DIARRHOEA-CAUSING ENTERIC PROTIST SPECIES IN INTENSIVELY AND EXTENSIVELY RAISED PIGS (SUS SCROFA DOMESTICUS) IN SOUTHERN SPAIN. PART II: ASSOCIATION WITH HEPATITIS E VIRUS SUSCEPTIBILITY

Diarrhoea-causing enteric protist species in intensively and extensively raised pigs (*Sus scrofa domesticus*) in Southern Spain. Part II: Association with Hepatitis E virus susceptibility

Running Head: Intestinal protists and HEV susceptibility in farmed pigs

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SUMMARY

Enteropathogenic parasites can infect a wide range of mammals, including humans, supposing an important zoonotic risk. Hepatitis E virus (HEV) is an emerging foodborne pathogen of increasing public health relevance, affecting both human and animal populations. Because both microorganisms share faecal-oral transmission route they may constitute an excellent model to evaluate the interplay between them. Thus, we aim to evaluate the viral-parasite interactions at the enteric interface in swine. We included pigs of two different breeds farming in South Spain under different production systems. We compared the HEV prevalence by the presence of *Giardia duodenalis*, *Cryptosporidium* spp., *Balantioides coli*, *Blastocystis* sp., and *Enterocytozoon bieneusi* in faecal samples. The HEV prevalence was 13.1 (62 out 475, 95% CI: 10.2–16.4). Those pigs infected with *Cryptosporidium* spp.

showed a higher prevalence of HEV (30.8% *vs.* 12%; $p = 0.012$). In the same way, animals bearing *E. bieneusi* seem to have a higher rate of HEV infection (24.2% *vs.* 12.2%; $p = 0.06$). According to their location in the gut, animals bearing intracellular enteroparasites showed a higher HEV prevalence than those uninfected (29.6% *vs.* 12.7%; $p = 0.038$), meanwhile those carrying extracellular enteroparasites had a lower likelihood to be infected by HEV than those uninfected (12.1% *vs.* 23.1%; $p = 0.071$). Those animals bearing both type of enteroparasites showed a similar prevalence of HEV infection than those exhibiting negative for both (20.8% *vs.* 26.1%; $p = 0.763$). Our study provides evidence that intracellular and extracellular enteroparasites modulate the susceptibility to HEV infection in pigs. Meanwhile, the presence of extracellular enteroparasites shows a protective effect on the risk of HEV acquisition in swine, whereas intracellular enteroparasites seems to have the opposite effect, favouring the HEV infection.

KEYWORDS: *Giardia duodenalis*; *Cryptosporidium*; *Balantioides coli*; *Blastocystis*; *Enterocytozoon bieneusi*; Large White pig; Iberian pig; Spain; transmission; Hepatitis E; modulation; coinfection.

INTRODUCTION

Coinfection by multiple parasites and viruses is frequent in animals (Yon et al., 2019; VanderWaal et al., 2018). In this situation an interaction process between cohabitant microorganisms might occur through physical competition or immune-mediated response. This competition may lead to favour or impair the host susceptibility to microorganisms, conditioning the disease progression. This complex interaction could modulate the epidemiology of several viruses and parasites, including their pathogenicity and virulence. Despite the potential large implication that the knowledge of this modulation between parasites and viruses might implies for our understanding of the course of infections and the occurrence and extent of clinical manifestations and pathology, the interplay among them remains poorly studied (Chudnovskiy et al., 2016; Mabbott, 2018).

