 50083 (Diamond 1968), adding preconditioned medium obtained from bacterial growth, adding heat-killed bacteria; and incubating the cultures under anaerobic conditions (Zierdt 1991), have failed. Perhaps *D. fragilis* can be axenized by eliminating the anaerobes first by repeated passage in the presence of low concentrations of metronidazole. Then it may be possible to eliminate *K. pneumoniae* with low concentrations of tetracycline or other antibiotics. We also tried to perform drug testing by the agar colony method (Gillin and Diamond 1978), but the necessity to maintain bacterial growth for sustaining the viability of *D. fragilis* made it impossible to quantify *D. fragilis* with this technique. In the absence of an axenic culture, we could achieve drug susceptibility testing results for *D. fragilis* only in the presence of the supporting bacteria. We noticed that earlier studies on drug testing with *D. fragilis* and other intestinal parasites were performed by Balamuth (1953) with bacterial cultures. We also believe that antiparasitic drug testing in the presence of bacteria is probably clinically relevant because it may simulate the in vivo situation.

There have been few studies on *D. fragilis*, even though the parasite was described more than 75 years ago, and no susceptibility studies of the drugs mentioned have been

done in the laboratory. Iodoquinol, paromomycin and tetracycline are listed as the drugs of choice for the treatment of *D. fragilis* infections (The Medical Letter 1992), but we were unable to find experimental data to substantiate this recommendation. Metronidazole is not recommended as a drug for the treatment of *D. fragilis* infections, but it was described by Spencer et al (1979) as an effective agent. However, the drug's mutagenicity and carcinogenic potential make it a less than ideal drug for use in children (Turner 1985). As with the other three drugs tested, experimental details on the susceptibility testing of metronidazole against *D. fragilis* are also lacking.

We demonstrated in the present study that, in the absence of an axenic culture, an in vitro drug susceptibility test can be performed on *D. fragilis* in the presence of growth-supporting bacteria. The minimal amoebicidal concentrations on *D. fragilis* determined in our study were: 128 μg/ml for iodoquinol, 16 μg/ml for paromomycin, 32 μg/ml (questionably) for tetracycline, and 32 μg/ml for metronidazole. It is difficult to correlate these minimal amoebicidal concentrations with the clinical responses reported in the literature because of the lack of knowledge on the levels of the antimicrobial agents in the gut. There is virtually no information on the concentrations of iodoquinol in the gastrointestinal tract, although it is reckoned that only about 5% of the drug is absorbed from the gastrointestinal tract (Searle Canada Inc., 1993). Paromomycin is poorly absorbed from the gastrointestinal tract, and most of an oral dose is excreted unchanged in the faeces. Unfortunately, there are no data on the levels of paromomycin in the gastrointestinal tract (Parke-Davis 1993). Tetracycline is a well-established antimicrobial agent, but a search of the literature published over the past 30 years did not reveal any

information on the concentration of the drug in the gut(Pfizer Canada Inc. 1993). In a study on metronidazole therapy for antibiotic-associated colitis caused by *Clostridium difficile*, Bolton and Culshaw (1986) reported a maximal metronidazole concentration in faeces of 24.2 μg/g and a maximal hydroxy-metronidazole concentration in faeces of 62.4 μg/g. This may explain the reported effective treatment of *D. fragilis* infections with metronidazole (Spencer et al 1979). While our study was performed on the ATCC strain of *D. fragilis* (which originated from a patient), the same procedure can be applied to a clinical isolate if it can be established in culture. Unfortunately, it is difficult to establish *D. fragilis* isolates from patients in culture and this may limit the application of the assay in the clinical management of *D. fragilis* infections. In the absence of a more refined protocol for susceptibility testing of *D. fragilis*, the approach described here can probably be used to test newer drugs as well, if they become available. If we consider the prevalence and pathogenic potential of *D. fragilis*, it is amazing to see that so little progress has been made on the study of this organism and that there is such a lack of experimental data on drug interaction with *D. fragilis*. As pointed out by Balamuth in 1953: "There is no question that a standardized technique is strongly needed and long overdue in the interest of accuracy and repeatability of claims. This advance has been impeded by our present inability to maintain these parasites in pure culture as well as by ignorance of other requirements existing in their complex environment." This situation has not changed over the past 40 years for *D. fragilis*. We described in this study the activities of antiparasitic agents against a dixenic *D. fragilis* culture. It should be

cautioned, however, that susceptibility testing results reported here were obtained on a single ATCC isolate, and it remains to be demonstrated how generalizable these results will be. Research towards a better understanding of the epidemiology, pathogenicity, diagnosis and treatment of this interesting organism is very much needed.

Table 5.1 **Minimal amoebicidal concentrations [a] of iodoquinol, paromomycin, tetracycline, and metronidazole for *D. fragilis* ATCC 30948 determined by viable counts performed daily over 3 days.**

Drug concentration (μg/ml)	Viable counts (x 10^4 per ml.) over 3 days											
	Iodoquinol			Paromomycin			Tetracycline			Metronidazole		
	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days
0 [b]	10	43	77	-	-	-	-	-	-	-	-	-
1	10	33	62	7.5	18	33	12	25	53	10	32	66
2	9.7	32	49	7.5	15	25	9.0	31	48	11	26	56
4	8.5	28	53	7.5	15	25	7.0	27	43	9.8	19	45
8	6.5	23	43	7.5	15	18	6.0	20	40	5.7	9.8	33
16	6.2	19	40	0	0	0	3.8	20	37	3.0	1.8	32
32	5.5	15	37	0	0	0	0	0	0	0	0	0
64	4.7	10	30	0	0	0	0	0	0	0	0	0
128	0	0	0	0	0	0	0	0	0	0	0	0
256	0	0	0	0	0	0	0	0	0	0	0	0

[a] Iodoquinol, 128 μg/ml, paromomycin, 16 μg/ml; tetracycline, 32 μg/ml (see text), and metronidazole, 32 μg/ml.

[b] Growth control tube with no drugs.

Table 5.2 **MICs of iodoquinol, paromomycin, tetracycline and metronidazole for *K. pneumoniae* and two variants of *B. vulgatus*.**

Organism	MIC (μg/ml)			
	Iodoquinol	Paromomycin	Tetracycline	Metronidazole
K. pneumoniae	>128	>16	≤1	>32
B. vulgatus (variant B1)	>128	>16	16	≤1
B. vulgatus (variant B2)	>128	>16	32	≤1

CHAPTER 6

PREVALENCE OF *DIENTAMOEBA FRAGILIS* ANTIBODIES IN INFANCY, CHILDHOOD AND ADOLESCENCE

A manuscript based on this chapter is being prepared for publication.

INTRODUCTION

Dientamoeba fragilis is a protozoan parasite first described by Jepps and Dobell in 1918. Its habitat is the human large intestine. It has been reported from most parts of the world where careful surveys have been conducted, with an estimated incidence of 1.4 to 53% (Burrows and Swerdlow 1956, Kean and Malloch 1966, Talis et al 1971, Yang and Scholten 1977 a, Bruckner et al 1979, Spencer et al 1979, Millet et al 1983). In selected population groups, *Dientamoeba* was reported to have a higher prevalence, ranging from 26.0% to 32.6% (Ockert 1990).

