AAC
Journals.ASM.org

In Vitro Susceptibility Testing of *Dientamoeba fragilis*

N. Nagata,[a,b] D. Marriott,[a,b] J. Harkness,[a,b] J. T. Ellis,[b,c] and D. Stark[a,b]

Division of Microbiology, SydPath, St. Vincent's Hospital, Darlinghurst, Australia[a]; University of Technology Sydney, School of Medical and Molecular Biosciences, Broadway, Australia[b]; and University of Technology Sydney, iThree Institute, Broadway, Australia[c]

***Dientamoeba fragilis* is a commonly encountered trichomonad which has been implicated as a cause of gastrointestinal disease in humans. Despite the frequency of reports recording infections with this parasite, little research has been undertaken in terms of antimicrobial susceptibility. The aim of this study was to evaluate the susceptibility of *D. fragilis* to several commonly used antiparasitic agents: diloxanide furoate, furazolidone, iodoquinol, metronidazole, nitazoxanide, ornidazole, paromomycin, secnidazole, ronidazole, tetracycline, and tinidazole. Antibiotic susceptibility testing was performed on four clinical strains of *D. fragilis*, designated A, E, M, and V, respectively. Molecular testing followed, and all strains were determined to be genotype 1. The activities of antiprotozoal compounds at concentrations ranging from 2 μg/ml to 500 μg/ml were determined via cell counts of *D. fragilis* trophozoites grown in dixenic culture. Minimum lethal concentrations (MLCs) were as follows: ornidazole, 8 to 16 μg/ml; ronidazole, 8 to 16 μg/ml; tinidazole, 31 μg/ml; metronidazole, 31 μg/ml; secnidazole, 31 to 63 μg/ml; nitazoxanide, 63 μg/ml; tetracycline, 250 μg/ml; furazolidone, 250 to 500 μg/ml; iodoquinol, 500 μg/ml; paromomycin, 500 μg/ml; and diloxanide furoate, >500 μg/ml. This is the first study to report the profiles of susceptibility to a wide range of commonly used treatments for clinical isolates of *D. fragilis*. Our study indicated 5-nitroimidazole derivatives to be the most active compounds *in vitro* against *D. fragilis*.**

Initially described by Jepps and Dobell (18), *Dientamoeba fragilis* is a protozoan parasite implicated as a cause of gastrointestinal diseases in both developed and developing regions of the world. Infection rates typically range from 0.5% to 16%, with higher rates seen in outbreaks or where personal hygiene is suboptimal (3, 17, 21, 26). The protozoan is recognized to cause chronic infections. In a prospective study, 6,750 patients were screened for *D. fragilis*, and 32% of patients presented with chronic symptoms (31), including abdominal pain, diarrhea, and nausea. Some authors have linked *D. fragilis* to irritable bowel syndrome (IBS)-like symptoms (4, 32).

Currently, a majority of evidence supports the pathogenic potential of *D. fragilis* (5). As such, it suggests the need for not only the correct diagnosis but also appropriate treatment (29, 30). The parasite responds to a number of antimicrobial compounds, with studies reporting the complete resolution and elimination of parasites following therapy with iodoquinol (8) metronidazole (24), paromomycin (13), and secnidazole (16). In an Australian study, complete resolution and eradication of the organism were observed for most patients following treatments with iodoquinol, paromomycin, or combination therapy, while treatment relapses/failures were recorded only with the use of metronidazole (29). It is of note that there are no current treatment guidelines for *D. fragilis* infections in place.

While *D. fragilis* can be readily cultured from clinical samples (4), long-term cultures are notoriously difficult to maintain (12), and this has hampered the *in vitro* study of this organism, including susceptibility testing. The objective of this study was to determine the *in vitro* susceptibility of a number of clinical isolates of *D. fragilis* to diloxanide furoate, furazolidone, iodoquinol, metronidazole, nitazoxanide, ornidazole, paromomycin, ronidazole, secnidazole, tetracycline, and tinidazole. The results obtained will assist in the development of recommendations for the treatment of dientamoebiasis.

MATERIALS AND METHODS

Parasite culture. Four strains of *D. fragilis*, A, E, M, and V, were isolated and propagated *in vitro* using Loeffler slopes, as described by Barratt et al. (4).

Antimicrobial agents/susceptibility testing. Susceptibility testing was performed for the following agents: diloxanide furoate, furazolidone, iodoquinol, metronidazole, nitazoxanide, ornidazole, paromomycin, ronidazole, secnidazole, tetracycline, and tinidazole. Metronidazole (Pfizer, NSW, Australia) in liquid form at 5 mg/ml was used as a stock solution and diluted with phosphate-buffered saline (PBS) buffer to cover a concentration range of 2 μg/ml to 500 μg/ml by doubling dilution. Tetracycline (Bioline, Alexandria, NSW, Australia) suspended in 90% ethanol at 12.5 mg/ml was diluted to 5 mg/ml and diluted in the same manner described for metronidazole. Ornidazole (Queensland Institute of Medical Research) in powder form was dissolved in 50% ethanol to 5 mg/ml and diluted thereafter. Paromomycin sulfate (Sigma-Aldrich, Sydney, NSW, Australia) and diloxanide furoate, furazolidone, iodoquinol, nitazoxanide, ronidazole, secnidazole, and tinidazole (all from West Lindfield Compounding Chemist, NSW, Australia) in powder form were suspended in 10% ethanol to make stock solutions at 5 mg/ml. Further dilutions were prepared by doubling dilution to cover from 2 μg/ml to 500 μg/ml. One milliliter of the respective dilutions was added to Loeffler slopes supplemented with rice starch and PBS overlay to contain a total liquid volume of 8 ml. A control consisting of 1 ml of 10% ethanol diluted into the same volume was performed in duplicate for all drugs in powder form to rule out any inhibitory effects on *D. fragilis*. As PBS buffer was used as a diluent for metronidazole and tetracycline, the same volume of PBS buffer was used as a control.