Enteropathogenic parasites can infect a wide range of mammals, including humans, supposing an important zoonotic risk (Ramirez et al., 2018). In the same way, Hepatitis E virus (HEV) is an emerging foodborne pathogen of increasing public health relevance, affecting both human and animal populations (Nimgaonkar et al., 2017). Because both HEV and enteropathogenic parasites share faecal-oral transmission route among animals through contact with infected hosts or their faecal material, they may constitute an excellent model to study the possible interplay between parasites and viruses. In this sense, in a seminal study conducted by our research group, we reported that the enteric extracellular protists *Giardia duodenalis* and *Blastocystis* sp., have a protective effect on HEV acquisition in pigs and sympatrically-living wild boars (Rivero-Juárez et al., 2020). This innovative finding needed further evaluation and confirmation in an independent cohort and population. By this reason and taking advantage of the animal population available for the Part I of this manuscript (see co-submission Dashti et al., 2021), we aim to expand the knowledge on viral-parasite interactions at the enteric interface in domestic pig.

MATERIALS AND METHODS

Ethical statement.

This study was carried out in accordance with Spanish legislation guidelines (RD 8/2003) and with the International Guiding Principles for Biomedical Research Involving Animals issued by the Council for International Organization of Medical Sciences and the International Council for Laboratory Animal Science (RD 53/2013).

Study area and sampling.

The study area and sampling strategy was fully described in the first paper of this series (Dasthi et al., 2021).

Molecular detection of HEV.

For the detection of HEV, RNA was extracted from 400 µL of serum using the QIAamp Mini Elute Virus Spin kit (QIAgen, Hilden, Germany) by an automated procedure (QIAcube, QIAgen). The purified RNA was eluted in a 50-µL volume. For RT qPCR, the QIAgen One-Step PCR Kit (QIAgen, Hilden, Germany) was used for 25 µL of template (50 µL of reaction volume) following a pan-genotypic in-house protocol targeting the ORF3 region developed and validated by our group with a detection limit set at 21 IU/mL (Frias et al., 2021). Samples yielding a cycle threshold value ≤40 were considered positive. HEV infection was defined as detectable HEV RNA in serum.

Molecular detection of enteric protists

Results about prevalence of both extracellular and intracellular enteroparasites were taken from Part I of this manuscript (Dasthi et al., 2021). A thorough description about the procedures applied for molecular detection and characterization of extracellular (*G. duodenalis, Blastocystis* sp.*,* and *Balantioides coli*) and intracellular (*Cryptosporidium* spp.*,* and *Enterocytozoon bieneusi*) protist species can be found there.

Statistics analysis

The prevalences of HEV, *G. duodenalis, Blastocystis* sp.*, B. coli*, *Cryptosporidium* spp.*,* and *E. bieneusi* in the global and different pig populations investigated were calculated using the proportion of positive samples with respect to the total number of samples examined with 95% confidence interval (95% CI). In Iberian pigs, the prevalence was also calculated by farming management system (intensive *versus* extensive). We used the χ^2 test when the expected values of at least 80% of the cells in a 2x2 contingency table to be greater than 5. When these conditions were not

verified, we compared the qualitative variables via the Fisher's exact test. To evaluate the possible interaction between HEV and the protist enteroparasites analysed in the study, a multivariate logistic regression model was constructed considering HEV infection as outcome variable. For this, three models were constructed: i) including the whole population, ii) including only Large White pigs, and iii) including only Iberian pigs. Odds ratios (OR) were calculated with 95% CIs. The Hosmer-Lemeshow test was used to test for goodness of fit for the logistic regression model. The statistical significance was established at a p-value of less than 0.05. Analyses were carried out using SPSS statistical software package version 18.0 (IBM Corporation, Somers, NY, USA). GraphPad Prism, version 6 (Mac OS X version; GraphPad Software; San Diego, California, USA) was used to Figures designed.

RESULTS

Study population and microorganism's prevalence

Four-hundred and seventy-five animals constituted the study population. The overall prevalence of HEV in the swine population surveyed was 13.1 (62 out 475, 95% CI: 10.2–16.4). All strains were consistent with genotype 3f. HEV was significantly less prevalent (p = 0.004) in intensively raised Iberian pigs (5.7%) than in their extensively raised counterparts (17.2%) or in intensively raised Large White pigs (15.2%). This finding was reported previously (Lopez-Lopez et al., 2018).

