Parasitol Res (2015) 114:1647–1659
DOI 10.1007/s00436-015-4423-1

REVIEW

Understanding drug resistance in human intestinal protozoa

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Received: 18 October 2014 / Accepted: 5 March 2015 / Published online: 19 March 2015

Abstract Infections with intestinal protozoa continue to be a major health problem in many areas of the world. The widespread use of a limited number of therapeutic agents for their management and control raises concerns about development of drug resistance. Generally, the use of any antimicrobial agent should be accompanied by meticulous monitoring of its efficacy and measures to minimize resistance formation. Evidence for the occurrence of drug resistance in different intestinal protozoa comes from case studies and clinical trials, sometimes with a limited number of patients. Large-scale field-based assessment of drug resistance and drug sensitivity testing of clinical isolates are needed. Furthermore, the association of drug resistance with certain geographic isolates or genotypes deserves consideration. Drug resistance has been triggered in vitro and has been linked to modification of pyruvate:ferredoxin oxidoreductase, nitroreductases, antioxidant defense, or cytoskeletal system. Further mechanistic studies will have important implications in the development of second generation therapeutic agents.

Keywords Intestinal protozoa · Drug · Resistance · Treatment

Introduction

Infections with intestinal protozoa are highly prevalent in developing countries where poor household hygienic practice, inadequate sanitation facilities, and low socioeconomic conditions favor their spread. Rural populations and those living in crowded urban or slum environments are at higher risk of infection. In countries of Europe and the North Americas, intestinal protozoa are uncommonly encountered due to better hygienic conditions. However, intestinal protozoa are repeatedly diagnosed in these areas in immigrants and refugee communities and have been also linked to contaminated recreational water and day care centers sporadic outbreaks (Savioli et al. 2006; Fletcher et al. 2012).

Pathogenic intestinal protozoa are responsible for significant intestinal disease in humans, with profound morbidity, and in some cases mortality. They depress appetite and reduce food intake, cause maldigestion, impair absorption, and increase nutrient loss. Subtle damage and impairment of intestinal functions can occur even in asymptomatic infection which represents a considerable portion of all infections. The small intestinal protozoa *Giardia intestinalis* and *Cryptosporidium* spp. have their major impact in children, while the large bowel pathogen *Entamoeba histolytica* infects all age groups but its most profound effects are observed in adults. Intestinal coccidian parasites have their major impact in immunosuppressed persons. Infection with *Balantidium coli* is associated with pig contact (Farthing et al. 2009; Becker et al. 2013). Both *Blastocystis hominis* and *Dientamoeba fragilis* are increasingly reported enteric protozoa with a possible role in the etiology of irritable bowel syndrome (Engsbro et al. 2014).

Chemotherapy is an important cost-effective approach used in combating infections with intestinal protozoa especially in areas where improvement of sanitary infrastructure is hindered by poor resources. In this context, chemotherapy is widely used to treat symptomatic patients and to reduce transmission through elimination of parasites in asymptomatically infected persons (Savioli et al. 2006; Escobedo et al. 2009;

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Alum et al. 2010). Moreover, it is likely that preventive chemotherapy programs for intestinal parasites will extend to cover common intestinal protozoa in the near future. Among factors that threaten the effectiveness of chemotherapy is the emergence of parasite strains resistant to available drugs (Hotez 2014).

Generally, development of antimicrobial resistance is enhanced by massive or indiscriminate drug use. Improper use through inadequate dosing, poor adherence, and sub-standard antimicrobials also have a role. The ability of microorganisms to multiply in the presence of drug concentrations higher than the concentrations in humans receiving curative doses arises through gene transfer or mutations (Cantón and Morosini 2011). Resistance is associated with inactivation of the antimicrobial agent, reduction of its uptake, active efflux, or modification of its target. Monitoring the magnitude and trends in resistance is essential for proper clinical management and should be used in updating therapeutic guidelines. Defining the molecular basis of resistance has important implications in identifying novel drug targets and development of more efficient therapeutic agents (Ouellette 2001; WHO 2014). This review discusses current treatment options for different human intestinal protozoa with a focus on existing evidences for the presence of drug-resistant strains. Available alternatives for management of intractable infections will be summarized. In addition, a comprehensive overview of factors mediating drug resistance and recent advances in explaining its biochemical and molecular basis will be presented.

G. intestinalis

G. intestinalis is the most commonly detected intestinal protozoan parasite worldwide (Fletcher et al. 2012). Giardiasis is treated primarily with 5-nitroimidazole (5-NI) drugs. A recent systematic review included 30 randomized clinical trials and concluded that 5-NI drugs continue to be the agents of choice in giardiasis treatment due to their high cure rate and reasonable safety profile (Pasupuleti et al. 2014). Metronidazole is a widely prescribed 5-NI derivative. Its recommended dose is 250 mg three times a day for 5–7 days for adults and 15 mg/kg three times a day for 5–7 days in children (Solaymani-Mohammadi et al. 2010; Kappagoda et al. 2011). Adverse effects of metronidazole are not uncommon and mainly include metallic taste, headache, anorexia, nausea, and abdominal pain (Alizadeh et al. 2006; Kappagoda et al. 2011). The other derivatives, tinidazoles and ornidazoles, are used in a single dose therapy with good tolerance which is considered an advantage in terms of patients' compliance (Ozbilgin et al. 2002; Fung and Doan 2005).