Received 16 July 2011 Returned for modification 3 August 2011
Accepted 13 October 2011
Published ahead of print 24 October 2011
Address correspondence to D. Stark, dstark@stvincents.com.au.

doi:10.1128/AAC.05125-11

TABLE 1 Bacterial species isolated from cultures of *D. fragilis*

Isolate	O_2 requirement	Organism identified
Clinical isolate V	Strict aerobe	*Pseudomonas aeruginosa*
	Facultative anaerobe	*Escherichia coli*
	Obligate anaerobes	*Clostridium tertium*
		Clostridium hathewayi
		Eubacterium limosum
		Collinsella aerofaciens
		Peptostreptococcus stomatis
		Peptostreptococcus micros
		Veillonella spp.
		Bacteroides ovatus
		Bacteroides fragilis
		Parabacteroides distasonis
		Prevotella oralis
		Arcobacter butzleri
		Eggerthella lenta
Clinical isolate E	Strict aerobe	*P. aeruginosa*
	Facultative anaerobe	*E. coli*
	Strict anaerobes	*P. micros*
		P. stomatis
		Veillonella spp.
		E. limosum
		Anaerococcus prevotii
		Bacteroides capillosus
		B. fragilis
		C. aerofaciens
		P. oralis
Clinical isolate M	Strict aerobe	*P. aeruginosa*
	Facultative anaerobe	*E. coli*
	Obligate anaerobes	*C. tertium*
		P. micros
		P. stomatis
		B. fragilis
		P. oralis
Clinical isolate A	Strict aerobe	*P. aeruginosa*
	Facultative anaerobe	*E. coli*
	Obligate anaerobes	*C. tertium*
		P. micros
		P. stomatis
		Veillonella spp.
		B. fragilis
		P. oralis

The cell concentration was determined using Kova slides viewed under a phase-contrast microscope at a magnification of ×400. As a decline in numbers of *D. fragilis* trophozoites occurs in negative controls after 92 h postexperiment, susceptibility testing with each compound was performed for only 4 days. Minimal lethal concentrations (MLCs) were determined to be the concentration of the drug at which no trophozoites were observed over the treatment period.

Characterization of bacterial flora and susceptibility testing from xenic culture. The bacterial flora present in dixenic cultures was characterized and identified. Supernatants from *D. fragilis* cultures were inoculated onto the following media: Columbia horse blood agar, Brilliance UTI agar, MacConkey agar, Sabouraud's agar, and anaerobic medium (Thermofisher Scientific Australia Pty Ltd., Scoresby, Victoria, Australia). The aerobic medium plates were incubated at 35°C for 24 to 48 h under aerobic conditions, while the anaerobic medium plates were incubated for 48 to 72 h under anaerobic conditions using an Anoxomat Mark II system (Mart Microbiology) with the following gas composition: 0.16% O_2, 5% H_2, 10% CO_2, and 85% N_2. All bacterial isolates were identified to species level using routine bacteriological procedures, including traditional phenotypic testing, biochemical testing, and RapID API strips (Biomeriéux, Baulkham Hills, Australia), according to the manufacturer's instructions.

Bacterial 16S rRNA gene sequencing. Cultured clinical isolates were used for DNA extraction using a QIAamp DNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. PCR amplifications were performed using pure-*Taq* Ready-To-Go (Amersham Biosciences, Rydalmere, Australia) PCR beads (each containing ~1.5 units *Taq* DNA polymerase, 10 mM Tris-HCl at pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM each deoxynucleoside triphosphate, and stabilizers, including bovine serum albumin), 1.0 μl of genomic DNA, and 0.5 μM universal primers, forward primer with the sequence of 5′-AGAGTTTGATCMTG GCTCAG, and reverse primer with the sequence of 5′-AAGGAGGTGW TCCARCC. The following thermocycling profile was used: 3 min denaturation at 94°C and 30 cycles of 1 min at 94°C, 1.5 min at 57°C, and 2 min at 72°C. Purification of PCR products was performed using QIAquick PCR purification kits (Qiagen), according to the manufacturer's instructions. Sequencing of purified bacterial DNA was then performed by the Australian Genome Research Facility (AGRF). The sequence data were then compared to other bacterial sequences using BLASTN (34) and the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/).

After the completion of susceptibility testing, the bacterial flora was again fully characterized and checked for the presence of bacterial species unaffected by the treatments.

Genotyping of *D. fragilis* isolates. Genotyping of the *D. fragilis* isolates were performed as previously described by Stark et al. (31).

RESULTS

***D. fragilis* genotyping.** All *D. fragilis* isolates were identified to be genotype 1 by 18S ribosomal DNA analysis (GenBank accession number AY730405).

Bacterial flora identified from four dixenic cultures. The bacterial flora identified from the dixenic cultures is shown in Table 1.

MLCs. The results are shown in Tables 2 to 9. Briefly, mean MLC values for the compounds were as follows: ornidazole, 8 to 16 μg/ml; ronidazole, 8 to 16 μg/ml; tinidazole, 31 μg/ml; metronidazole, 31 μg/ml; secnidazole, 31 to 63 μg/ml; nitazoxanide, 63 μg/ml; tetracycline, 250 μg/ml; furazolidone, 250 to 500 μg/ml; iodoquinol, 500 μg/ml; paromomycin, 500 μg/ml; and diloxanide furoate, >500 μg/ml (as minimal or no effects were found with diloxanide furoate, iodoquinol, and paromomycin, tables for these compounds have been excluded). Minor differences in the MLCs between the clinical isolates were observed for a number of drugs, including furazolidone, nitazoxanide, ornidazole, ronidazole, and secnidazole, with isolate V showing slightly higher MLCs than those of the other isolates in general (Tables 2 and 4 to 7).

Bacterial flora identified posttreatment. The bacterial flora identified posttreatment are presented in Table 1.

(i) Ronidazole. At the concentrations above the MLC, ronidazole treatment led to the removal of the majority of the bacterial flora, while it left *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium* spp., and *Bacteroides fragilis* unaffected.

(ii) Tinidazole. Tinidazole treatment led to the removal of *Eubacterium limosum*, *Collinsella aerofaciens*, *Veillonella* spp., and *Parabacteroides distasonis*, while it left *E. coli*, *P. aeruginosa*, *Clostridium* spp., *Bacteroides* spp., *Peptostreptococcus* spp., and *Eggerthella lenta* unaffected.