D. fragilis has been reported in Canada (Porter 1934, Fantham and Porter 1936, Miller 1939, Saunders 1949), but the recovery rates have been very low except for the Toronto area (Scholten and Yang 1975). There is no reason to suppose that *D. fragilis* is more prevalent in the Toronto area than elsewhere, and its low incidence or absence in other areas probably reflects a lack of awareness of this parasite and the use of techniques unsuitable for its recovery (Scholten and Yang 1974).

Although *D. fragilis* was described more than 75 years ago (Jepps and Dobell 1918), there have been few studies on this organism. Before our first report (Chan et al 1993) on the application of an indirect immunofluorescence (IIF) technique, the only available method of laboratory diagnosis of *D. fragilis* infections is microscopic examination of stained smears of preserved faecal specimens for the characteristic trophozoites (Scholten and Yang 1974, Grendon et al 1991). We searched the literature and could not find information on the seroprevalence of this organism. The objective of this study was to determine the prevalence of antibodies to *D. fragilis* in infancy,

childhood and adolescence in the National Capital Region of Canada, by using the IIF technique.

MATERIALS AND METHODS

Review of records of parasitology. We reviewed the records of the past 5 years (1989-1993) on parasitology examination at the Children's Hospital of Eastern Ontario (CHEO). The frequencies of identification of 3 organisms (*D. fragilis*, *Giardia lamblia* and *Cryptosporidium parvum*) associated with diarrhoea and known to be endemic in our region were compared.

Serum specimens. (A) Clinically symptomatic patients: 3 patients (F 10 y, M 12 y and F 16 y) attended the out-patient clinic at CHEO because of gastrointestinal symptoms. Laboratory investigations did not reveal any enteric pathogens except a "pure" infection of *D. fragilis* on parasitological examinations using sodium acetate-acetic acid-formalin fixative (Yang and Scholten 1977b) and haematoxylin staining method. Blood was drawn for this study with consent from the patients and/or their parents. (B) Age and sex-matched controls: for each of the 3 patients 4 age- and sex- matched healthy controls participating in a vaccine immunogenic study in January 1992 were found. None of these 12 well controls had any gastrointestinal symptoms in the preceding month. (C) Population study: Sera were obtained from 189 children aged from 6 months to 19 years who had participated in a vaccine immunogenic study. Each child was healthy and informed consent was obtained prior to collection of the blood. The study was approved by the Ethics Review Committee of CHEO. Questionnaires on these healthy subjects were reviewed, and demographic information including the age, sex and place of birth was

recorded. All sera were stored at -20°C until this seroprevalence study for *D. fragilis* antibodies commenced.

***D. fragilis* strain and maintenance of culture.** Please refer to Chapter 2 (page 47).

Bacteria in *D. fragilis* culture. Please refer to Chapter 2 (page 50).

Preparation of *D. fragilis* antigen slides. Please refer to Chapter 4 (page 79).

IIF assay. Twofold serial doubling dilutions of sera were made in FA buffer, starting at a dilution of 1 in 10 and ending at 1 in 320. Twenty-five μl of serum dilutions were applied to the antigen slides for 1 h at 35°C in a moist chamber. After three washings in FA buffer (5 min each) a blocking solution of 3% fetal calf serum in FA buffer was applied for 30 min. This was followed by three washings in FA buffer, and 25 μl of fluorescein labelled goat anti-human polyvalent immunoglobulins (Sigma Chemical Company, St. Louis, MO.) diluted 1 in 100 in FA buffer with Evans blue (dilution of 1 in 20,000 in buffer) were applied for 1 h. After three washings, the air-dried slides were mounted with FA mounting fluid (Difco Laboratories, Detroit, MI) and were examined with a 25x objective on a Leitz epifluorescence microscope with a 490-nm exciter filter, a 510-nm dichromatic beam splitter, and a 520-nm barrier filter. *D. fragilis* trophozoites showed a pink-red color with negative sera (Fig. 6.1A) and a bright yellow-green color with positive sera (Fig. 6.1B). Positive sera were graded (4+, 3+, 2+ and 1+) by their intensity of fluorescence from 4+ (strong reaction) to 1+ (weak reaction). A reading of 2+ (medium but definite fluorescence) was taken as the end-point of the dilution series for a positive serum sample. A serum sample giving a titer of 10 or higher was regarded as positive (Tzipori and Campbell 1981, Jokipii et al 1988). Geometric mean titres

(GMT) were calculated from the end-point dilutions for each sex and age group distribution. Sera negative at 1 in 10 dilutions were assigned a GMT value of 1 for calculation. As quality control for the test, a positive and a negative serum sample were included in each run.

In addition to measurement of whole immunoglobulins using polyvalent antiserum, sera from the three symptomatic patients and the 12 matched well controls were also tested for IgG, IgM and IgA antibodies individually by IIF with fluorescein-labelled reagents (Wellcome Diagnostics, Temple Hill, Dartford, England). The fluorescein labelled anti-human immunoglobulin class reagents were used at a dilution of 1 in 40 in FA buffer.

Absorption of patient sera before immunoblotting. An immunoblot assay was carried out on 20 representative absorbed serum samples to demonstrate the validity of the IIF test. Because human sera may have natural antibodies to the bacteria (*K. pneumoniae* and *B. vulgatus*) in the *D. fragilis* dixenic culture, we used those bacteria to perform an absorption test to enhance the specificity of the immunoblot assay. *K. pneumoniae* as well as B1 and B2 variants of *B. vulgatus* were grown in TYGM-9 medium overnight at 35°C. They were washed three times in FA buffer by centrifugation at 1,000xg for 10 min. Equal volumes of patient sera and bacterial suspension (to which 0.15 ml of rice starch suspension had been added after the final washing) were mixed and allowed to react for 1 h at 35°C, with gentle mixing every 10 min. At the end of 1 h, the mixture in 1.5 ml Eppendorf tubes was spun in an Eppendorf microcentrifuge at 10,000x g for 3 min to pellet the bacteria and the starch particles. The supernatant was removed and used as a 1 in 2 serum dilution for further dilution tests. Sera from the three symptomatic patients,

the 12 well controls, two children with high IIF titres (titres of 160), and three children with negative IIF readings were selected as representative samples for this absorption test before they were subjected to the immunoblot assay.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