In vitro exposure of *Giardia* trophozoites to 5-NI drugs causes apoptotic-like morphological changes, loss of motility, swelling of the cell body, and severe damage of the dorsal surface membrane (Müller et al. 2006; Bagchi et al. 2012). 5-NI drugs are activated inside *Giardia* trophozoites to toxic radical intermediates that also damage DNA causing death of the parasite. Activation depends on the presence of the electron acceptor ferredoxin and the activity of pyruvate:ferredoxin oxidoreductase (PFOR), a system with low redox potential electron transfer not found in mammalian cells. Activation occurs also via the flavin-dependent thioredoxin (trx) reductase pathway (Leitsch et al. 2011). A *Giardia* nitroreductase enzyme, designated GlNR1, is thought to participate in the activation since its expression level correlates with susceptibility to metronidazole (Nillius et al. 2011).

Several studies demonstrated occurrence of clinical resistance to the standard anti-giardial treatment (Table 1). A cure rate of 84 % was reported by Baqai et al. (2001) on using metronidazole 400 mg three times a day for 5 days to treat 21 patients with confirmed *G. intestinalis* infection. The efficacy of tinidazole, 50 mg/kg in a single dose, in treatment of 61 children with giardiasis was 82 % (Cañete et al. 2006). Failure to respond to repeated courses of metronidazole was described in two patients with no defined immune deficiency (Nash et al. 2001). Metronidazole and tinidazole were ineffective in treating 22 % of giardiasis patients attending a travel clinic in Spain, and the efficacy of a second course of treatment was only 17 %. (Muñoz Gutiérrez et al. 2013).

In clinical settings, treatment failure does not necessarily imply drug resistance. Apparently, reduced efficacy may be caused by inadequate drug levels, reinfection in highly endemic areas, immunosuppression, or sequestration in the gallbladder (Nash et al. 2001). Furthermore, the possibility of spread of clinically significant resistant strains might be low. Tejman-Yarden et al. (2011) observed that three out of five metronidazole-resistant cell lines exhibited marked defects of attachment to plastic inert surfaces and to monolayers of Caco-2 human intestinal epithelial cells, and this was accompanied by impaired ability to induce infection in experimental animals. Furthermore, resistant isolates that could establish in vivo infection showed significantly lower parasite loads than their metronidazole-sensitive parental cells with an apparently shorter duration of infection (Tejman-Yarden et al. 2011).

Factors favoring emergence of drug resistance to standard anti-*Giardia* treatment are not clearly understood. Indeed, continuous exposure of *Giardia* trophozoites to sublethal doses of metronidazole in culture media has been used to obtain resistant lines after a period of slow growth (Tejman-Yarden et al. 2011). The wide use of 5-NI drugs for several other indications such as treatment of infections with *Helicobacter pylori* and gingivitis, and as prophylaxis in colorectal surgery may enhance emergence of 5-NI-resistant *Giardia* (Löfmark et al. 2010; Pasupuleti et al. 2014).

Studies on differential gene expression patterns in wild-type and resistant lines indicated that the expression levels of a variety of genes are affected in resistant strains and that

Table 1 Drugs used in the treatment of *G. intestinalis* with an overview of evidences for treatment failure/drug resistance

Drug	Recommended dose	Evidence of treatment failure/drug resistance	Reference
Metronidazole	250 mg three times a day for 5–7 days for adults and 15 mg/kg three times a day for 5–7 days in children (Kappagoda et al. 2011)	Treatment failure in 16 % of 21 patients	Baqai et al. 2001
		Failure to respond to repeated courses in two patients with no immune deficiency	Nash et al. 2001
		Among outbreak cases, 42 patients failed to respond to between one and three courses	Mørch et al. 2008
		Of 170 patients, ten had persistent infection after one or more courses	Lopez-Velez et al. 2010
Tinidazole	Single dose of 2 g in adults and 50 m/kg in children (Kappagoda et al. 2011)	Treatment failed in 18 % of 61 treated children	Cañete et al. 2006
		Treatment failure in three out of four family members. *Giardia* assemblage B was characterized	Requena-Méndez et al. 2014
Albendazole	400 mg/day for 3–5 days (Solaymani-Mohammadi et al. 2010)	Resistance of two isolates obtained from patients who did not respond to treatment was confirmed in a mouse model	Lemée et al. 2000
Nitazoxanide	500 mg twice a day for 3 days for adults (Kappagoda et al. 2011) 7.5 mg/kg of body weight twice daily for 3 days in children (Escobedo et al. 2008)	Treatment was ineffective in 22 % of 74 treated children.	Escobedo et al. 2008

resistance formation takes place on a multigenic rather than a monogenic level (Müller et al. 2008; Nillius et al. 2011). Downregulation of PFOR and reduced level of its related electron transport peptide, ferrodoxin, were reported in metronidazole-resistant *Giardia* (Liu et al. 2000; Müller et al. 2007a, b). Antisense inhibition of PFOR mRNA using hammerhead ribozyme causes transfected *Giardia* organisms to become metronidazole resistant (Dan et al. 2000). However, *Giardia* cell lines that are highly resistant to a structurally modified generation of 5-NI were found to have normal levels of PFOR, suggesting the implication of an alternative mechanism of resistance (Dunn et al. 2010). Decreased GlNR1 mRNA level in *G. intestinalis* confers metronidazole resistance. Furthermore, another nitroreductase, designated GlNR2, was characterized in *G. intestinalis* trophozoites and its overexpression was linked to reduced susceptibility to metronidazole, denoting participation in drug inactivation (Müller et al. 2013). Suppression of flavin reduction and impairment of flavin metabolism was observed in highly metronidazole-resistant *Giardia* (Leitsch et al. 2011). Resistance formation to metronidazole has been also linked to changes in expression of open reading frames encoding major surface antigens such as the variant surface protein. Moreover, expression patterns of genes involved in stress response such as cytosolic heat-shock protein are correlated with resistance (Müller et al. 2008).