(iii) Ornidazole. At ornidazole concentrations above the MLC, *E. coli*, *P. aeruginosa*, and *B. fragilis* were unaffected by the treatment, while ornidazole led to the removal of *Clostridium* spp., *E. limosum*, *C. aerofaciens*, *Veillonella* spp., *Bacteroides ovatus*, *Pep-*

TABLE 3 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with metronidazole

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day					Day					Day					Day				
	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days
0[b]	1 (0)	1.3 (0.53)	6.7 (4.42)	12 (6.72)	7.2 (4.07)	1 (0)	1.9 (3.01)	4.7 (16.79)	4.9 (26.52)	0.75 (1.06)	1 (0)	4.3 (3.18)	9.3 (1.94)	9.3 (1.15)	3.7 (2.12)	1 (0)	6 (2.47)	30.3 (5.48)	40.2 (12.73)	17.9 (6.54)
2	1 (0)	1.1 (1.41)	1.4 (3.01)	5.9 (1.94)	7.3 (4.6)	1 (0)	1.5 (3.54)	3.8 (3.89)	4.3 (22.63)	0.42 (0.35)	1 (0)	3.1 (1.24)	9.3 (3.18)	10 (3.09)	4.8 (4.07)	1 (0)	4.6 (2.47)	46.6 (2.12)	42.2 (1.77)	15.9 (6.54)
4	1 (0)	0.68 (1.59)	4 (5.13)	8.5 (2.65)	11 (0.35)	1 (0)	2.3 (7.78)	4.5 (17.85)	3.9 (17.85)	0.57 (0)	1 (0)	3.2 (1.41)	8.1 (5.3)	12 (0.62)	5.5 (1.77)	1 (0)	1.2 (0)	26.9 (7.25)	43.7 (3.01)	12.9 (1.59)
8	1 (0)	0.18 (0.53)	0.86 (1.06)	2.6 (3.01)	10 (3.36)	1 (0)	1.7 (2.65)	1.3 (3.71)	2.4 (3.01)	1.1 (1.77)	1 (0)	1.2 (0.53)	5.9 (3.71)	9.9 (4.24)	4.5 (2.92)	1 (0)	0.4 (0)	15.5 (9.72)	57.7 (16.44)	20 (1.06)
16	1 (0)	0 (0)	0.03 (0.18)	0.25 (0.71)	4.1 (2.3)	1 (0)	1.9 (6.89)	1.3 (0.71)	2.4 (1.59)	3.3 (11.84)	1 (0)	1.25 (0.18)	4.2 (1.77)	5.4 (3.09)	2.3 (0.35)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
63	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
125	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where phosphate-buffered saline, used for dilution, was added into the medium.

TABLE 2 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with furazolidone

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day					Day					Day					Day				
	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days
0[b]	1 (0)	0.79 (2.65)	1.5 (4.07)	3.7 (10.43)	0.014 (0.18)	1 (0)	1.4 (0)	3.7 (9.02)	2.6 (38.54)	0.67 (1.59)	1 (0)	1.7 (2.3)	4.4 (2.3)	6.6 (4.95)	3.2 (9.37)	1 (0)	1.3 (1.06)	2 (2.83)	6.7 (5.13)	2.2 (1.94)
2	1 (0)	0.99 (1.59)	1.9 (1.59)	5 (3.01)	0.6 (5.3)	1 (0)	0.72 (3.01)	3.5 (6.01)	2 (16.09)	0.59 (7.07)	1 (0)	1.9 (1.06)	4.6 (8.13)	7 (4.77)	2.8 (3.36)	1 (0)	1.3 (1.94)	2 (1.06)	6.3 (7.07)	1.8 (3.89)
4	1 (0)	1.2 (2.3)	1.4 (3.89)	4 (0.88)	0.12 (1.06)	1 (0)	1 (4.24)	5.2 (4.07)	4.3 (26.16)	0.39 (0)	1 (0)	2 (0.88)	4.3 (3.01)	6.5 (3.89)	2 (2.65)	1 (0)	1.3 (0.71)	1.9 (2.12)	6.2 (2.83)	1.6 (3.18)
8	1 (0)	0.5 (0.53)	0.68 (1.59)	0.98 (1.77)	0.058 (0.71)	1 (0)	0.68 (1.59)	1.9 (7.25)	1.1 (6.19)	0.23 (0.35)	1 (0)	1.6 (1.59)	3.9 (2.65)	5.9 (4.24)	2.7 (5.13)	1 (0)	1.5 (1.41)	2.1 (1.41)	5.6 (7.78)	1.7 (0.88)
16	1 (0)	0.53 (1.24)	0.65 (1.94)	2.3 (3.01)	0.029 (0.35)	1 (0)	0.75 (7.07)	1.3 (5.66)	2.3 (15.2)	0.78 (8.84)	1 (0)	1.5 (2.83)	2.8 (7.25)	4.3 (6.36)	1.3 (4.77)	1 (0)	1.2 (1.41)	2 (3.36)	3.3 (1.59)	1.5 (1.41)
31	1 (0)	0.32 (0.71)	0.2 (1.41)	1.3 (5.48)	0.56 (5.48)	1 (0)	0.62 (3.01)	1.2 (13.26)	1.9 (12.9)	0.43 (1.06)	1 (0)	0.41 (0.53)	2.1 (2.83)	3 (1.59)	0.93 (1.41)	1 (0)	0.63 (2.12)	1.8 (1.41)	2.7 (4.95)	1.1 (2.12)
63	1 (0)	0.35 (0)	0.1 (0.18)	0.29 (1.06)	0.56 (1.59)	1 (0)	0.27 (0.35)	0.71 (0.71)	2.7 (9.02)	0.74 (9.19)	1 (0)	0.11 (0)	0.18 (0.35)	0.04 (0.35)	0 (0)	1 (0)	0.06 (0.18)	1.1 (2.12)	0.2 (0.71)	0.06 (0.18)
125	1 (0)	0.17 (1.1)	0.18 (0.71)	0.13 (0.2)	0.23 (1.1)	1 (0)	0.2 (0.18)	0.1 (0.71)	0.42 (1.24)	0.62 (1.77)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviations are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where 10% ethanol, used for dilution, was added into the medium.