Interaction between HEV and enteroparasites individually

As it was described previously (Dasthi et al., 2021), the prevalence of *G. duodenalis* was 10.7% (51 out 475), 47.8% for *Blastocystis* sp. (227 out 475), 45.5% for *B. coli* (216 out 475), 5.5% for *Cryptosporidium* spp. (26 out 475), and 6.9% for *E. bieneusi* (26 out 475). In Table 1, we show the prevalence of HEV infection by the five enteroparasites analysed in the study. Those animals infected

with *Cryptosporidium* spp. showed a higher prevalence of HEV (30.8% *vs.* 12%; $p = 0.012$). In the same way, animals bearing *E. bieneusi* tended to have a higher rate of HEV infection, almost achieving statistically significant (24.2% *vs.* 12.2%; $p = 0.06$).

Introducing all the variables in a multivariate analysis, the presence of *Cryptosporidium* spp. was associated with a higher risk to be infected by HEV (Table 2).

Effect of extracellular and intracellular enteroparasites on the HEV prevalence

For this analysis, *G. duodenalis*, *Blastocystis* sp., and *B. coli*, were grouped as extracellular parasites, meanwhile *Cryptosporidium* spp., and *E. bieneusi* were grouped as intracellular parasites. The rate of HEV infection was higher in those animals bearing at least one intracellular enteroparasite than in those uninfected individuals (11.9% *vs.* 24.4%; p = 0.032). When analysed by breed and farming system, Large White pigs carrying intracellular enteroparasites showed a higher HEV infection rate than animals not carrying these protists (Figure 1). In contrast, those Large White pigs bearing extracellular parasites showed a trend to have a lower prevalence of HEV infection (Figure 1), almost reaching statistical significance. Because of the low number of Iberian pigs bearing intracellular enteroparasites ($n = 4$), this comparison was rule out.

To analyse the possible additive or agonist effect between extracellular and intracellular enteroparasites in Large White pigs, animals were sorted in four categories: i) negative for both, ii) only positive for extracellular enteroparasites, iii) only positive for intracellular enteroparasites, and iv) positive for both. Those animals bearing intra- and extracellular enteroparasites showed a similar prevalence of HEV infection than those exhibiting negative results for both (20.8% *vs.* 26.1%; $p = 0.763$) (Figure 2). In contrast, the prevalence of HEV was significantly higher among those animals only bearing intracellular enteroparasites compared to those bearing only extracellular parasites (50.0% *vs.* 9.2%; $p = 0.055$) (Figure 2). Nevertheless, it should be noted that because of the low number of individuals infected by intracellular parasites only, this direct comparison analysis could lack sufficient statistical power.

DISCUSSION

The interaction between extracellular enteroparasites and susceptibility to viral infection has been suggested by our group and others (Bilenko et al., 2004; Rivero-Juarez et al., 2020). In this sense, we have previously reported that *G. duodenalis* and *Blastocysts* sp. seem to modulate the prevalence of HEV infection in swine (Rivero-Juarez et al., 2020). In that survey we hypothesized that the simultaneous presence of both extracellular enteroparasites might confer protection, by a still unknown mechanism, against HEV infection. In the present study, we investigated further this possible association in an independent pig population, including other farming systems (intensive) and breeds (fast-growing Large White pigs). Confirming our previous findings, this study shows a clear interaction between extracellular enteroparasites and susceptibility to HEV infection in pigs. Remarkably, our study provides scientific ground for a novel modulatory effect involving intracellular enteroparasites. Meanwhile, the presence of the extracellular enteroparasites shows a protective effect on the risk of HEV acquisition, whereas intracellular enteroparasites seems to have the opposite effect, favouring the HEV infection. This effect can be clearly confirmed by the fact that animals only infected by extracellular enteroparasites showed a lower rate of HEV infection than animals only infected by intracellular enteroparasites. These findings suggest a strong interaction between enteroparasites and HEV at the enteric interface.