D. fragilis culture was prepared as described under "Preparation of *D. fragilis* antigen slides". Trophozoites washed with FA buffer were resuspended in 0.5 ml PBS - 0.05% Tween 20 instead of sodium acetate-acetic acid-formalin fixative. The bacteria in the *D. fragilis* dixenic culture were also propagated for comparative study in SDS-PAGE and immunoblotting. *K. pneumoniae* was cultured aerobically on 5% sheep blood agar and incubated at 35°C for 24 hours. B1 and B2 variants of *B. vulgatus* were grown on 5% sheep blood agar and incubated anaerobically at 35°C for 48 hours. These bacteria were suspended and washed 3 times in FA buffer at 10,000xg for 3 minutes in an Eppendorf centrifuge. After the final wash the bacteria were suspended in 0.5 ml PBS-0.05% Tween 20. *D. fragilis* and bacterial preparations were sonicated 6 times for 10 seconds each time in ice cold water. They were then frozen overnight at -20°C. After thawing they were centrifuged at 12,000xg for 6 minutes. Protein concentrations were determined for the supernatants which were mixed with sample buffer (0.5M Tris-HCl [pH 6.8], 10% glycerol, 10% SDS, 5% ß-mercaptoethanol, and 0.05% bromophenol blue) and then boiled for 5 minutes. A preparation of 68 μg of *D. fragilis* protein was loaded per gel track for electrophoresis. A 10-μl sample of a boiled prestained solution of molecular mass standards with the following molecular masses (in kilodaltons): phosphorylase b,

106; bovine serum albumin, 80; ovalbumin, 49.5; carbonic anhydrase, 32.5; soybean trypsin inhibitor, 27.5; lysozyme, 18.5 (Bio-Rad Laboratories Inc., Mississauga, Ontario, Canada) was loaded in one track of each gel. SDS-PAGE was performed according to procedures of Laemmli (1970), and Harlow and Lane (1988) with 15% separating gel in a mini-Protean II cell apparatus (Bio-Rad) at 100 V for 2 hours. Electrophoresed proteins were transferred to nitrocellulose (BA-S NC supported nitrocellulose, poresize 0.45 μm, Schleicher and Schuell, distributed by Mandel Scientific Co. Ltd., Guelph, Ontario, Canada) as described by Towbin et al (1979), at 100 V for 1 hour. Replica gels were stained with Coomassie brilliant blue. Nitrocellulose was blocked with 3% gelatin and 1% bovine serum albumin in Tris-buffered saline (TBS), (60 minutes at 35°C), washed (once for 10 minutes in 0.05% Tween 20-Tris buffered saline [TTBS]), and probed with absorbed patient serum samples (1/80 dilution, overnight at 35°C). After 3 washings (10 minutes each) with TTBS, the membrane was exposed to a 1/500 dilution of peroxidase-conjugated goat anti-human whole molecule IgG (Sigma) in TBS-1% gelatin (2 hours at 35°C). The membrane was washed 3 times in TTBS, and the protein bands were developed in TBS-5% 4-chloro-1-naphol (Pierce, Rockford,IL)-0.015% H_2O_2 for 30 minutes.

RESULTS

Review of records of parasitology. The frequencies of identification of *D. fragilis*, *G. lamblia* and *C. parvum* at CHEO from 1989 - 1993 are listed in Table 6.1. *D. fragilis* was the most frequently found parasite in 4 of 5 years. In the combined total of 5 years, *D. fragilis* was found in 151 of 5774 specimens (2.6%) followed by *G. lamblia* at 2.0% and

C. parvum at only 0.2%.

Positive control. A positive serum sample gave an initial 2+ endpoint reading for *D. fragilis* antibodies at a dilution of 1 in 40. This serum was used as a positive control on each of the 9 days of IIF testing. The daily endpoint reading fluctuated from a titer of 20 to a titre of 80 (Table 6.2), i.e. a difference of 2 tubes of dilution, but it was within one dilution factor when compared to the initial titer of 40. The geometric mean titer of 9 runs was 29, which was considered as either the same, or within one tube of dilution of the initial titer.

Sera from symptomatic patients. Sera from the three symptomatic patients (F 10 y, M 12 y and F 16 y) infected with *D. fragilis* gave IIF titres of 80 for whole immunoglobulins (Table 6.3). There was no difference (either same titer or within one tube of dilution) in the IIF reading for *D. fragilis* antibodies with the pre-absorbed and absorbed serum samples of these three symptomatic patients. Antibodies to *D. fragilis* were mainly of the IgG class, but IgM antibodies were also detected in lower titres. Tests for serum IgA antibodies were negative at 1 in 10 dilutions (Table 6.3).

Sera from age- and sex- matched well controls.

Results of IIF readings for *D. fragilis* antibodies with the serum samples from 12 age- and sex- matched well controls are shown in Table 6.3. Four healthy controls were matched for each of the three symptomatic patients (F 10 y, M 12 y and F 16 y) with IIF titres for whole immunoglobulins ranging from 20 to 160. The GMT was 48. Similar to the symptomatic patients, serum antibodies to *D. fragilis* from these matched controls

belonged mainly to the IgG class of immunoglobulins (Table 6.3). Of 12 controls, 5 were negative for IgM antibodies, 6 were positive with low titres, and 1 (control no. 5) had an IgM titre of 160 (Table 6.3). Individual IgG or IgM antibody titres were either the same, or lower than that of whole immunoglobulins except for control no. 2,3,5,7,8 and 9, which had IgG and/or IgM readings higher than that of whole immunoglobulins. The tests were repeated, and similar results were obtained. Serum IgA antibodies were not detected in any of the 12 controls.

Sera from random-selected healthy infants, children and adolescents

One hundred and eighty-nine serum specimens from random-selected healthy infants, children and adolescents were obtained for this study. The sex and age distributions are listed in Table 6.4. The ratio of male to female was 85 (0.8):104(1). The age distribution was from 6 months to 19 years (median 3.2 years). Of 189 subjects, 177 (93.7%) were born in Canada, 2 (1.1%) in the United States, 1 (0.5%) in Europe, 1 (0.5%) in Asia, 1 (0.5%) in a country or region not mentioned above, and 7 with no information given.

Of the 189 serum samples tested, 172 (91.0%) were seropositive for *D. fragilis* antibodies. The distribution of sex and age seroprevalence is shown in Table 6.5 and Figure 6.2. At 6 months of age, 60.0% of infants had antibodies towards *D. fragilis* with a GMT of 5.6, and at the age of 1 year, 87.5% of infants were seropositive with a GMT of 11.1. Seropositivity steadily increased to 89.5% (GMT=14.1) at 18 months, and to 88.9% (GMT=15.9) at 2 years. The highest seropositive age groups in infancy were 3 and 4 years, when 96.8% (GMT=23.6) and 94.7% (GMT=24.6) of seropositivity were

observed. Collectively the seropositivity was 89.3% for infants <5 years of age, 92.3% for young children 5-13 years of age and 96.4% for adolescents (Table 6.5).

There were no appreciable differences in seroprevalence between males and females (93.0% vs 86.2% among children <5 years of age, 88.9% vs 95.3% among children 5-13 years of age, 90.0% vs 100.0% among adolescents 14-19 years of age, and 91.8% vs 90.4% for the entire group). In the male population the GMT rose from 4.5 at 6 months of age to 35.6 at 4 years, and it remained steady at 16.7 through late childhood and at 18.3 through adolescence. Likewise for the female population the GMT rose from 6.6 at 6 months to 20.7 at 4 years, then it remained relatively constant at 24.9 and 22.5 through the two later stages in life (Table 6.5 and Fig. 6.2).