TABLE 4 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with nitazoxanide

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day					Day					Day					Day				
	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days
0[b]	1 (0)	0.93 (0.88)	4.3 (11.31)	1.5 (1.41)	0.16 (0)	1 (0)	1 (1.59)	2.1 (15.2)	3.1 (36.06)	0.55 (3.89)	1 (0)	1.8 (1.06)	4.6 (4.95)	6.3 (9.19)	2.6 (1.41)	1 (0)	1.9 (1.24)	6.1 (5.3)	8.5 (6.54)	3.8 (2.12)
2	1 (0)	0.87 (0.18)	3 (10.25)	0.55 (3.01)	0.22 (1.06)	1 (0)	0.47 (1.77)	2.1 (13.97)	1.5 (18.21)	0.1 (3.01)	1 (0)	2.1 (1.24)	5.5 (1.77)	6.5 (4.42)	2.4 (2.12)	1 (0)	1.3 (1.77)	5 (9.19)	8.1 (5.3)	3 (2.12)
4	1 (0)	0.73 (1.06)	2.6 (4.6)	1.1 (8.31)	0.21 (0.18)	1 (0)	0.63 (4.42)	1.9 (7.25)	1.9 (7.6)	0.07 (0.35)	1 (0)	1.9 (1.41)	5.3 (2.3)	6.7 (2.12)	2.2 (3.89)	1 (0)	1.2 (0.35)	2.8 (3.36)	7.4 (7.25)	3.2 (2.65)
8	1 (0)	0.46 (0.18)	0.62 (0.46)	0.58 (1.59)	0.08 (0.18)	1 (0)	0.18 (1.77)	0.89 (2.47)	1.3 (3.89)	0.12 (0.88)	1 (0)	1.4 (2.83)	2.9 (5.66)	4.7 (8.31)	2 (2.83)	1 (0)	1.1 (3.54)	1.8 (1.41)	5.6 (5.13)	2.4 (3.89)
16	1 (0)	0.063 (0)	0.047 (0.18)	0.17 (0.53)	0.063 (0.35)	1 (0)	0.065 (0.53)	0.095 (0.35)	1.1 (1.59)	1.2 (0)	1 (0)	0.41 (1.56)	1.8 (0.88)	0.38 (0.21)	0 (0)	1 (0)	0.21 (1.59)	1.1 (2.69)	5.5 (9.05)	1.9 (0.14)
31	1 (0)	0.13 (0)	0.047 (0.53)	0.22 (2.12)	0.032 (0.35)	1 (0)	0.017 (0.53)	0.017 (0.53)	0.25 (0.18)	0.31 (9.19)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
63	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
125	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where 10% ethanol was added into the medium.

TABLE 5 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with ornidazole

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day					Day					Day					Day				
	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days
0[b]	1 (0)	1.6 (0.53)	6.1 (5.13)	0.28 (0.18)	0 (0)	1 (0)	1.3 (0.53)	11 (14.5)	4.6 (1.24)	0.58 (0.35)	1 (0)	3.8 (1.06)	8 (4.95)	20.5 (9.19)	2.3 (1.41)	1 (0)	2.5 (0.7)	5.5 (6.19)	3.3 (0.7)	2.1 (1.77)
2	1 (0)	1.1 (1.94)	4.6 (7.25)	0 (0)	0 (0)	1 (0)	1.1 (1.06)	11 (6.36)	6.7 (5.83)	1.3 (0.71)	1 (0)	3.5 (0.53)	7.9 (10.08)	18.5 (3.89)	2 (0.53)	1 (0)	1.5 (1.59)	4.2 (1.06)	2.8 (2.3)	1.9 (2.47)
4	1 (0)	0.36 (0.88)	3.7 (2.65)	0.24 (0.35)	0 (0)	1 (0)	0.33 (0.71)	10 (0.88)	5.9 (2.47)	0.62 (0.88)	1 (0)	2.3 (1.41)	6.7 (9.55)	14.9 (5.83)	2.1 (0.35)	1 (0)	0.4 (0.35)	1.1 (0.53)	2.9 (1.41)	1.8 (3.01)
8	1 (0)	0 (0)	2.5 (3.89)	2.9 (9.37)	0.2 (0.53)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
16	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
63	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
125	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where 10% ethanol was added into the medium.

TABLE 7 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with secnidazole

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day 0	1 day	2 days	3 days	4 days	Day 0	1 day	2 days	3 days	4 days	Day 0	1 day	2 days	3 days	4 days	Day 0	1 day	2 days	3 days	4 days
0[b]	1 (0)	1.1 (1.77)	4 (3.89)	5.8 (16.79)	3.5 (0.71)	1 (0)	0.82 (11.84)	3.8 (52.5)	0.34 (1.77)	0.52(5.48)	1 (0)	2.2 (2.83)	5 (6.19)	6.1 (7.42)	2.2 (0.53)	1 (0)	2.1 (1.23)	6.2 (3.89)	8.3 (3.36)	3 (1.06)
2	1 (0)	1.4 (3.01)	5.7 (0.88)	4.7 (7.78)	4.6 (10.08)	1 (0)	0.9 (3.71)	1.4 (0.53)	0.01 (0)	0.15 (1.06)	1 (0)	1.8 (1.24)	4.6 (8.49)	6 (3.89)	2.6 (3.36)	1 (0)	1.5 (1.41)	5.8 (7.78)	7.8 (3.54)	3.2 (11.31)
4	1 (0)	1.3 (4.07)	5.7 (4.07)	5.1 (1.24)	2.3 (4.07)	1 (0)	0.51 (0.71)	1.5 (17.5)	0.07 (1.41)	0.35 (1.41)	1 (0)	1.5 (2.83)	4.3 (3.18)	5.9 (2.65)	2.1 (2.83)	1 (0)	1.1 (0.71)	4 (4.42)	6.9 (6.36)	2.8 (2.3)
8	1 (0)	0.84 (0.18)	5.2 (5.83)	5 (0.88)	2.2 (4.42)	1 (0)	0.14 (1.94)	0.5 (3.36)	0 (14.85)	0.78 (7.95)	1 (0)	0.54 (2.47)	2.2 (4.07)	3 (1.94)	1.3 (4.95)	1 (0)	0.57 (1.59)	2.3 (2.83)	4.1 (5.3)	2 (3.54)
16	1 (0)	0.7 (2.3)	7.4 (6.01)	6.1 (0.88)	3.2 (17.15)	1 (0)	0.075 (1.06)	0.14 (1.24)	0.085 (3.18)	0.97 (29.79)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31	1 (0)	0.45 (2.47)	5.9 (1.06)	7.1 (10.43)	3.1 (4.42)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
63	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
125	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where 10% ethanol, used for dilution, was added into the medium.