Apart from 5-NI drugs, giardiasis can be treated with albendazole, an antihelmithic benzimidazoles (Granados et al. 2012). The anti-giardial action of albendazole is mediated through inhibition of the polymerization of cytoskeleton proteins resulting in reduction in the rate and amount of microtubule assembly (MacDonald et al. 2004). A meta-analysis of randomized trials showed that the effectiveness of albendazole given in a dose of 400 mg/day for 5 days was comparable to that of metronidazole. Side effects of albendazole are less than that of metronidazole (Solaymani-Mohammadi et al. 2010). The therapeutic efficacy of albendazole and restoration of normal mucosal architecture can be enhanced by co-administration of the probiotic, *Lactobacillus casei* (Shukla et al. 2013). The lower toxicity, relative insolubility, poor absorption from the gut, and lack of significant effects on the intestinal microflora make albendazole an ideal substitute for metronidazole (Solaymani-Mohammadi et al. 2010). Albendazole is used in mass treatment campaigns of helminth control programs (El-Setouhy et al. 2007; Mwinzi et al. 2012). It has to be noted that the lower doses and/or shorter duration of albendazole treatment in such programs have lower anti-giardial efficacy and does not significantly reduce the prevalence of *G. intestinalis* (Swanson et al. 2012), a situation that may predispose to development of albendazole-resistant strains. Noteworthy, the efficacy of mebendazole, another benzimidazole drug, in the treatment of giardiasis was found to be significantly low in comparison to tinidazole (Cañete et al. 2006).

In the clinical context, documentations of *Giardia* resistance to albendazole are scarce (Table 1). Lemée et al. (2000) described two *Giardia* isolates obtained from patients who did not respond to treatment with albendazole (0.4 g/day for 5 days) and confirmed their resistance to albendazole in a neonatal mouse model. For research purposes, induction of albendazole-resistance is achieved in vitro by repeated subculture of *Giardia* trophozoites in increasing sublethal concentrations of the drug. Comparison of the resulting resistant clone

with a non-resistant one is used to elucidate potential drug targets (Paz-Maldonado et al. 2013). Sequence alignments of the β giardin gene amplified from albendazole-sensitive and albendazole-resistant strains of *Giardia* revealed that resistance is accompanied by several amino acid mutations which result in altered protein folding in the three-dimensional structure of β-giardin, one of the cytoskeletal proteins (Jiménez-Cardoso et al. 2009). The mutation in the β-tubulin gene which has been linked to benzimidazole resistance in nematode parasites of animals (Ghisi et al. 2007) was not detected in albendazole-resistant *Giardia* (Argüello-García et al. 2009).

Protein electrophoresis and reverse transcription PCR were used to elucidate the proteomic and molecular basis of albendazole resistance in *G. intestinalis*. Eight proteins implicated in antioxidant defense, energy metabolism, and cytoskeletal system formation were found to be differentially regulated in *Giardia*-sensitive and *Giardia*-resistant clones. Moreover, gene expression analysis of mRNA levels correlated well the proteomic study (Paz-Maldonado et al. 2013).

An alternative treatment option in giardiasis is nitazoxanide, a nitrothiazolide derivative with broad-spectrum antiparasitic activity (Rossignol 2010). The typical dose for treating giardiasis and cryptosporidiosis in adults is 500 mg orally every 12 h for 3 days (Kappagoda et al. 2011). In children infected with *G. intestinalis*, the cure rate of nitazoxanide used in a dose of 7.5 mg/kg of body weight twice daily for 3 days was 78 % (Escobedo et al. 2008). In another study, a 7-day treatment resulted in a cure rate of 93 % and this increased to 100 % with a second course (Zumaquero-Ríos et al. 2013). In a clinical trial, nitazoxanide reduced the duration of diarrheal illness in patients infected with *G. intestinalis* as well as in those with multiple etiologies or no identified enteropathogen. These results make nitazoxanide particularly suitable for empiric treatment of diarrheal illness in children where the etiology is unknown or presumed to be of infectious origin (Rossignol et al. 2012).

In vitro exposure of *Giardia* trophozoites to nitazoxanide for 3 h induces alterations in the ventral disk surface membrane, formation of cytoplasmic vacuoles, and disintegration of the cytoplasmic compartments (Müller et al. 2006). Nitazoxanide also damages the cyst wall, impairs cyst viability, and exhibits superior inhibitory effects on excystation of *Giardia* cysts in comparison to metronidazole and albendazole (Bernal-Redondo et al. 2004). In vivo, nitazoxanide is rapidly deacetylated to tizoxanide which was shown to be more active than metronidazole against susceptible strains and twice as active against resistant isolates. (Adagu et al. 2002).

Several mechanisms have been proposed for the anti-giardial action of nitazoxanide. First, nitazoxanide is a specific inhibitor of PFOR enzyme of *Giardia* trophozoites. It abstracts a proton from the activated thiamine pyrophosphate vitamin B1 cofactor of PFOR, thereby inhibiting the production of acetyl-coenzyme A and CO_2 necessary for energy metabolism. This mechanism is not susceptible to mutation-based drug resistance as the vitamin would not be functional if mutated (Hoffman et al. 2007). Second, nitazoxanide binds to and inhibits nitroreductases which might disturb the redox balance of the cell (Müller et al. 2007b). Third, disulfide isomerases from *G. intestinalis* were identified as a potential nitazoxanide target (Müller et al. 2007a).

Reports on clinical isolates of nitazoxanide-resistant *Giardia* are lacking (Table 1). However, *Giardia* resistance to nitazoxanide was successfully triggered in vitro (Müller et al. 2008). Resistance formation is associated with upregulation of disulfide isomerases expression that compensate for nitazoxanide-induced loss of activity. Pronounced alterations in the expression of genes encoding variant surface proteins also confer nitazoxanide resistance (Müller et al. 2007a, b).