SDS-PAGE and immunoblotting

Results of SDS-PAGE are presented in Figure 6.3. There were some minor differences in the protein banding pattern of *D. fragilis* in this preparation when compared to the preparation presented in Chapter 4, Fig. 4.3. At least 9 protein bands of molecular masses 89,62,48,41,39,30,28,27 and 24 kDa were visualized in this preparation. As with previous preparations, the protein band of 39 kDa was the most prominent.

The immunoblot of a representative IIF-positive serum and an IIF-negative serum is shown in Figure 6.4. The IIF-positive serum gave a strong reaction with the 39 kDa band, a weak reaction with the 48 kDa band, and a very weak reaction with the 41 kDa band. The IIF-negative serum did not give any band on immunoblot with *D. fragilis* proteins.

The immunoblot with serum samples from the 3 symptomatic patients is presented

in Figure 6.5. All of these 3 IIF- positive serum samples gave a strong reaction with the 39 kDa band, and a very weak reaction with the 48 kDa band.

The immunoblot with sera from the 12 well controls is shown in Figure 6.6. Of these 12 well controls, who were all IIF-positive, 10 (lanes 2 to 11) gave definite bands with the 39 kDa protein. A few serum samples gave a weak reaction with the 48 kDa band. One serum sample (control no. 5) gave a reaction with the 62 kDa band. Controls no. 1 and no. 12 were negative on immunoblot. They both had low IIF IgG titres (titres of 40, Table 6.3). On a previous immunoblotting assay with a serum dilution of 1 in 40, control no. 1 gave a very weak reaction, and control no. 12 gave a definite reaction, with the 39 kDa *D. fragilis* protein band (Figure 6.7).

The immunoblot of serum samples from the 2 children with high IIF titres (titres of 160) is shown in Figure 6.8. These IIF-positive sera showed a very strong reaction with the 39 kDa *D. fragilis* protein band, and weak reactions with the 48,41 kDa and lower molecular size proteins. Sera from the 3 children with negative IIF readings either did not show any band at all (Figure 6.4), or a very faintly visible band with the 39 kDa protein.

DISCUSSION

This study is the first report of the detection of antibodies to *D. fragilis* in symptomatic patients and healthy subjects. The IIF assay used in this study resembled the one used for the detection of antibodies to *G. lamblia* (Jokipii et al 1988), and for the detection of antibodies to *C. parvum* (Tzipori and Campbell 1981, Campbell and Current 1983). As in those studies, a titer of 10 or higher was regarded as a positive. The IIF assay used in

the current study was targeted to detect human whole immunoglobulins for all serum samples. Individual serum IgG, IgM and IgA antibodies were also assayed for 3 symptomatic patients infected with *D. fragilis*, and for 12 age-and sex-matched well controls. In order to verify the validity of the IIF assay, the sera from the 3 symptomatic patients, 12 well controls, 2 children with high IIF titres, and 3 children with negative IIF readings were absorbed with the bacteria cocultured with *D. fragilis* trophozoites, and then assessed for their specificity by immunoblotting against *D. fragilis*. All 17 IIF-positive serum samples identified the 39 kDa *D. fragilis* protein band, suggesting that this could be an important immunodominant protein. Some serum samples identified additional bands. The 3 IIF-negative serum samples either did not give any reaction on immunoblot, or only a very faint reaction with the 39 kDa band, thereby strengthening the specificity of the IIF assay. The reproducibility of the IIF assay was reinforced by concurrent testing of a known positive and a known negative control serum on a daily basis, and demonstration of a fluctuation of end-point reading usually within one tube and definitely not more than two tubes for the positive control serum.

In this study all 3 symptomatic patients had IIF titres of 80. Antibodies to *D. fragilis* identified by IIF were mainly those of the IgG class, while IgM antibodies were also detected in lower titres. Unfortunately, we were unable to obtain follow-up specimens from these 3 patients to demonstrate if there were rising antibody titres. Tests for serum IgA antibodies were negative at 1 in 10 dilutions for all 3 patients.

All of the 12 age- and sex- matched well controls exhibited antibodies to *D. fragilis*. The range of antibody titres was from 20 to 160, which overlapped with the titres

of 80 of the symptomatic patients. The GMT of the matched controls was 48, which was within one tube of dilution when compared with the titer of the symptomatic patients. It is therefore not possible to establish a serological diagnosis on *D. fragilis* infections based on a single titer by IIF on human whole immunoglobulins, even though we have not ruled out the possibility of seroconversion or rising titres on paired sera. Similar to the symptomatic patients, antibodies to *D. fragilis* in the matched well controls were mainly those of the IgG class, and IgM antibodies were either absent, or detected only in low titres. A few controls exhibited higher individual IgG or IgM antibody titres than those of whole immunoglobulins on repeated tests. This could possibly be due to the use of reagents from different manufacturers, different working dilutions of the reagents as recommended by the different manufacturers, the subjective interpretation of the IIF assay, and a fluctuation of up to 2 tubes of dilution in the end-point reading of the test, as we have experienced in the monitoring of quality control for the IIF assay in this study. Perhaps some of these problems could be solved by developing an ELISA for *D. fragilis* antibodies, in light of the fact that enzyme immunoassays for IgG and IgM antibodies to *G. lamblia* and *C. parvum* have been developed (Smith et al 1981, Goka et al 1986, Ungar et al 1986). As in the case of symptomatic patients, serum IgA antibodies were not detected in any of the 12 matched well controls.

A survey of 189 healthy infants, children and adolescents demonstrated an age-related increase in seroprevalence of antibodies to *D. fragilis*. Although the number of subjects surveyed in this study was relatively small, the trend of increasing seroprevalence with age was evident. Sixty percent of infants at the age of 6 months had antibodies to *D.*

fragilis with a GMT of 5.6. At the age of 1 year, 87.5% of infants were seropositive with a GMT of 11.1. A seropositivity rate of 89.5% (with an increased GMT value to 14.1) at 18 months, and 88.9% (increased GMT to 15.9) at 2 years may indicate increased exposure to the environment as the range of intake of foods is broadened. The highest seropositivity rate (96.8%) was seen at 3 years of age with a high GMT value of 23.6. Similar results were observed at 4 years of age, when 94.7% of children were seropositive with the highest GMT value of 24.6. Collectively the seropositive rate for infants <5 years of age was 89.3%, and a seropositivity of 92.3% (GMT=20.7) was observed in young children 5-13 years of age. Almost all adolescents (96.4%, GMT=20.8) were seropositive towards *D. fragilis*. The distribution of antibody titres among different age groups (Table 6.5 and Fig. 6.2) suggests that antibody titres increased through infancy before they stabilized, probably between 5 and 13 years of age.