TABLE 6 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with ronidazole

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day 0	1 day	2 days	3 days	4 days	Day 0	1 day	2 days	3 days	4 days	Day 0	1 day	2 days	3 days	4 days	Day 0	1 day	2 days	3 days	4 days
0[b]	1 (0)	1.2 (0.2)	3.2 (0.22)	0.84 (0.18)	0 (0)	1 (0)	1.6 (0.23)	3.1 (0.08)	1.1 (0.18)	0 (0)	1 (0)	3.8 (1.06)	8 (4.95)	20.5 (9.19)	2.3 (1.41)	1 (0)	2.5 (0.7)	5.5 (6.19)	3.3 (0.7)	2.1 (1.77)
2	1 (0)	1 (0.14)	2.2 (0.77)	1.1 (0.2)	0 (0)	1 (0)	1.5 (0.51)	2.7 (0.61)	0.96 (0.18)	0 (0)	1 (0)	2.7 (3.01)	7.3 (7.42)	17 (14.14)	2.6 (2.12)	1 (0)	2.3 (2.65)	5.3 (10.08)	2.8 (3.01)	1.9 (1.94)
4	1 (0)	0.3 (0.07)	1.3 (0.28)	0.7 (0.37)	0 (0)	1 (0)	1 (0)	0.9 (0.02)	0.2 (0.05)	0 (0)	1 (0)	0.7 (0.88)	6.7 (4.6)	10.5 (3.01)	1.7 (1.24)	1 (0)	1.2 (1.94)	3.9 (12.37)	2.7 (3.36)	1.8 (1.77)
8	1 (0)	0.1 (0.06)	0.2 (0.02)	0.4 (0.13)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0.8 (1.06)	3.3 (4.07)	3 (9.37)	1.5 (1.59)
16	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
31	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
63	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
125	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where phosphate-buffered saline, used for dilution, was added into the medium.

TABLE 8 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with tetracycline

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day					Day					Day					Day				
	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days
0[b]	1 (0)	2.3 (0.53)	15 (0.71)	22 (3.71)	12 (13.61)	1 (0)	2.3 (5.48)	8.8 (3.54)	2.3 (7.25)	0.07 (1.24)	1 (0)	1.8 (1.06)	4.6 (4.95)	6.3 (9.19)	2.6 (1.41)	1 (0)	1.9 ( )	6.1 (5.3)	8.5 (6.54)	3.8 (2.12)
2	1 (0)	1.4 (3.36)	11 (1.59)	22 (1.59)	24 (2.83)	1 (0)	0.95 (0.71)	7.4 (9.55)	2.4 (12.2)	0.1 (1.06)	1 (0)	1.4 (3.36)	11 (1.59)	22 (1.59)	24 (2.83)	1 (0)	0.95 (0.71)	7.4 (9.55)	2.4 (12.2)	0.1 (1.06)
4	1 (0)	1.5 (0.35)	5.4 (4.95)	11 (10.78)	12 (5.48)	1 (0)	1.1 (0.18)	6.9 (9.54)	0.89 (1.94)	0.14 (0.18)	1 (0)	1.5 (0.35)	5.4 (4.95)	11 (10.78)	12 (5.48)	1 (0)	1.1 (0.18)	6.9 (9.54)	0.89 (1.94)	0.14 (0.18)
8	1 (0)	0.66 (0)	3.8 (2.3)	4.4 (6.19)	8.2 (9.37)	1 (0)	0.78 (0.18)	2.5 (4.95)	2.9 (16.79)	0.09 (1.59)	1 (0)	0.66 (0)	3.8 (2.3)	4.4 (6.19)	8.2 (9.37)	1 (0)	0.78 (0.18)	2.5 (4.95)	2.9 (16.79)	0.09 (1.59)
16	1 (0)	0.73 (1.41)	2.5 (7.25)	6.2 (7.78)	9.1 (11.67)	1 (0)	0.96 (0.88)	2.7 (11.14)	2.7 (21.57)	0.21 (0.88)	1 (0)	0.73 (1.41)	2.5 (7.25)	6.2 (7.78)	9.1 (11.67)	1 (0)	0.96 (0.88)	2.7 (11.14)	2.7 (21.57)	0.21 (0.88)
31	1 (0)	0.73 (1.06)	2.1 (5.83)	4.5 (3.54)	4.4 (2.12)	1 (0)	0.99 (1.06)	0.7 (0.53)	2.1 (10.08)	0.04 (0.35)	1 (0)	0.73 (1.06)	2.1 (5.83)	4.5 (3.54)	4.4 (2.12)	1 (0)	0.99 (1.06)	0.7 (0.53)	2.1 (10.08)	0.04 (0.35)
63	1 (0)	0.36 (0.88)	0.6 (1.06)	1.1 (2.47)	1.3 (5.3)	1 (0)	1 (2.83)	1.4 (5.83)	0.98 (0.18)	0.57 (9.02)	1 (0)	0.34 (0.18)	0.7 (0.18)	1.1 (3.18)	0.16 (1.23)	1 (0)	0.32 (0.71)	0.74 (1.2)	0.12 (0.02)	0 (0)
125	1 (0)	0.13 (0.88)	0.07 (0.18)	0.03 (0)	0.13 (0.18)	1 (0)	0.99 (3.18)	0.62 (0.71)	1 (5.66)	0.8 (9.37)	1 (0)	0.18 (0.35)	0.06 (0)	0 (0)	0 (0)	1 (0)	0.14 (1.06)	0.12 (0.18)	0.16 (0.88)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where phosphate-buffered saline, used for dilution, was added into the medium. Concentrations below the MLC have been omitted.