Understanding the issue of cross-resistance between anti-giardial drugs is essential for proper management of drug-resistant *Giardia* infections. Laboratory-developed metronidazole-resistant *Giardia* lines were found to be almost completely cross-resistant to tinidazole (Tejman-Yarden et al. 2011). Cross-resistance between 5-NI drugs is expected as they share common mechanism of activation by *Giardia* trophozoites but it has to be noted that variations in pharmacokinetics and bioavailability of drugs influence their efficacy in clinical setting (Fung and Doan 2005). Moreover, cross-resistance among diverse 5-NI drugs is not absolute. Valdez et al. (2009) synthesized several 5-NI derivatives that could markedly overcome metronidazole resistance partly due to their more positive redox activation potential and ease of reduction.

Interestingly, in vitro-triggered nitazoxanide resistance is accompanied by metronidazole resistance (Müller et al. 2007a; Tejman-Yarden et al. 2011). The anti-giardial action of drugs possessing a 5-nitro group such as nitazoxanide and furazolidone are lower for metronidazole-resistant *Giardia* than for metronidazole-sensitive isolates, denoting some degree of cross-resistance. Albendazole, which does not possess a nitro group, is equally effective against all metronidazole-sensitive and metronidazole-resistant lines (Dunn et al. 2010). Cross-resistance between metronidazole and albendazole was rarely reported in clinical isolates of *Giardia* (Lemée et al. 2000; Abboud et al. 2001). When resistance is suspected, it is generally recommended to prescribe a structurally unrelated anti-giardial agent (Tejman-Yarden et al. 2011).

A variety of other chemotherapeutic options are less commonly used in the therapy of giardiasis. Furazolidone is an effective alternative but must be administered four times a day for 7 to 10 day (Talari et al. 2006). Despite low efficacy, paromomycin is preferred for treatment of pregnant women as it is poorly absorbed in the intestine (Mørch et al. 2008; Muñoz Gutiérrez et al. 2013). Quinacrine can achieve cure

in patients with 5-NI-resistant giardiasis (Requena-Méndez et al. 2014). Generally, these drugs should be reserved for the treatment of cases refractory to the first-line drugs (Mørch et al. 2008).

Combination of drugs is recommended when monotherapy fails in the treatment of giardiasis. A combination of albendazole and metronidazole showed 79 % efficacy in patients with persistent giardiasis following 1–3 courses of metronidazole treatment. Quinacrine in combination with metronidazole was effective in treating patients that failed to respond to the former albendazole-metronidazole combination (Mørch et al. 2008). Metronidazole or tinidazole in combination with quinacrine was effective in eradicating *G. intestinalis* infection in five out of six patients with refractory giardiasis (Nash et al. 2001). In a case series of 10 patients with giardiasis refractory to one or more courses of 5-NI drugs, good response to a combination regimen of a 5-NI agent with one or two other drugs (paromomycin, quinacrine, or albendazole) was observed (Lopez-Velez et al. 2010). However, in another study, nitazoxanide-albendazole combination (1000–400 mg), with each drug given separately on two consecutive days, showed a low cure rate of 42 % in *G. intestinalis*-infected children (Speich et al. 2013). This might be attributed to using both drugs in sub-optimal doses (Baqai et al. 2001).

Whether *Giardia* assemblages differ in their susceptibility to drugs is not clear. One study found no difference in drug susceptibility between field isolates of assemblages A and E. However, assemblage A laboratory strains were more susceptible to the benzimidazoles and less susceptible to 5-NI and furazolidone than assemblage B laboratory strain (Bénéré et al. 2011). A study in Spain showed a relatively high rate of 5-NI refractory giardiasis among travelers visiting south and east of the Mediterranean basin and the Asian continent, suggesting that resistance is associated with infection by distinct genotypes (Muñoz Gutiérrez et al. 2013). Genetic analysis of *Giardia* cysts isolated from patients with metronidazole refractory giardiasis detected after a waterborne outbreak revealed that the isolates had the same genotypes as glutamate dehydrogenase and β-giardin genes within assemblage B although sequence profiles from the isolates at the peak of the outbreak were more heterogenous. These findings suggest that resistance is limited to certain genotypes (Mørch et al. 2008).

E. histolytica

5-NI drugs are the mainstay of amoebiasis treatment. Metronidazole is used in a dose of 500–750 mg orally three times a day for 10 days. Other 5-NI drugs such as tinidazole, secnidazole, and ornidazole have a much longer half-life than metronidazole, allowing for shorter duration of treatment without affecting their efficacy (Table 2) (Salles et al. 1999; Fung and Doan 2005; Mackey-Lawrence and Petri 2011). Because 5-NI drugs are readily and almost completely absorbed after oral administration, they lack activity against luminal stages of the parasite. Therefore, luminal amoebicides, such as diloxanide furoate, paromomycin, or iodoquinol, are recommended to treat asymptomatic patients and those with intestinal disease following 5-NI therapy (Kappagoda et al. 2011; Kikuchi et al. 2013). Emetine and dehydroemetine had been previously used in the treatment of intestinal amoebiasis. However, their adverse effects preclude their widespread use (Khaw and Panosian 1995).