Because there is no other seroprevalence study on *D. fragilis* for comparison, we attempted to compare our results with those reported for *G. lamblia* and *C. parvum*, two commonly found intestinal protozoan parasites associated with diarrhea in North America and other parts of the world. In a study on antibodies to cysts of *G. lamblia* by an IIF assay, Jokipii et al (1988) reported that titres of 10 or higher were found in 85.6% of adults who had probably never had clinical giardiasis. Using an enzyme immunoassay, Miotti et al (1986) studied the age- related rate of seropositivity of antibody to *G. lamblia* in four diverse populations. Similar prevalence rates and quantitative levels of antibody were found in adults living in Arizona (44%), Panama (48%) and Peru (46%) but a significantly lower ($p<0.05$) percentage of the adults living in Baltimore (18%) displayed

serological evidence of infection by *G. lamblia*. In the United States, children living in Baltimore had low levels of seropositivity to *G. lamblia* throughout childhood, whereas children living on an Indian reservation in Arizona showed a progressive acquisition of antibody early in childhood, with adult levels achieved by 8 years of age. In Latin America, children living in Panama attained adult levels of seropositivity to *G. lamblia* between 9 and 20 years of age, whereas children in Peru displayed adult levels of seropositivity in the first 6 months of life (Miotti 1986). Tzipori and Campbell (1981) first described the detection of serum antibodies against *C. parvum* in 10 animal species (including humans) by an IIF assay. A positive rate of 86% was found with randomly-selected human sera at a dilution of 1 in 10. Recently, Kuhls et al (1994) reported the seroprevalence of cryptosporidial antibodies during infancy, childhood and adolescence by means of an ELISA. Thirteen percent of children younger than 5 years of age, 38% of children 5-13 years of age and 58% of adolescents (14-21 years of age) were seropositive for antibodies to *C. parvum* (Kuhls et al 1994).

These independent seroprevalence studies by different techniques (IIF vs enzyme immunoassay) demonstrated the widespread presence of antibodies to *G. lamblia* and *C. parvum* at different ages and locales. Results of seropositivity were higher in studies by IIF for both organisms (85.6% for *G. lamblia* and 86% for *C. parvum*). In the current study we used a similar IIF assay to detect antibodies to *D. fragilis* with sera from infants, children and adolescents. A high level of seroprevalence was determined for all age groups of children, with an overall seropositivity of 91.0%. We have reviewed the records of the past 5 years on parasitology examination at CHEO (Table 6.1). *D. fragilis*

was the most frequently found parasite in 4 of 5 years. In the combined total of 5 years from 1989 to 1993, *D. fragilis* was found in 2.6% of specimens followed by *G. lamblia* at 2.0% and *C. parvum* at only 0.2%. The high seropositivity of 91.0% for *D. fragilis* (compared to reported 85.6% for *G. lamblia* and 86% for *C. parvum*) determined by IIF thus seems to be a logical finding.

To conclude, we have described the detection of *D. fragilis* antibodies in symptomatic patients and healthy subjects by an IIF assay. While further studies are needed to assess the diagnostic value, if any, of seroconversion, rising titres or levels of IgM antibodies, the high seroprevalence even at a young age found in this study suggests that *D. fragilis* is widespread and that exposure to this organism occurs at a very early age in our region.

Table 6.1 Detection of *Dientamoeba fragilis, Giardia lamblia* and *Cryptosporidium parvum* in routine parasitological examination at the Children's Hospital of Eastern Ontario, 1989 - 1993.

Year	1989	1990	1991	1992	1993	Combined total of 5 years
No. of specimens	1469	1330	1135	917	923	5774
No. of positive patients *(%)						
Dientamoeba fragilis	41(2.8)	38(2.9)	25(2.2)	31(3.4)	16(1.7)	151(2.6)
Giardia lamblia	20(1.4)	23(1.7)	33(2.9)	23(2.5)	16(1.7)	115(2.0)
Cryptosporidium parvum	2(0.1)	4(0.3)	2(0.2)	4(0.4)	1(0.1)	13(0.2)

*Repeat specimens not counted.

Table 6.2 Reproducibility of daily reading of the same positive *D. fragilis* control serum (titre of 40) by indirect immunofluorescence assay.

Day of test	Titre
1	40
2	80
3	80
4	20
5	20
6	20
7	20
8	20
9	20
Geometric mean = 29	

Table 6.3 Indirect immunofluorescence serum titres of D. fragilis antibodies of 3 symptomatic children and 12 age- and sex-matched healthy controls.

Symptomatic patient no.	Age (years)	Sex	Titre				Matched Control no.	Titre			
			Whole Immunoglobulins	IgG	IgM	IgA		Whole Immunoglobulins	IgG	IgM	IgA
1	12	M	80	80	10	-*	1	40	40	10	-
							2	40	80	-	-
							3	40	160	-	-
							4	80	80	20	-
2	16	F	80	40	10	-	5	40	160	160	-
							6	160	40	10	-
							7	40	160	-	-
							8	20	40	-	-
3	10	F	80	40	40	-	9	40	80	10	-
							10	80	40	-	-
							11	40	40	10	-
							12	40	40	10	-
Geometric mean titre			80					48			

*- = negative at a dilution of 1 in 10

Table 6.4 Age and sex distribution of 189 healthy infants, children and adolescents whose sera were assayed for *D. fragilis* antibodies.

Age	No. of subjects	
	Male	Female
<5 years		
Birth to 6 months	4	6
6-12 months	8	8
12-18 months	11	8
18 months - 2 y	11	16
3 y	17	14
4 y	6	13
Subtotal	57	65
5-13 y		
5y	4	3
6y	1	1
7y	2	4
8y	0	4
9y	4	2
10y	2	1
11y	1	2
12y	2	2
13y	2	2
Subtotal	18	21
14-19y		
14y	1	4
15y	2	5
16y	4	6
17y	1	1
18y	1	2
19y	1	0
Subtotal	10	18
Total	85 (45%)	104 (55%)

Table 6.5 Antibodies to *D. fragilis* in 189 healthy infants, children and adolescents

Age Group (years)	Male			Female			Combined No.(%) seropositve/ no. of children	Titre*
	n	No. (%) seropositive/ no. of males	Titre*	n	No. (%) seropositive/ no. of females	Titre*		
0.5	4	2/4 (50.0)	4.5	6	4/6 (66.7)	6.6	6/10 (60.0)	5.6
1	8	7/8 (87.5)	8.2	8	7/8 (87.5)	15.0	14/16 (87.5)	11.1
1.5	11	11/11 (100.0)	20.0	8	6/8 (75.0)	8.7	17/19 (89.5)	14.1
2	11	11/11 (100.0)	18.8	16	13/16 (81.3)	14.2	24/27 (88.9)	15.9
3	17	16/17 (94.1)	19.0	14	14/14 (100.0)	20.0	30/31 (96.8)	23.6
4	6	6/6(100.0)	35.6	13	12/13 (92.3)	20.7	18/19 (94.7)	24.6
<5	57	53/57 (93.0)		65	56/65 (86.2)		109/122 (89.3)	
5-13	18	16/18 (88.9)	16.7	21	20/21 (95.3)	24.9	36/39 (92.3)	20.7
14-19	10	9/10 (90.0)	18.3	18	18/18 (100.0)	22.5	27/28 (96.4)	20.8
All	85	78/85 (91.8)		104	94/104 (90.4)		172/189 (91.0)	

*Geometric mean titre

Figure 6.1 ***D. fragilis*** **visualized by indirect immunofluorescence staining with a negative serum (no fluorescence)** (A) and a positive serum (strong fluorescence) (B). Both serum samples were diluted 1 in 10. Magnification, X2000.