TABLE 9 *In vitro* cell growth of the four isolates of *D. fragilis* during treatment with tinidazole

Drug concn (μg/ml)	Viable count (10^4 cells/ml) over 4 days (SD)[a]																			
	Isolate V					Isolate E					Isolate A					Isolate M				
	Day				4	Day				4	Day					Day				
	0	1 day	2 days	3 days	days	0	1 day	2 days	3 days	days	0	1 day	2 days	3 days	4 days	0	1 day	2 days	3 days	4 days
0[b]	1 (0)	3.25 (0.64)	6.4 (1.84)	1.8 (0.07)	0 (0)	1 (0)	5.1 (0.66)	3.2 (0.59)	0.36 (0.52)	0 (0)	1 (0)	3.8 (1.06)	8 (4.95)	20.5 (9.19)	2.3 (1.41)	1 (0)	2.5 (0.7)	5.5 (6.19)	3.3 (0.7)	2.1 (1.77)
2	1 (0)	1.95 (0.64)	7.3 (0.99)	1.4 (0.14)	0 (0)	1 (0)	0.8 (0.29)	2 (1.03)	0.5 (0.15)	0 (0)	1 (0)	2.9 (1.94)	7.7 (1.59)	17.8 (2.65)	2 (1.06)	1 (0)	2.4 (3.71)	4.9 (8.49)	3.3 (1.94)	1.9 (4.24)
4	1 (0)	1.6 (0.14)	5.7 (0.49)	1.3 (0.28)	0 (0)	1 (0)	1.1 (0)	1 (0.15)	0 (0)	0 (0)	1 (0)	2.6 (1.41)	7.1 (5.48)	16.8 (13.79)	2.2 (2.12)	1 (0)	1.4 (0.35)	3 (2.12)	3.1 (4.6)	2.6 (1.24)
8	1 (0)	2.3 (0.85)	4.5 (0.64)	2.8 (1.06)	0 (0)	1 (0)	1.1 (0.66)	1.1 (1.33)	0 (0)	0 (0)	1 (0)	2 (1.06)	6.1 (2.65)	9.4 (6.36)	1.2 (1.41)	1 (0)	1.4 (1.77)	2.7 (4.24)	3.2 (5.48)	2.7 (2.83)
16	1 (0)	0.95 (0.35)	3.55 (1.2)	1.2 (0.57)	0 (0)	1 (0)	1.8 (1.55)	1.5 (0.44)	0 (0)	0 (0)	1 (0)	0.7 (0.18)	2.4 (2.65)	4.7 (3.54)	1 (0)	1 (0)	0.3 (0.18)	1.2 (2.47)	2.3 (3.18)	2.6 (9.02)
31	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
63	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
125	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
250	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)
500	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (0)	0 (0)	0 (0)	0 (0)	0 (0)

[a] The values for standard deviation are given in two decimal places. Two replicates were used for each concentration tested per isolate. All values have been divided by original values of day 0 (x) to produce the same values ($x = 1$) for initial concentration.

[b] A drug concentration of 0 μg/ml refers to negative control, where phosphate-buffered saline, used for dilution, was added into the medium.

tostreptococcus spp., *P. distasonis*, *Prevotella oralis*, *Arcobacter butzleri*, and *E. lenta.*

(iv) Metronidazole/secnidazole. The following bacterial species were identified after treatment with either metronidazole or secnidazole: *E. coli*, *P. aeruginosa*, *Bacteroides* spp., *Peptostreptococcus* spp., *P. oralis*, and *C. aerofaciens*. Treatment at high concentrations resulted in the removal of the following bacterial species: *Clostridium* spp., *Veillonella* spp., *P. distasonis*, *E. limosum*, *Anaerococcus prevotii*, *P. distasonis*, *A. butzleri*, and *E. lenta.*

(v) Iodoquinol. The following bacteria were isolated after treatment with iodoquinol: *Clostridium* spp., *Bacteroides* spp., *C. aerofaciens*, *Peptostreptococcus* spp., *P. oralis*, and *Veillonella* spp., as well as both *E. coli* and *P. aeruginosa*. A total of five species of bacteria were removed following iodoquinol treatment: *E. limosum*, *P. distasonis*, *A. prevotii*, *A. butzleri*, and *E. lenta*.

(vi) Paromomycin. *B. fragilis*, *B. ovatus*, *C. aerofaciens*, *E. coli*, and *P. aeruginosa* were present in cultures for four isolates, while *E. limosum*, *Peptostreptococcus* spp., *A. prevotii*, *Veillonella* spp., *Bacteroides merdae*/*B. caccae*, *P. distasonis*, *P. oralis*, *A. butzleri*, and *E. lenta* were removed by treatment with paromomycin.

(vii) Tetracycline. Following tetracycline treatment, the following bacterial species were present: *E. coli*, *P. aeruginosa*, *Bacteroides* spp., *Clostridium* spp., *A. prevotii*, *Peptostreptococcus* spp., and *Veillonella* spp. Treatment removed *E. limosum*, *P. distasonis*, *P. oralis*, *A. butzleri*, and *E. lenta*.

(viii) Furazolidone. The bacterial species *E. coli*, *P. aeruginosa*, *Clostridium* spp., *Bacteroides* spp., *Peptostreptococcus* spp., *P. oralis*, and *Veillonella* spp. were present in cultures treated with furazolidone, and furazolidone treatment removed *E. limosum*, *C. aerofaciens*, *P. distasonis*, *A. butzleri*, and *E. lenta* for four isolates.

(ix) Nitazoxanide. Following treatment with nitazoxanide, *E. coli*, *P. aeruginosa*, *Bacteroides* spp., *C. aerofaciens*, *E. limosum*, *A. prevotii*, *Peptostreptococcus* spp., and *Veillonella* spp. were present, and treatment removed *E. limosum*, *P. distasonis*, *P. oralis*, *A. butzleri*, and *E. lenta*.

(x) Diloxanide furoate. Treatment with diloxanide furoate had no effect on the bacterial flora, as all bacterial species were identified in all isolates.

DISCUSSION

D. fragilis has been a frequently encountered pathogenic protozoon; however, very little research on the susceptibility of this organism has been conducted. This study suggests that the 5-nitromidazole derivatives (metronidazole, ornidazole, ronidazole, and tinidazole) are the most active components against *D. fragilis* trophozoites, with lethal concentrations ranging from 8 to 63 μg/ml.

Metronidazole, a common oral synthetic antimicrobial compound, was found to have an MLC of 31 μg/ml (Table 3), and this is in correlation with the minimal amoebicidal concentration of 32 μg/ml obtained by Chan et al. (10). The efficacy of metronidazole treatment for *D. fragilis* ranges from 70 to 80% (24).

Secnidazole, the first of 5-nitroimidazole derivatives known to offer a 3-day antiprotozoal activity from a single dosage due to its longer half-life, was shown to be effective in the treatment of dientamoebiasis by Girginkardesler et al. (16). The data presented here support such findings, as the minimum amoebicidal concentration was found to be 63 μg/ml (Table 7).