The amoebicidal activity of metronidazole was confirmed in a mouse model of intestinal amoebiasis; complete cure following metronidazole therapy was documented using tests for amoebic antigen detection and culture of caecal contents (Becker et al. 2011). Similar to *G. intestinalis*, *E. histolytica* induces intracellular reductive activation of 5-NI drugs. This occurs through ferredoxin, PFOR, trx reductase, and nitroreductases (Leitsch et al. 2007; Pal et al. 2009). In addition, *E. histolytica* possesses two oxidoreductases that participate in metronidazole activation. *E. histolytica* transformants overexpressing either of these enzymes are more sensitive to metronidazole (Jeelani et al. 2010). The amoebicidal activity of activated metronidazole is attributed to generation of reactive oxygen species and formation of covalent adducts with several components of the antioxidant defense system including superoxide dismutase, purine nucleoside phosphorylase, and trx as well as the non-protein thiols, cysteine. Reduced metronidazole also binds to and modifies trx and trx reductase. This interferes with reduction of peroxiredoxins, which have a crucial redox regulatory function in *E. histolytica*. The ultimate result would be impairment of cellular redox-dependent responses and oxidative stress (Leitsch et al. 2007; Schlosser et al. 2013).

From the clinical point of view, it appears that drug resistance is not a common problem in amoebiasis (Table 2). High metronidazole and tinidazole sensitivity was demonstrated in 15 clinical isolates of *E. histolytica* maintained in monoxenic culture medium (Bansal et al. 2004). Intestinal amoebiasis treatment failure is mostly attributed to recurring infection or ignoring the subsequent use of a luminal agent rather than actual drug resistance (Fujishima et al. 2010). However, in vitro development of metronidazole-resistant *E. histolytica* cell lines was successfully achieved. Although the parasite was unable to withstand high metronidazole concentrations tolerated by resistant strains of other protozoan species, it can be manipulated to tolerate drug levels comparable to those found in serum following recommended doses (Wassman et al. 1999). Lower drug susceptibility of an Indian clinical isolate compared to a standard laboratory strain was recently reported (Iyer et al. 2014). These observations together with the indiscriminate use of metronidazole and its poor quality in some less-developed countries, with less than adequate contents of active ingredients, raise concern about development of metronidazole resistance in *E. histolytica*.

Table 2 Drugs used in the treatment of *E. histolytica* and *D. fragilis* with an overview of evidences for treatment failure/drug resistance

Protozoa	Drug	Recommended dose	Evidence of treatment failure/drug resistance	Reference
E. histolytica	Metronidazole	500–750 mg three times a day for 10 days (Kappagoda et al. 2011)	*E. histolytica* can be manipulated to tolerate drug levels comparable to those found in serum following recommended doses	Wassman et al. 1999
			Lower susceptibility of an Indian clinical isolate compared to a standard laboratory strain	Iyer et al. 2014
	Nitazoxanide	500 mg for adults. 7.5 mg/kg for children, twice daily for 3–7 days Rossignol et al. 2001; Zumaquero-Ríos et al. 2013)	31 % of 36 diarrheic patients were not cured	Rossignol et al. 2001
			Among 61 children, 13 % required a second course	Zumaquero-Ríos et al. 2013
D. fragilis	Metronidazole	500 mg three times daily for 10 days (Engsbro et al. 2012)	Of 25 treated cases, ten were not cured	Engsbro et al. 2012
			Of seven treated patients, infection persisted in three cases	van Hellemond et al. 2012
	Secnidazole or ornidazole	Single dose of 30 mg/kg for children 2 g for adults (Girginkardeşler et al. 2003; Kurt et al. 2008)	Of four clinical isolates, one was less susceptible to ornidazole and secnidazole compared to the others	Nagata et al. 2012
	Iodoquinol	650 mg three times a day for 20 days (Kappagoda et al. 2011)	Weak effects on trophozoite counts in vitro	Nagata et al. 2012
	Clioquinol	Three daily doses of 250 mg for 7 days (van Hellemond et al. 2012)	Treatment failure occurred in 17 % of 12 patients	van Hellemond et al. 2012

In contrast to *Giardia*, reduced PFOR activity cannot account for metronidazole resistance in *E. histolytica*. Moreover, no relationship was found between metronidazole sensitivity and nitroreductase mRNA expression and/or the presence of nonsense mutations in *E. histolytica* nitroreductase genes (Pal et al. 2009). Exposure of *E. histolytica* trophozoites to metronidazole induces, within 1 h, modest increase in levels of mRNA coding for the antioxidant enzymes peroxiredoxin and superoxide dismutase. However, this gene overexpression is not accompanied by changes in protein levels (Tazreiter et al. 2008). Establishment of metronidazole resistance in *E. histolytica* is accompanied by pronounced increase in the expression of peroxiredoxin and superoxide dismutase (Samarawickrema et al. 1997; Wassman et al. 1999). Recently, immuno-localization studies revealed that *E. histolytica* trophozoites cope with metronidazole-induced stress by altering the cellular pattern of peroxiredoxin as manifested by recruitment to the cell surface and less nuclear staining in metronidazole-treated compared to untreated cells. These changes were not found in metronidazole-adapted cells (Iyer et al. 2014).

Additional factors may contribute to metronidazole resistance. A marked decrease in the expression of ferredoxin-specific RNA and downregulation of ferredoxin occurs in *E. histolytica* trophozoites that are resistant to high concentrations of metronidazole (Wassman et al. 1999). *E. histolytica* also possesses nitroimidazole reductases which are capable of inactivating metronidazole and detoxification of its radicals. These enzymes were found to confer strong metronidazole resistance to transformed *E. coli* cells (Pal et al. 2009). Drug resistance in *E. histolytica* was also linked to the presence of high levels of a 170-kDa membrane molecule known as P-glycoprotein (PGP), an energy-dependent pump that extrudes drugs from the cells. Elevated levels of PGP are related to transcriptional activation of specific genes and increased mRNA stability (López-Camarillo et al. 2003).