Figure 6.1 (A) negative serum (no fluorescence)

Figure 6.1 (B) positive serum (strong fluorescence)

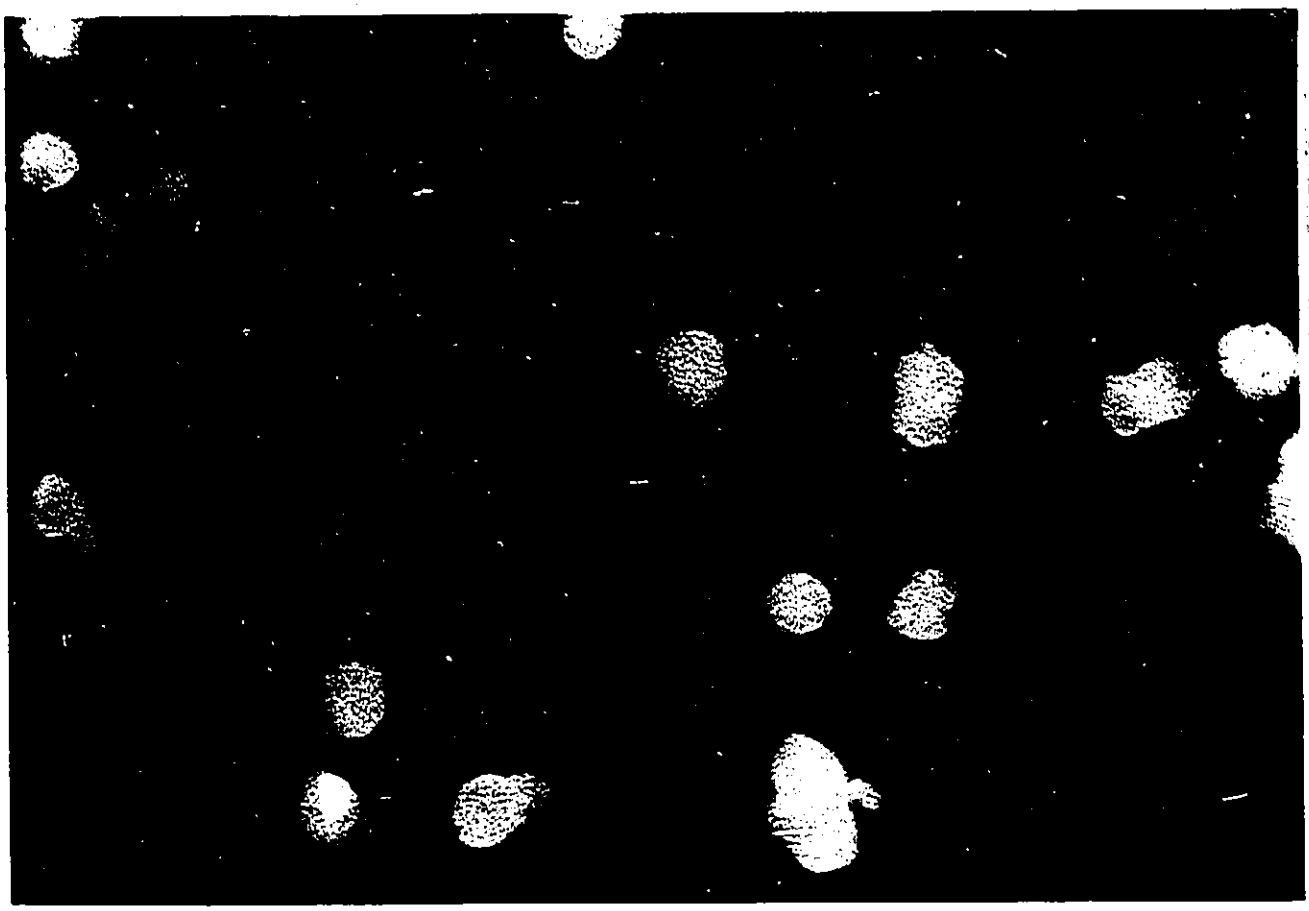
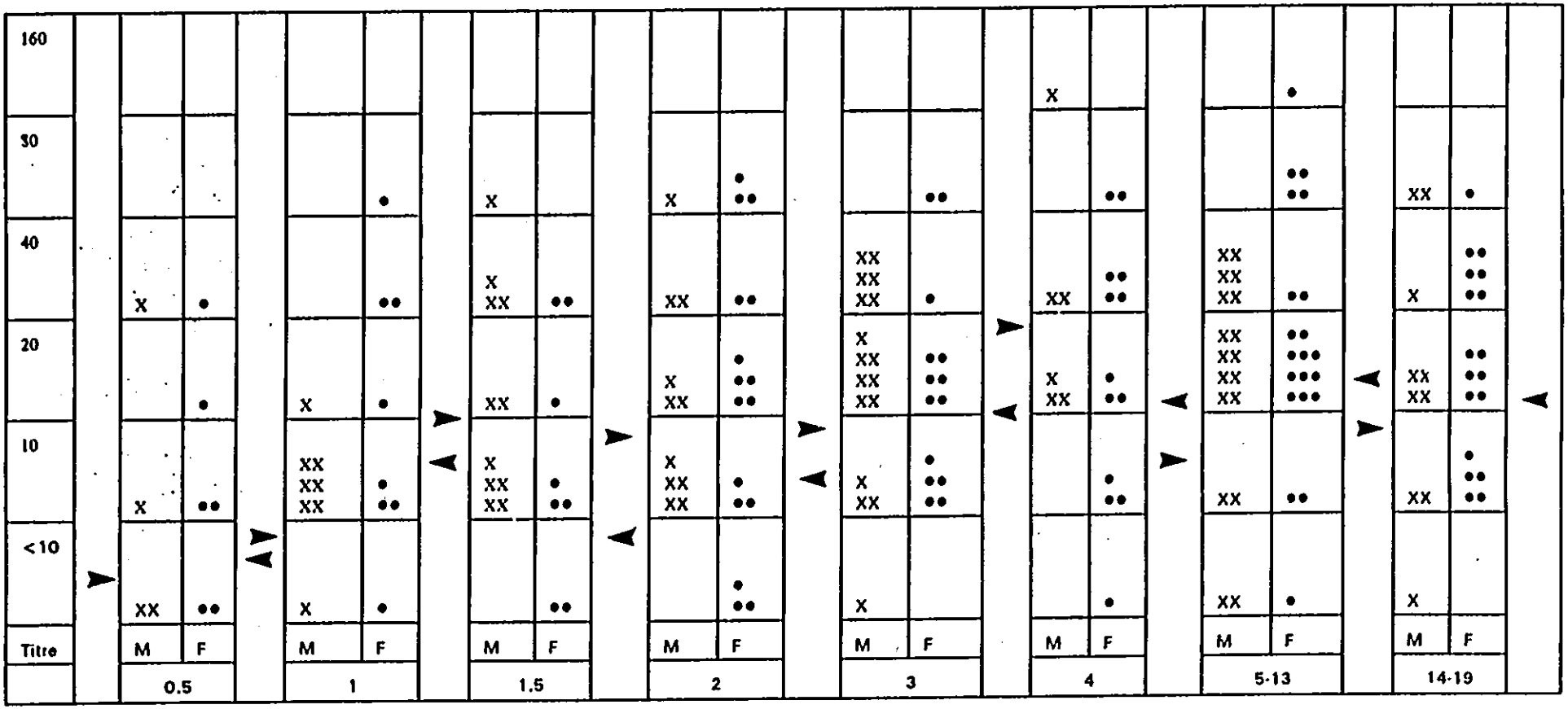
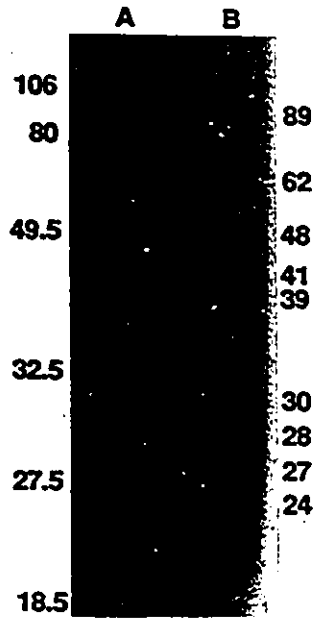