Tinidazole is another known antiprotozoal compound effective for a number of protozoal infections (14). This study suggests the use of tinidazole to be effective for inhibition of *D. fragilis* trophozoites *in vitro*, with MLCs being as low as 31 μg/ml.

Ronidazole has been show to be approximately five times more active than metronidazole in inhibition of *Giardia intestinalis* (7). Another study using a closely related enteric pathogen of felines, *Tritrichomonas foetus*, revealed that ronidazole had a higher lethal activity against *T. foetus* trophozoites than metronidazole (20). The data presented here agree with the high lethal activity of ronidazole, as the MLC was determined to be as low as 8 μg/ml.

Ornidazole was shown to be highly active against *Dientamoeba*, with MLCs from this study found to be as low as 8 μg/ml. Previously, a comparison of metronidazole and ornidazole was undertaken by Kurt et al. (23). Significant differences were found, with ornidazole showing higher parasite clearance and clinical improvement along with fewer side effects.

Furazolidone is a synthetic nitrofuran derivative used for the treatment of a broad range of bacterial and protozoal infections (26a). It is evident from our data that the treatment of *D. fragilis* with furazolidone displays only minimal inhibitory effects at best, and MLCs were as high as 250 μg/ml for clinical isolates. No studies to date have tested furazolidone for the purpose of treating *D. fragilis* infections.

Nitazoxanide was first introduced in 1984 as a cestocidal drug (27), and subsequent studies have demonstrated that it has inhibitory effects against *Trichomonas vaginalis* (2, 9) and other diarrhea-causing protozoa. Our study demonstrated that the use of nitazoxanide at a concentration of 63 μg/ml is lethal for *D. fragilis* and thus may be a possible treatment option; however, no clinical studies to date have reported on the use of nitazoxanide for the treatment of dientamoebiasis.

Tetracycline is a broad-spectrum antimicrobial which has been recommended as a possible treatment option for *D. fragilis* (26). In agreement with the findings of Chan et al. (10), the data presented here suggest that treatment of dientamoebiasis with tetracycline should be reconsidered, as relatively high MLC values of 250 μg/ml were obtained.

Iodoquinol is a halogenated hydroxyquinoline which has been used extensively to treat dientamoebiasis (22). This study suggests that it may be ineffective for the treatment of *D. fragilis*, as the MLC for all isolates was 500 μg/ml.

Paromomycin is a poorly absorbed aminoglycoside antibiotic that is also currently recommended by the Centers for Disease Control and Prevention (CDC) for the treatment of *D. fragilis* infections (1). It was shown here that the use of paromomycin as a therapeutic option may be questionable, as the MLCs were 500 μg/ml. The data presented here and in the previous study by Chan et al. (10), who reported a minimal amoebicidal concentration of 16 μg/ml for paromomycin, differ significantly.

Diloxanide furoate is a luminal amoebicide, and it would appear that diloxanide furoate has minimal to no inhibitory effects on *D. fragilis* infections *in vitro*, as the MLC for diloxanide furoate was found to be greater than 500 μg/ml. However, it should be noted that the hydrolysis reaction involved in the activation of diloxanide furoate appears to be temperature and $[OH^-]$ dependent, so it is possible that *in vivo* results may differ (15). No studies to date have tested diloxanide furoate for treatment of dientamoebiasis.

There is only one previously reported experiment of *in vitro* susceptibility testing of *D. fragilis*, and that study used an ATCC strain (ATCC 30948). In this study, the minimal amoebicidal con-

centrations for iodoquinol, paromomycin, tetracycline, and metronidazole were determined to be 128, 16, 32, and 32 μg/ml, respectively (10), in contrast to our data, which were 500, 500, 250, and 31 μg/ml, respectively. The previous study used the *D. fragilis* strain ATCC 30948 (genotype 2), which has not been found to be associated with gastrointestinal diseases to date and is rarely encountered in human samples (11, 25, 28). While the majority of studies have shown genotype 1 in nearly all cases (6, 31, 33, 36), testing of this genotype may be more appropriate in terms of clinical significance and may be a possible reason for the different MLC values between the two studies.

The true complexity of the bacterial flora contained within these clinical isolates of *D. fragilis* is summarized in Table 1. Elimination of certain species and/or the majority of the bacterial flora present in the cultures may indirectly result in detrimental effects to the *D. fragilis* trophozoites, as the parasite has long been known to utilize them as a food source. However, it was observed that the treatments did not affect the majority of the bacterial flora present. This supports the notion that elimination of *D. fragilis* in these is the result of the antiparasitic effects of the drug and not due to the antibacterial effects on the bacterial populations.

The results of this study show that the inhibitory effects of a number of antimicrobials currently used as recommended treatments, including iodoquinol, paromomycin, and tetracycline, make them inappropriate for treatment of *D. fragilis* infections. The use of newer antiprotozoal compounds with far fewer known side effects or combination therapies derived from current treatment options for *D. fragilis* infections appears to be a viable option for consideration. In summary, the data presented here indicated that the use of 5-nitroimidazoles is the most effective option for treating *D. fragilis* infections, with ornidazole and ronidazole being the most active *in vitro* compounds.

ACKNOWLEDGMENTS

We thank West Lindfield Compounding Chemist for providing diloxanide furoate, furazolidone, iodoquinol, nitazoxanide, and secnidazole and J. Upcroft of the Queensland Institute of Medical Research.