Interplay between virus and enteroparasites can be explained by two mechanisms: by a cross immune impair or by mechanical competition. In the case of intracellular enteroparasites, the effect on HEV infection increased susceptibility could be related with the induction of immune evasion by these enteroparasites. A key component of immune response against HEV, *Cryptosporidium* and microsporidia is IFN gamma (IFNγ). This cytokine produced during the infection is essential for controlling parasite´s infection, increasing the STAT-1 transcription factor at intestinal epithelial cells, and leading to the expression of interferon-stimulated genes (ISGs) (Laurent et al., 2017). In the same way, IFNγ production by dendritic cells seems to be essential for parasite control (Moretto et al.,

2007). Immune response against HEV and infection control are also related with IFNγ production by CD8+ T cells (Bremer et al., 2021). Because of this, both microorganisms employed as immune evasion strategy the blockage of IFNγ production. *Cryptosporidium* reduces STAT-1 production, impairing the immune response and allowing the parasite replication (Choudhry et al., 2009). By its part, the HEV open-reading frame 1 (ORF1) and 2 (ORF3), inhibit the NF-KB JAK-STAT and JNK-MAPK pathways induced by TNF-α and IFNγ, promoting viral escape and, consequently, viral replication and disease progression (Lei et al., 2018; Li et al., 2019). Because this interplay mechanisms between HEV and intracellular enteroparasites, the immune evasion triggered by the infection of any of them could favour the co-infection of the other. In addition, other mechanism that could favours the HEV infection is the disruption of the enteric layer as a consequence of intracellular infection of enterocytes by these enteroparasites, leading to a loss of barrier function (Certad et al., 2017). In this sense, a study conducted in cattle in India suggests that initial primary infection with *Cryptosporidium* spp. leads to severe epithelial damage allowing secondary infections over time (Brar et al., 2017).

On the other hand, the mechanism whereby extracellular enteroparasites decrease the susceptibility to HEV infection might be also immune-related. Thus, immune-mediated process has been also described in extra-intestinal infections by trematode parasites such as *Schistosoma* spp. (Bullington et al., 2021). This helminth infection is associated with a worst prognosis in those individuals co-infected with human immunodeficiency virus or hepatitis C virus, a higher reactivation rate among Herpesvirus-infected patients, or a lower likelihood to reach immunity after vaccination against measles and hepatitis B virus (Bullington et al., 2021). This effect is also related with impairment of the immune system (Bullington et al., 2021), characterized by a decrease on Th1 response. In contrast, this effect is associated with a subsequent increase on Th2 responses, which lead to a better immune control. In this sense, patients with influenza infection showed a lower rate of mortality and morbidity when were co-infected with *Schistosoma* parasites (Bullington et al., 2021). In the case of extracellular enteroparasites, such as *G. duodenalis* or *Blastocystis* sp., we have previously discussed the possible indirect effect of an immune-mediated mechanism in protecting the

host against HEV infection (Rivero-Juarez et al., 2020). In this sense, during the early stage of *G. duodenalis* infection, there is an early induction of Th1 response (increased levels of IFN-γ, IL-1β, IL-6, and TNF-α) followed by a predominant Th2 response (Serradell et al., 2018). In the same way, it was demonstrated that *Blastocystis* sp. (ST1-ST5) produces an upregulation of Th2 cytokines (IL-6, IL-8, and TGF-β) (Kumarasamy et al., 2013). Nevertheless, these protist enteroparasites might also be acting as mechanical barrier, limiting the introduction of microorganism (such as HEV) in the enterocytes, therefore decreasing the rate of the infection or associated comorbidities. This mechanism has been previously suggested by others to explain the less severe outcome of children infected by rotavirus when they were also coinfected with *G. duodenalis* (Bilenko et al., 2004). Independently of the mechanism of action, the modulation effect of extracellular and intracellular enteroparasites has a great value and could be useful to design preventive measures for HEV at farm level.