Figure 6.2 Antibodies to *D. fragilis* detected by indirect immunofluorescence in the sera of 189 random healthy infants, children and adolescents. Each X represents a male (M) individual and each ● represents a female (F) individual with the titre indicated by the line at the bottom of the box. The arrows indicate the geometric means.



AGE (YEARS)

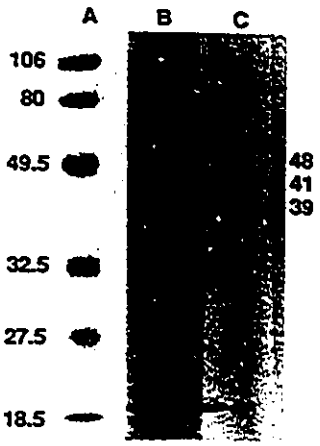
Figure 6.3 SDS-PAGE of *Dientamoeba fragilis* with 68μg of protein. Lane A: molecular size marker in kilodaltons, Lane B: *D. fragilis* with at least 9 discernable bands of molecular masses 89,62,48,41,39,30,28,27 and 24 kDa.



SDS-Page of Df (68 μg protein)

Figure 6.4 Immunoblot of *Dientamoeba fragilis* (DF) with a representative IIF-positive serum and an IIF-negative serum (absorbed sera, dilutions of 1 in 80).

Lane A: molecular size marker in kilodaltons,
Lane B: IIF-positive serum identifying a
39 kDa band (strong reaction),
48 kDa band (weak reaction, and)
41 kDa band (very weak reaction)
Lane C: IIF negative serum (no reaction)



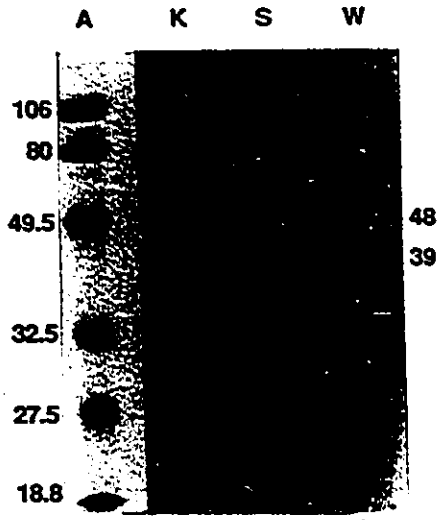
Immunoblot of Df from a positive and a negative serum (absorbed sera, dilution 1 in 80)

Figure 6.5 Immunoblot of *Dientamoeba fragilis* (DF) with absorbed serum samples at a dilution of 1 in 80 from 3 symptomatic patients (IIF-positive).

Lane A: molecular size marker in kilodaltons,

Lanes K, S and W: patient serum

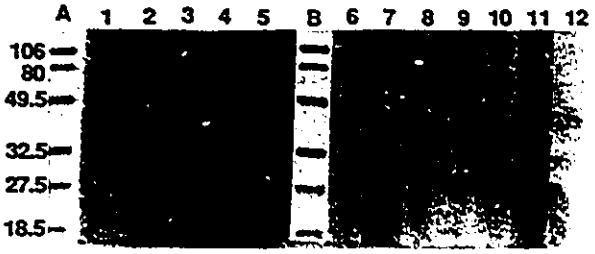
Samples giving a strong reaction with the 39 kDa band, and a very weak reaction with the 48 kDa band.



Immunoblot of Df with absorbed sera (dilution 1 in 80) from 3 symptomatic patients

Figure 6.6 Immunoblot of *Dientamoeba fragilis* (DF) with absorbed serum samples (dilutions of 1 in 80) from 12 well controls (all IIF-positive).

Lanes A and B: molecular size marker in kilodaltons
Lanes 1 to 12: controls
Lanes 2 to 11 gave definite bands with the 39 kDa protein.
Lane 1 and Lane 12 were negative on immunoblot, both controls with low IIF IgG titres (titres of 40).



Immunoblot of Df with absorbed sera (dilution 1 in 80) from 12 well controls

Figure 6.7 Immunoblot of *Dientamoeba fragilis* (DF) with control no. 1 and control no. 12 at a serum dilution of 1 in 40.

Control no. 1: very weak reaction with 39 kDa band
Control no. 12: definite reaction with 39 kDa band.
These controls were run on separate gels previously, and therefore the 39 kDa bands did not appear to be at the same position.

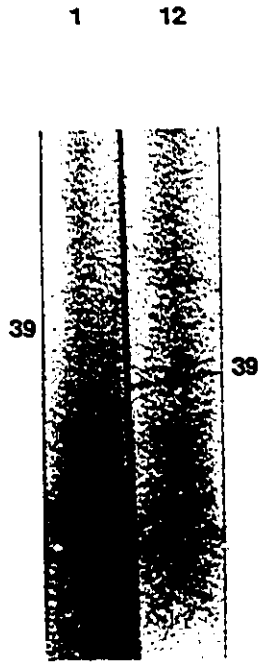
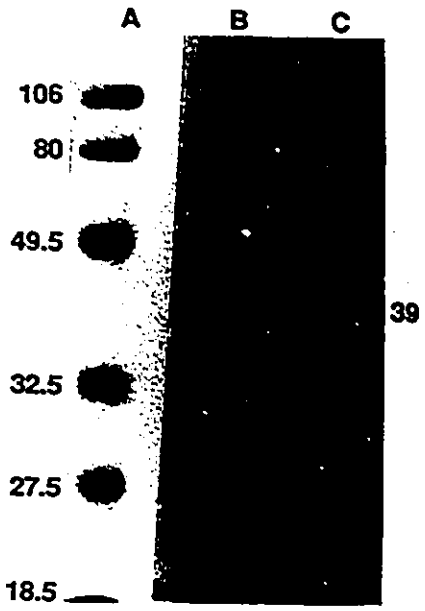

1
12
39
39

Figure 6.8 Immunoblot of *Dientamoeba fragilis* (DF) with absorbed sera (dilutions of 1 in 80) from the 2 children with high IIF titres (titres of 160).

