Evaluation of a multiplex Real-Time PCR assay for the diagnosis of human intestinal protozoa in Azienda Ospedaliero Universitaria Pisana.

R. Fonnesu[1], R. Fais[1], G. Trillocco[1], E. Balestri[1], A. Cara[1], M. Prato[1], A. Lupetti[1,2], V. Mangano[1,2]

[1]Dipartimento di Ricerca Traslazionale e delle Nuove Tecnologie in Medicina e Chirurgia, Università di Pisa;
[2]SD Microbiologia Universitaria, AOUP

Introduction: The gold standard for diagnosis of human infection with intestinal protozoa is microscopic examination of stool specimens following concentration. However, this method has limited sensitivity at low parasite densities, requiring observation of multiple specimens and specific staining. Furthermore, morphologically identical taxa with different pathogenicity such as species of the *Entamoeba histolytica* complex cannot be identified. This study aims to evaluate a new multiplex Real-Time PCR assay (Allplex™ GI-Parasite Assay, Seegene) for the detection of DNA of *Giardia duodenalis*[1], *E. histolytica* sensu stricto[2], *Cryptosporidium* spp.[3], *Cyclospora cayetanensis*[4], *Diantamoeba fragilis*[5] and *Blastocystis hominis*[6] (**Figure 1**).

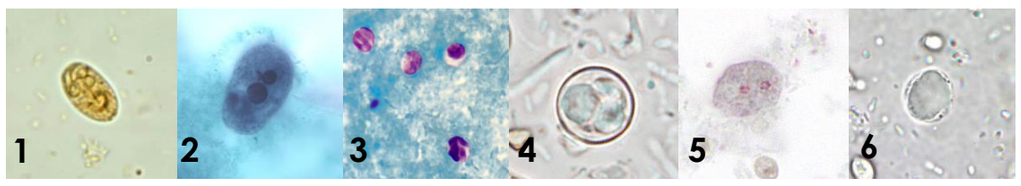
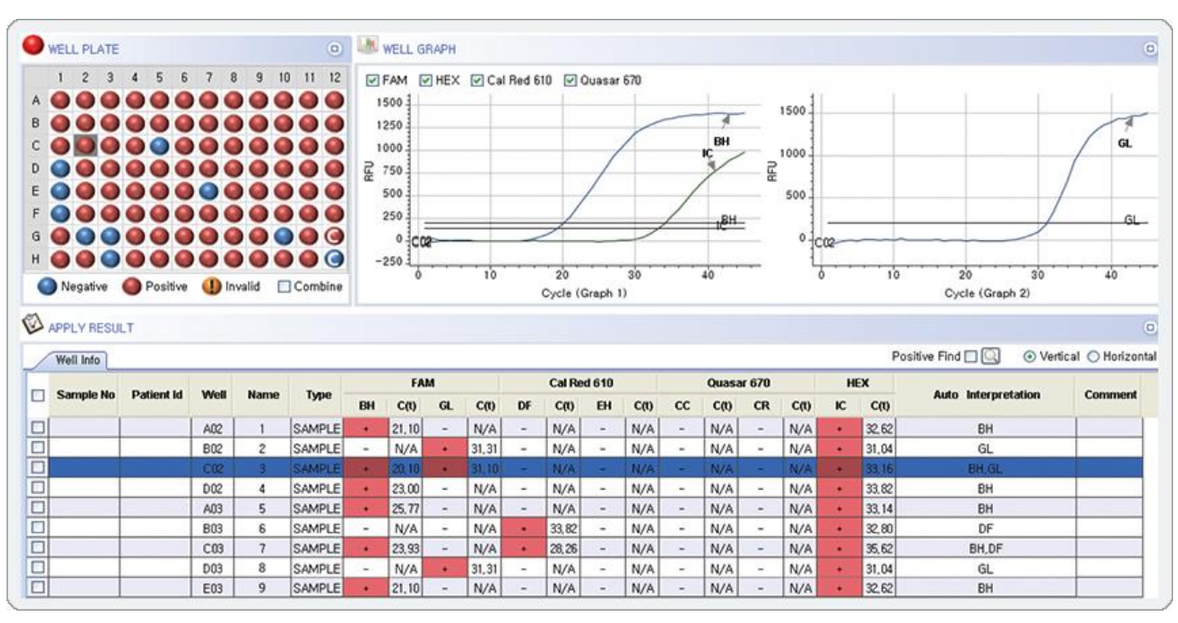


Figure 1: Microscopy images of intestinal protozoa (from CDC- DPDX website)

Materials and Methods: Multiplex Real-Time PCR (**Fig 2a**) was performed on:
1. positive controls stool samples (N=9) to evaluate performance in alternative storage conditions (EtOH, formalin and Ecofix, RT, 4 °C, -20 °C,) from the validated one;
2. stool samples from patients (N=100) previously tested with an immunochromatographic test (ImmunoCard STAT! CGE, Meridian Bioscience **Figure 2b**) for antigen detection of *G. duodenalis, E. histolytica* complex and *Cryptosporidium* spp. to compare results of the two methods;
3. stool samples from patients (N=54) following introduction of the new method in the diagnostic routine to assess prevalence of infection.