REFERENCES

1. **Abramowicz M.** 1995. Drugs for parasitic infections. Med. Lett. Drugs Ther. **37**:99–108.
2. **Adagu IS, Nolder D, Warhurst DC, Rossignol JF.** 2002. In vitro activity of nitazoxanide and related compounds against isolates of Giardia intestinalis, Entamoeba histolytica and Trichomonas vaginalis. J. Antimicrob. Chemother. **49**:103–111.
3. **Balcioglu IC, et al.** 2007. Rural life, lower socioeconomic status and parasitic infections. Parasitol. Int. **56**:129–133.
4. **Barratt JL, et al.** 2010. Newly defined conditions for the in vitro cultivation and cryopreservation of Dientamoeba fragilis: new techniques set to fast track molecular studies on this organism. Parasitology **137**: 1867–1878.
5. **Barratt JLN, Harkness J, Marriott D, Ellis JT, Stark D.** 2010. A review of Dientamoeba fragilis carriage in humans: several reasons why this organism should be considered in the diagnosis of gastrointestinal illness. Gut Microbes **2**:3–12.
6. **Bart A, et al.** 2008. Intragenomic variation in the internal transcribed spacer 1 region of Dientamoeba fragilis as a molecular epidemiological marker. J. Clin. Microbiol. **46**:3270–3275.
7. **Boreham P, Phillips R, Shepherd R.** 1985. A comparison of the in-vitro activity of some 5-nitroimidazoles and other compounds against Giardia intestinalis. J. Antimicrob. Chemother. **16**:589.
8. **Butler WP.** 1996. Dientamoeba fragilis. An unusual intestinal pathogen. Dig. Dis. Sci. **41**:1811–1813.
9. **Cedillo-Rivera R, Chavez B, Gonzalez-Robles A, Tapia A, Yepez-Mulia L.** 2002. In vitro effect of nitazoxanide against Entamoeba histolytica, Giardia intestinalis and Trichomonas vaginalis trophozoites. J. Eukaryot. Microbiol. **49**:201–208.
10. **Chan FT, Guan MX, Mackenzie AM, Diaz-Mitoma F.** 1994. Susceptibility testing of Dientamoeba fragilis ATCC 30948 with iodoquinol, paromomycin, tetracycline, and metronidazole. Antimicrob. Agents Chemother. **38**:1157–1160.
11. **Clark CG.** 2000. Cryptic genetic variation in parasitic protozoa. J. Med. Microbiol. **49**:489–491.
12. **Clark CG, Diamond LS.** 2002. Methods for cultivation of luminal parasitic protists of clinical importance. Clin. Microbiol. Rev. **15**:329.
13. **Cuffari C, Oligny L, Seidman EG.** 1998. Dientamoeba fragilis masquerading as allergic colitis. J. Pediatr. Gastroenterol. Nutr. **26**:16–20.
14. **Fung HB, Doan TL.** 2005. Tinidazole: a nitroimidazole antiprotozoal agent. Clin. Ther. **27**:1859–1884.
15. **Gadkariem EA, Belal F, Abounassif MA, El-Obeid HA, Ibrahim KEE.** 2004. Stability studies on diloxanide furoate: effect of pH, temperature, gastric and intestinal fluids. Il Farmaco **59**:323–329.
16. **Girginkardesler N, Coşkun S, Cüneyt Balcioğlu I, Ertan P, Ok UZ.** 2003. Dientamoeba fragilis, a neglected cause of diarrhea, successfully treated with secnidazole. Clin. Microbiol. Infect. **9**:110–113.
17. **Girginkardesler N, Kurt O, Kilimcioglu AA, Ok UZ.** 2008. Transmission of Dientamoeba fragilis: evaluation of the role of Enterobius vermicularis. Parasitol. Int. **57**:72–75.
18. **Jepps M, Dobell C.** 1918. Dientamoeba fragilis ng, n. sp., a new intestinal amoeba from man. Parasitology **10**:352–367.
19. Reference deleted.
20. **Kather EJ, Marks SL, Kass PH.** 2007. Determination of the in vitro susceptibility of feline Tritrichomonas foetus to 5 antimicrobial agents. J. Vet. Intern. Med. **21**:966–970.
21. **Kean B, Malloch C.** 1966. The neglected ameba: Dientamoeba fragilis. A report of 100 "pure" infections. Am. J. Dig. Dis. **11**:735.
22. **Khaw M, Panosian CB.** 1995. Human antiprotozoal therapy: past, present, and future. Clin. Microbiol. Rev. **8**:427–439.
23. **Kurt O, Girginkardesler N, Balcioglu IC, Ozbilgin A, Ok UZ.** 2008. A comparison of metronidazole and single-dose ornidazole for the treatment of dientamoebiasis. Clin. Microbiol. Infect. **14**:601–604.
24. **Norberg A, Nord C, Evengård B.** 2003. Dientamoeba fragilis—a protozoal infection which may cause severe bowel distress. Clin. Microbiol. Infect. **9**:65–68.
25. **Peek R, Reedeker FR, van Gool T.** 2004. Direct amplification and genotyping of Dientamoeba fragilis from human stool specimens. J. Clin. Microbiol. **42**:631–635.
26. **Preiss U, Ockert G, Brömme S, Otto A.** 1990. Dientamoeba fragilis infection, a cause of gastrointestinal symptoms in childhood. Klin. Padiatr. **202**:120–123.

26a. **Raether W, Hänel H.** 2003. Nitroheterocyclic drugs with broad speetrum activity. Parasitol. Res. **90**(Suppl 1):S19–S39.

27. **Rossignol JF, Maisonneuve H.** 1984. Nitazoxanide in the treatment of Taenia saginata and Hymenolepis nana infections. Am. J. Trop. Med. Hyg. **33**:511–512.
28. Reference deleted.
29. **Stark D, et al.** 2010. A review of the clinical presentation of dientamoebiasis. Am. J. Trop. Med. Hyg. **82**:614–619.
30. **Stark D, Beebe N, Marriott D, Ellis J, Harkness J.** 2005. Detection of Dientamoeba fragilis in fresh stool specimens using PCR. Int. J. Parasitol. **35**:57–62.
31. **Stark D, Beebe N, Marriott D, Ellis J, Harkness J.** 2005. Prospective study of the prevalence, genotyping, and clinical relevance of Dientamoeba fragilis infections in an Australian population. J. Clin. Microbiol. **43**:2718–2723.
32. **Stark D, van Hal S, Marriott D, Ellis J, Harkness J.** 2007. Irritable bowel syndrome: a review on the role of intestinal protozoa and the importance of their detection and diagnosis. Int. J. Parasitol. **37**:11–20.
33. **Vandenberg O, et al.** 2006. Clinical and microbiological features of dientamoebiasis in patients suspected of suffering from a parasitic gastrointestinal illness: a comparison of Dientamoeba fragilis and Giardia lamblia infections. Int. J. Infect. Dis. **10**:255–261.
34. **Wheeler D, Bhagwat M.** 2007. BLAST QuickStart: example-driven web-based BLAST tutorial. Methods Mol. Biol. **395**:149–176.
35. Reference deleted.
36. **Windsor JJ, Clark CG, Macfarlane L.** 2004. Molecular typing of Dientamoeba fragilis. Br. J. Biomed. Sci. **61**:153–155.