Lane A: molecular size marker in kilodaltons

Lanes B and C:IIF-positive sera showing a strong reaction with the 39 kDa band and weaker reactions with other protein bands.



Immunoblot of Df with 2 absorbed sera of high titres

CHAPTER 7

CONCLUSION

The studies described in this thesis and the resulting publications are the first reports of *Dientamoeba fragilis* related to the characterization of the ATCC dixenic culture, advances in laboratory diagnosis, immunological aspects of the organism, susceptibility testing and seroprevalence in a paediatric population of the National Capital Region.

Using a polyclonal antiserum produced in a rabbit and absorbed with the characterized bacteria, an indirect fluorescent antibody assay was developed to examine for the presence of *D. fragilis* trophozoites in preserved faecal specimens. This is the first report that has used immunological techniques to identify *D. fragilis* in clinical samples. The test proved to be sensitive and specific and it might be a useful screening method because of its speed in reading (less than 1 minute of examination time per specimen, versus 15 minutes in routine parasitological examination procedures). The success with immunological techniques also indicates that enzyme immunoassays could be developed for antigen detection tests to increase sensitivity of current detection methods, and to provide a better understanding of the pathogenicity and epidemiology of *D. fragilis* by facilitating the diagnosis.

Two monoclonal antibodies (MAbs) against *D. fragilis* were developed and characterized. MAb 8B2 was produced from a frozen-and-thawed *D. fragilis* antigen preparation, and MAb 1H11 was produced from a sodium acetate-acetic acid-formalin fixed *D. fragilis* antigen preparation. Both MAbs were IgG1 isotypes with kappa light chains. MAb 8B2 did not react with clinical *D. fragilis* strains and it did not bind in the immunoblot. MAb 1H11 identified a *D. fragilis* 39 kDa protein on immunoblot, and it showed some promise in its possible clinical application by giving a positive indirect immunofluorescence test with *D. fragilis* trophozoites identified in clinical specimens.

Susceptibility testing was performed on *D. fragilis* ATCC 30948 in a dixenic culture with *Klebsiella pneumoniae* and *Bacteroides vulgatus*. The activities of four antiparasitic drugs (iodoquinol, paromomycin, tetracycline and metronidazole) were assessed by counting viable *D. fragilis* trophozoites with a haemacytometer by trypan blue exclusion. The minimal amoebicidal concentrations of the four drugs were determined. This is the first report of laboratory susceptibility testing of these antiparasitic drugs correlating with the recommendations for the treatment of *D. fragilis* infections.

Sera from 3 symptomatic patients (F10y, M12y and F16y), 12 age- and sex- matched controls, and 189 random healthy individuals (age 6 months to 19 years) were tested by an indirect immunofluorescence (IIF) assay for antibodies against *D. fragilis*. All 3 symptomatic patients infected with *D. fragilis* gave IIF titres of 80, and all 12 matched controls were also positive (titre ranges from 20 to 160, geometric mean titre (GMT) was 48). Of 189 healthy children, 172 (91.0%) were positive at a serum dilution of 1 in 10, or higher dilutions. The specificity of the IIF assay was confirmed by immunoblotting 20 representative serum samples against *D. fragilis* (3 negative and 17 positive sera). All 17 IIF-positive serum samples identified the 39 kDa band, suggesting that this could be an important immunodominant protein of *D. fragilis*. GMT was 5.6 at 6 months of age, 11.1 at 1 year, 14.1 at 1.5 yr, 15.9 at 2 y, 23.6 at 3 y, 24.6 at 4y, 20.7 for the age group of 5-13y, and 20.8 for 14-19y. The high seroprevalence and increasing GMT through infancy may indicate that exposure to *D. fragilis* during childhood is common in our region.

There have been few studies on *D. fragilis*, even though the organism was described

more than 75 years ago. The research carried out for this thesis has contributed to the advances in the study of the epidemiology, immunology, laboratory diagnosis and treatment of this ubiquitous but mysterious organism.

Future studies should focus on the 39 kDa protein, which appears to be an immunodominant protein as demonstrated by the studies on MAbs and seroprevalence. A more detailed characterization of this protein and cDNA cloning experiments on the gene expression of this protein will likely help to unravel some of the mysteries of *D. fragilis*.

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APPENDIX

PUBLICATIONS

1. Chan, F.T.H., M.X. Guan and A.M.R. Mackenzie. 1993. Application of indirect immunofluorescence to detection of *Dientamoeba fragilis* trophozoites in fecal specimens. Journal of Clinical Microbiology 31:1710-1714.

2. Chan, F.T.H., M.X. Guan, A.M.R. Mackenzie and F. Diaz-Mitoma. 1994. Susceptibility testing of *Dientamoeba fragilis* ATCC 30948 with iodoquinol, paromomycin, tetracycline and metronidazole. Antimicrobial Agents and Chemotherapy 38:1157-1160.

3. Chan, F.T.H., M.X. Guan, N. Stewart, I. Robb, F. Diaz-Mitoma and A.M.R. Mackenzie. 1995. Development and characterization of monoclonal antibodies in the study of *Dientamoeba fragilis*. Manuscript being prepared for publication.

4. Chan, F.T.H., N. Stewart, L.A. Fuite, I.O.L. Chan, F. Diaz-Mitoma, W.J. King, N.E. MacDonald and A.M.R. Mackenzie. 1995. Prevalence of *Dientamoeba fragilis* antibodies in infancy, childhood and adolescence. Manuscript being prepared for publication.

ABSTRACTS AND PRESENTATIONS

1. Chan, F.T.H., M.X. Guan and A.M.R. Mackenzie. 1992. Detection of *Dientamoeba fragilis* in faecal specimens by indirect immunofluorescence. Conjoint Meeting on Infectious Diseases, Toronto, Ontario. (I was the invited chairman for the session of Parasitology at this meeting).

2. Chan, F.T.H., M.X. Guan, A.M.R. Mackenzie and F. Diaz-Mitoma. 1994. Activities of diiodohydroxyquinoline, paromomycin, tetracycline and metronidazole on *Dientamoeba fragilis* ATCC 30948. 94th General Meeting, American Society for Microbiology, Las Vegas, Nevada.

3. Chan, F.T.H., M.X. Guan, N. Stewart, I. Robb, F. Diaz-Mitoma and A.M.R. Mackenzie. 1994. Development and characterization of monoclonal antibodies in the study of *Dientamoeba fragilis*. 94th General Meeting, American Society for Microbiology, Las Vegas, Nevada.

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5. Chan, F.T.H., N. Stewart, L.A. Fuite, I.O.L. Chan, F. Diaz-Mitoma, W.J. King, N.E. MacDonald and A.M.R. Mackenzie. 1995. Prevalence of *Dientamoeba fragilis* antibodies in children. 95th General Meeting, American Society for Microbiology, Washington, D.C..