2a



Sample No	Patient Id	Well	Name	Type	FAM		Cal Red 610		Quasar 670		HEX		Auto Interpretation	Comment
					BH	C(t)	GL	C(t)	CC	C(t)	IC	C(t)		
		A02	1	SAMPLE	+	21,10	-	N/A	-	N/A	+	32,62	BH	
		B02	2	SAMPLE	-	N/A	+	31,31	-	N/A	+	31,04	GL	
		C02	3	SAMPLE	+	20,10	+	31,10	-	N/A	+	33,16	BH,GL	
		D02	4	SAMPLE	+	23,00	-	N/A	-	N/A	+	33,82	BH	
		A03	5	SAMPLE	+	25,77	-	N/A	-	N/A	+	33,14	BH	
		B03	6	SAMPLE	-	N/A	-	N/A	+	33,82	+	32,80	DF	
		C03	7	SAMPLE	+	23,93	-	N/A	+	28,26	+	35,62	BH,DF	
		D03	8	SAMPLE	-	N/A	+	31,31	-	N/A	+	31,04	GL	
		E03	9	SAMPLE	+	21,10	-	N/A	-	N/A	+	32,62	BH	

2b

Figure 2a: Allplex™ GI-Parasite Assay results interpretation software;
Figure 2b: ImmunoCard STAT! CGE, Meridian Bioscience device.

Results 1: Preservation in EtOH showed 100% sensitivity and was the best alternative to freezing, allowing storage at RT and thereby avoidance of the cold chain (**Figure 3**).

Figure 3 : Results obtained from testing positive controls, preserved in different condition.

G. duodenalis and *E. histolitica ss*. samples give positive results in every condition tested, while for both *Cryptosporidium spp*. and *B. hominis* formalin preservation results in a negative outcome. *D. fragilis* were detected only when preserved at -20°C or in ethanol.

Results 2: Compared to ICT, multiplex Real-Time PCR was equally sensitive but allowed to discriminate between *E. dispar/moskovskii* and *E. histolytica* ss: in fact, 2 positive results for *E. histolytica* complex given by ICT weren't confirmed by multiplex Real-Time PCR.

Results 3: Prevalence of infection are shown in **Figure 4.**

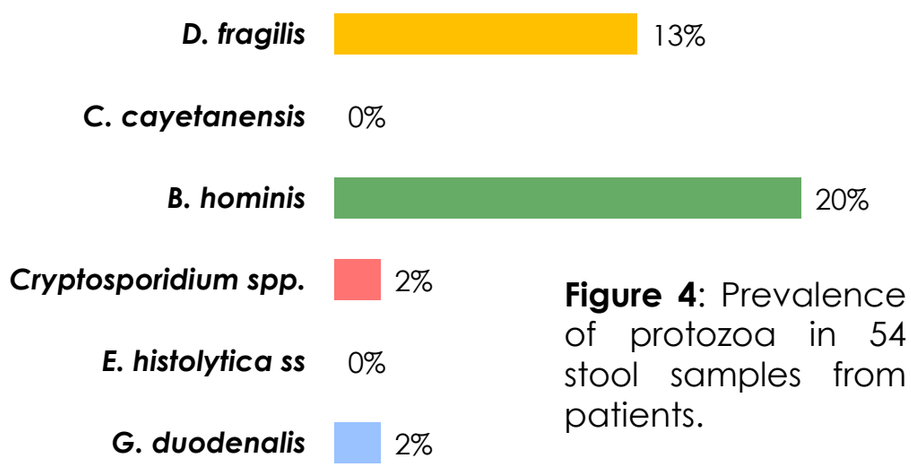


Figure 4: Prevalence of protozoa in 54 stool samples from patients.

The observed prevalence of *B. hominis* and *D. fragilis* is in line with data from Italy and other European countries reported in the literature. Detection of these protozoa is fundamental to gain new insights into their uncertain pathogenetic role.

In particular, *B. hominis* seems to have a pathogenic role in immunodeficient patients (Gabrielli et al., 2020 Parasite Epidemiol Control), as HIV+ subjects: by our data, 2 of the 10 patients positive for *B. hominis* (20%), were found to be HIV+.

Conclusions: Multiplex Real-Time PCR is a useful diagnostic and investigation tool, allowing to detect 6 different protozoa species at the same time with higher specificity and sensibility than previous used method.

Acknowledgments: The authors thanks Simone Cacciò e Anna Rosa Sanella from Istituto Superiore di Sanità and Francesca Perandin from IRCSS Ospedale Sacro Cuore Don Calabria for positive controls samples, and all of the laboratory technicians for the help provided.