CONCLUSIONS

Results reported here show an interaction between protist enteroparasites and HEV in swine. The presence of the extracellular enteroparasites *Giardia duodenalis*, *Blastocystis* sp., and *Balantioides coli*, might protect against HEV infection in this species, significantly reducing its prevalence at reservoir level. In contrast, the intracellular enteroparasites *Cryptosporidium* spp., and *Enterocytozoon bieneusi* favour the HEV infection. These findings insight an interesting interplay between viruses and enteroparasites at intestine interface.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available within the main body of the manuscript.

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FIGURE CAPTIONS

Figure 1. Hepatitis E virus prevalence in Large White pigs according to extracellular (*Giardia duodenalis*, *Blastocystis* sp., and *Balantioides coli*) and intracellular (*Cryptosporidium* spp., and *Enterocytozoon bieneusi*) enteroparasites.

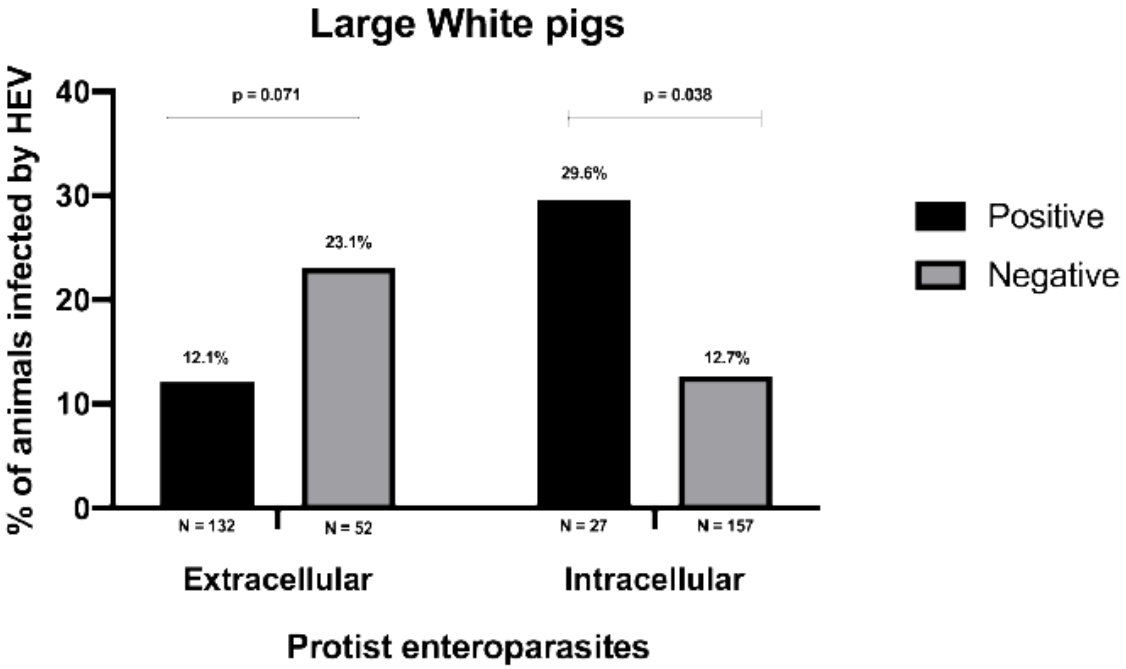

Figure 2. Comparison of Hepatitis E virus prevalence in Large White pigs carrying neither extracellular (*Giardia duodenalis*, *Blastocystis* sp., and *Balantioides coli*) nor intracellular (*Cryptosporidium* spp., and *Enterocytozoon bieneusi*) enteroparasites, only extracellular or intracellular enteroparasites, or both intracellular and extracellular enteroparasites.