Though mainly approved for use against giardiasis and crytosporidiosis, nitazoxanide was also shown to possess an antiamoebic action (Rossignol et al. 2007). Clinical trials showed great variability in its efficacy. In a randomized, double-blind, placebo-controlled study conducted on adults and children with invasive intestinal amoebiasis in Egypt, nitazoxanide twice daily for 3 days at doses of 500 mg (age>or =12 years), 200 mg (age 4–11 years), or 100 mg (age 1–3 years) achieved a cure rate of 94 % (Rossignol et al. 2007). In another study in Egypt, the same nitazoxanide adult dose produced a parasitological response rate of 69 % among diarrheic patients with *E. histolytica* and/or *Entamoeba dispar* cysts or trophozoites in stool samples (Rossignol et al. 2001). In Mexico, nitazoxanide (7.5 mg/kg of body weight twice daily for 7 days) was used to treat children infected with *E. histolytica/dispar*. A cure rate of 87 % was recorded with successful treatment of all uncured cases after a second drug course (Zumaquero-Ríos et al. 2013).

Exposure of *E. histolytica* trophozoites to nitazoxanide induces cell swelling and distortion of cell shape, redistribution

of vacuoles, plasma membrane damage, and formation of extensive empty areas in the cytoplasm with marked reduction in the percentage of viable parasites (Cedillo-Rivera et al. 2002). Nitazoxanide has been shown to directly inhibit PFOR of *E. histolytica* (Hoffman et al. 2007). In vitro studies documented that nitazoxanide is more active than metronidazole and albendazole against *E. histolytica* isolates. This is related to the fact that activation of nitazoxanide occurs at a less negative redox potential than that required for metronidazole (Adagu et al. 2002). On the contrary, lower efficacy of nitazoxanide compared to metronidazole was observed in experiments conducted in vivo (Becker et al. 2011). Such discrepancy between in vitro and in vivo studies may be related to variation in systemic bioavailability of drugs (Becker et al. 2011). Studies evaluating the antiamoebic efficacy of nitazoxanide versus metronidazole in humans are needed.

It is now well documented that *E. histolytica* is morphologically identical to, but genetically distinct from, two non-pathogenic *Entamoeba* species, namely *E. dispar* and *E. Moshkovskii* (Ali et al. 2008). The relative distribution of these species shows wide geographic variations (Ngui et al. 2012; Hooshyar et al. 2012). However, most clinical trials that have been conducted so far relied on microscopic diagnosis without including specific tests for differentiation of *Entamoeba* species (Gonzales et al. 2009). Interestingly, one in vitro study documented greater susceptibility of *E. histolytica* clinical isolates to antiamoebic drugs compared to the *E. dispar* isolates (Bansal et al. 2004). Further studies are warranted to reassess the efficacy of antiamoebic drugs on the basis of pathogenecity of the infecting *Entamoeba* species.

D. fragilis

D. fragilis is a trichomonad parasite originally thought to be uncommonly encountered in human fecal samples. The fragile nature of *D. fragilis* trophozoites, the sole parasite form, makes it easily missed in routine stool examination. Prompt stool examination or fixation, cultivation, and molecular methods greatly enhance its detection. Using such diagnostic techniques, prevalence rates exceeding that of *G. intestinalis* have been reported in various areas and population groups (Stark et al. 2010; Barratt et al. 2011). This parasite has been associated with acute and chronic gastrointestinal symptoms and has been also found in asymptomatic individuals (Stark et al. 2010). Several studies investigated the association of *D. fragilis* with inflammatory bowel diseases but the results are conflicting (Yakoob et al. 2010; Jimenez-Gonzalez et al. 2012).

Metronidazole in a dose of at least 500 mg three times daily for 10 days is recommended as a primary therapeutic regimen for *D. fragilis*. Treatment failure occurs in about 40 % of cases (Engsbro et al. 2012). 5-NI derivatives with long half-life such as secnidazole and ornidazole (each in a dose of 30 mg/kg for children, and 2 g for adults) achieve cure rates over 90 % when used in a single-dose regimen (Girginkardeşler et al. 2003; Kurt et al. 2008). An in vitro study evaluating the efficacy of 5-NI derivatives against four *D. fragilis* clinical isolates documented that all of the isolates were equally susceptible to metronidazole but one isolate was less susceptible to ornidazole and secnidazole compared to the others (Nagata et al. 2012). A retrospective cohort study showed that paromomycin in three daily doses of 500 mg for 7–10 days had superior eradication rate (98 %) compared to the same regimen of metronidazole (57 %) (van Hellemond et al. 2012).

Nitazoxanide was shown to have minimum lethal concentration on *D. fragilis* trophozoites grown in culture media, suggesting that it might be a suitable treatment option (Nagata et al. 2012). Another frequently prescribed drug in dientamoebiasis is clioquinol, an iodoquinol structurally related compound. In a retrospective study, analysis of clinical data of *D. fragilis*-infected children revealed that parasitologic success, defined by a negative post-treatment PCR, was 58.1 % with clioquinol treatment versus 52.4 % with metronidazole treatment with no statistically significant difference (Schure et al. 2013).

A study on a limited number of patients suggested the use of iodoquinol for the treatment of *D. fragilis.* However, the spontaneous clearance of infection, which occurs in about 40 % of patients, makes interpretation of the study quite difficult (Stark et al. 2010). In a case report, a patient with *D. fragilis* infection failed to respond to metronidazole but was successfully treated with doxycycline and iodoquinol (Stark et al. 2009). However, weak effects of iodoquinol and tetracycline on trophozoite counts were demonstrated in vitro, indicating that their use in patients with *D. fragilis* should be reassessed (Nagata et al. 2012). A summary of drugs prescribed for treatment of *D. fragilis* with an overview of studies documenting treatment failure or drug resistance is provided in Table 2.

B. coli

Balantidiosis is an uncommon human zoonotic infection acquired mainly via the fecal-oral route from the normal host, the pig. Infected individuals may be asymptomatic or may develop a fulminant infection with bloody and mucus-containing diarrhea (Schuster and Ramirez-Avila 2008). Balantidiosis is usually treated with either tetracyclines 500 mg four times a day or metronidazole 750 mg three times a day for 5 to 10 days. The organism responds well to the recommended antimicrobial agents with no reports on *B. coli* drug resistance or treatment failure (Farthing et al. 2009; Bellanger et al. 2013) (Table 3). Research on *B. coli* has been generally sparse with no attempts to develop drug-resistant mutants.

Table 3 Drugs used in the treatment of *B. coli*, *B. hominis*, and intestinal coccidia with an overview of evidences for treatment failure/drug resistance

Protozoa	Drug	Recommended dose	Evidence of treatment failure/ drug resistance	Reference
B. coli	Tetracyclines	500 mg four times a day for 10 days (Farthing et al. 2009)	–	–
	Metronidazole	750 mg three times a day for 5 to 10 days (Schuster and Ramirez-Avila 2008; Bellanger et al. 2013)	–	–
B. hominis	Nitazoxanide	500 mg orally twice a day for 3 days (Kappagoda et al. 2011)	Treatment with a combination of nitazoxanide, furazolidone, and secnidazole failed in five patients	Roberts et al. 2014
	Sulfamethoxazole-trimethoprim	30 mg/kg/day and 6 mg/kg/day, respectively, in two doses per day 7 days (Heyland et al. 2012)	Eradication rate in 37 patients was 35 %	Heyland et al. 2012
	Metronidazole	400 mg three times daily for 7–10 days (Roberts et al. 2014)	Subtype seven isolates are more resistant to metronidazole compared to subtype 4 isolates	Mirza et al. 2011
			Eradication rate was 44 % in patients who failed to respond initially to sulfamethoxazole-trimethoprim	Heyland et al. 2012
			Infection persisted in eight treated patients	Roberts et al. 2014
Cryptosporidium spp.	Nitazoxanide	500 mg twice a day for 3 days (Kappagoda et al. 2011)	–	–
C. cayetanensis	Sulfamethoxazole-trimethoprim	800 and 160 mg, respectively, twice daily for 7–10 days Kappagoda et al. 2011)	–	–
	Ciprofloxacin	500 mg orally twice a day for 7 days (Kappagoda et al. 2011)	–	–
C. belli	Sulfamethoxazole-trimethoprim	800 and 160 mg, respectively, twice daily for 10 days (Kappagoda et al. 2011)	Drug resistance was proposed to be one of the causes of relapse in eight AIDS patients despite immune reconstitution	Boyles et al. 2012
	Ciprofloxacin	500 mg twice a day for 7 days (Kappagoda et al. 2011)	–	–

B. hominis

B. hominis is one of the most common microscopically detected organisms in human fecal samples (Roberts et al. 2013; El Safadi et al. 2014). On the basis of phylogenic data, it is classified as an unusual member of a complex group of protists called stramenopiles (Hoevers and Snowden 2005). The pathogenic potential of *B. hominis* is a matter of debate, with data suggesting that it might be subtype dependent (Roberts et al. 2013). *B. hominis* has been detected in asymptomatic individuals as well as in patients with diarrhea, abdominal pain, flatulence, or vomiting. About 20 % of infections may be eradicated spontaneously. Specific treatment of *B. hominis* is recommended in patients with gastrointestinal symptoms and high parasite burden especially when no other etiologic agent can be identified (Coyle et al. 2012; van Hellemond et al. 2013).

Several antimicrobial agents have been described for treatment of *B. hominis* but without a consensus on the most effective one (Table 3). Metronidazole was the suggested drug of choice. Two clinical trials confirmed the efficacy of metronidazole treatment in inducing clinical remission and parasitologic eradication (Nigro et al. 2003; Dinleyici et al. 2011). Likewise, there was some evidence that nitazoxanide, trimethoprim-sulfamethoxazole, and the probiotic *Saccharomyces boulardii* are suitable alternatives for treatment of *Blastocystis* infection (Rossignol et al. 2005; Dinleyici et al. 2011). In one study, paromomycin produced even a significantly higher parasitological eradication rate in comparison to metronidazole (77 versus 38 %) (van Hellemond et al. 2013). A rapid throughput assay demonstrated that metronidazole-resistant *Blastocystis* isolates were sensitive to ornidazole, furazolidone, mefloquine, and trimethoprim-sulfamethoxazole. The study recommended the use of these drugs as effective therapeutic alternatives in cases of metronidazole treatment failure (Mirza et al. 2011).

In contrast to the previous studies, a recent study highlighted lack of efficacy of several commonly used antimicrobial

regimens in the treatment of *Blastocystis* spp. infection. Persistence of symptoms and failure of parasite eradication were observed in 18 patients following treatment with either metronidazole, iodoquinol, or a combination of nitazoxanide, furazolidone, and secnidazole (Roberts et al. 2014). Another study reported that eradication rates achieved with trimethoprim-sulfamethoxazole or metronidazole in *Blastocystis*-positive children were not significantly different from the spontaneous clearance rate observed in the placebo-treated group (Heyland et al. 2012).

Susceptibility to antimicrobial agents differs between subtypes and even between isolates of the same subtype. *Blastocystis* isolates belonging to subtype 7 exhibit extensive variations in metronidazole susceptibility but generally they are more resistant to metronidazole compared to subtype 4 parasites. Subtypes 4 and 7 are resistant to paromomycin (Mirza et al. 2011). A case report illustrated that a combination of paromomycin and metronidazole resulted in resolution of symptoms and eradication of the parasite in a patient infected with *Blastocystis* subtype 2 (Vogelberg et al. 2010).

As has been demonstrated for *Giardia*, metronidazole-resistant *Blastocystis* strains had lower pathogenic potential, evidenced by lower tolerance to nitrosative stress and impaired ability to attach to human enterocytes (Tejman-Yarden et al. 2011; Wu et al. 2014). Studies investigating drug targets and mechanisms of resistance in *Blastocystis* species are limited. The enzyme PFOR responsible for metronidazole activation in other organisms has been identified in *B. hominis* (Denoeud et al. 2011). Yet, correlation of its level to metronidazole sensitivity has not been explored in *Blastocystis*. Development of two metronidazole-resistant *Blastocystis* lines were achieved by exposing the parasite to incremental increases in drug concentration, by repeated periods of exposure to 4 μM metronidazole alternating with periods of no exposure or by a combination of the two methods. The resulting isolates were not similarly susceptible to other antiprotozoal drugs suggesting development of variable mechanisms of drug resistance (Dunn et al. 2012). Further in-depth drug sensitivity assays and large-scale clinical trials are still needed. In such studies, incorporating suitable methods for subtyping *Blastocystis* isolates is indispensible.

Intestinal coccidia

The coccidian parasites, *Cryptosporidium* spp., *Cyclospora cayetanensis*, and *Cystoisospora belli* (previously known as *Isospora belli)* are well-recognized agents of enteric disease in man. They induce severe protracted gastrointestinal illness in immunosuppressed individuals and asymptomatic or mild self-limited illness in persons with intact immunity. *Cryptosporidium* spp. is also an important contributor to childhood diarrhea and malnutrition in developing countries (Farthing et al. 2009). Despite trials of several antimicrobial agents, treatment options for cryptosporidiosis are still limited. This intrinsic drug resistance is attributed to the unique parasite location in the host cell, being within an intracellular but extracytoplasmic parasitophorous vacuole. Moreover, the parasite posses transport proteins which act as efflux pumps that transport drugs out of the parasite (Bonafonte et al. 2004). Expression of mRNA of these transporters was detected in infected cell cultures at different time points corresponding to the development of the sexual and asexual stages of the parasite (Benitez et al. 2007).

The current optimal therapy for cryptosporidiosis is nitazoxanide. It reduces parasite load and shorten the duration of cryptosporidial diarrhea in immunocompetent individuals (Hussien et al. 2013). In contrast to extracellular protozoa, the nitro group is not required for the activity of nitazoxanide against *Cryptosporidium parvum*, implying a different mechanism of action (Gargala et al. 2010). Treatment of immunosuppressed patients is remarkably difficult even with higher doses and longer duration of treatment (Kelly 2011). Paromomycin and azithromycin are partially effective, and they are not routinely prescribed as anti-cryptosporidial agents in practice (Kappagoda et al. 2011; Hussien et al. 2013). The use of highly active antiretroviral therapy (HAART) in HIV-infected patients is associated with dramatic improvement of cryptosporidial diarrhea. Improvement is likely to result from immune reconstitution as well as inhibition of the parasite proteases (Alfonso and Monzote 2011).

Infections with *C. cayetanensis* or *C. belli* are best treated with oral co-trimoxazole (sulfamethoxazole 800 mg and trimethoprim 160 mg) twice daily for 7–10 days. This regimen eliminates the parasite from stool and interrupts diarrhea in most cases. HIV-infected patients should receive larger doses followed by secondary prophylactic doses for extended periods. Ciprofloxacin is anther therapeutic option suitable for patients who cannot tolerate co-trimoxazole (Kappagoda et al. 2011; Verdier et al. 2000). This drug targets the parasite DNA gyrase and inhibits the apicoplast DNA replication leading to parasite death (Wiesner et al. 2008). A 7-day regimen with nitazoxanide has been proposed as an effective alternative for persons having a history of sulfa allergy who do not respond to therapy with ciprofloxacin (Zimmer et al. 2007).

To date, there is no direct clinical or biochemical evidence for emergence of drug resistance in human intestinal coccidia (Table 3). Severe malabsorption and cholangitis in a patient infected with *C. belli* resulted in sub-therapeutic drug levels in the plasma and bile and predisposed to treatment failure. The good response to intravenous rather than orally administered drugs excluded initially suspected resistance (Bialek et al. 2001). Results of a case series study involving eight patients suggested that resistance to co-trimoxazole might be a factor, among others, responsible

for relapsing *C. belli* infections in AIDS patients despite immune reconstitution (Boyles et al. 2012).

Conclusion

Accumulating reports on treatment failure and in vitro susceptibility testing of clinical isolates suggest the existence of resistance to commonly used drugs in some isolates of *G. intestinalis*, *B. hominis*, and *D. fragilis*. On the other hand, there is still no strong evidence for emergence of drug resistance in *E. histolytica*, *B. coli*, and intestinal coccidia. Some advances have been achieved in understanding the biochemical and molecular basis of such resistance. Nevertheless, association of drug susceptibility/resistance with certain geographic isolates or genotypes is a major issue that still deserves further consideration. Rational use of available antiparasitic agents is essential to maintain their usefulness. Pharmacoepidemiological studies, repeated monitoring of drugs efficacy, and surveillance for possible emergence of resistance are essential. Search for effective alternative therapeutics is warranted to be used if spread of drug-resistant strains threatens the containment of infections with intestinal protozoa.

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