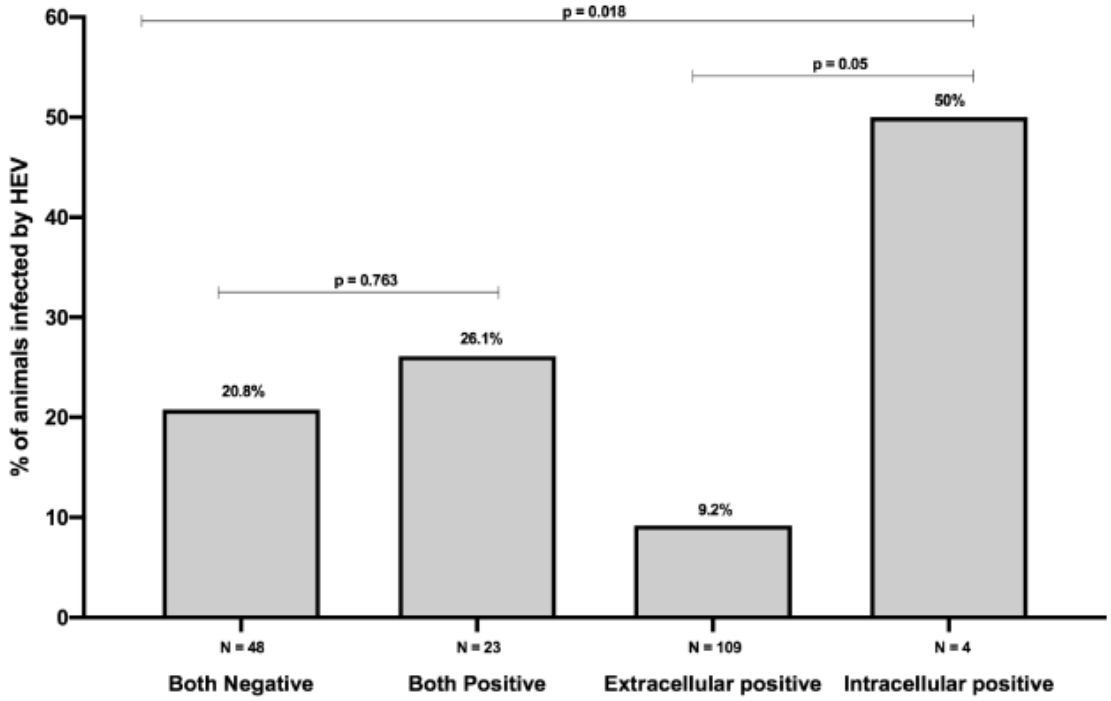

Table 1. Prevalence of HEV based on protist enteroparasites prevalence.

Protists		**Global**			**Large White**			**Iberian pig intensive**			**Iberian pig extensive**		
Type	**Results**	**HEV neg.**	**HEV pos.**	***p***	**HEV neg.**	**HEV pos.**	***p***	**HEV neg.**	**HEV pos.**	***p***	**HEV neg.**	**HEV pos.**	***p***

Extracellular													
G. duodenalis *n* (%)	*Negative*	366 (86.3)	58 (13.7)	0.376	131 (84)	25 (16)	0.579	121 (94.5)	7 (5.5)	0.521	114 (81.4)	26 (18.6)	0.213
	Positive	47 (92.2)	4 (7.8)		25 (89.3)	3 (10.7)		11 (91.7)	1 (8.3)		11 (100)	0	
Blastocystis sp. *n* (%)	*Negative*	217 (87.5)	31 (12.5)	0.785	53 (80.3)	13 (19.7)	0.208	101 (93.5)	7 (6.5)	0.682	63 (85.1)	11 (14.9)	0.521
	Positive	196 (86.3)	31 (13.7)		103 (87.3)	15 (12.7)		31 (96.9)	1 (3.1)		62 (80.5)	15 (19.5)	
B. coli *n* (%)	*Negative*	221 (85.3)	38 (14.7)	0.276	74 (82.2)	16 (17.8)	0.413	77 (91.7)	7 (8.3)	0.144	70 (82.4)	15 (17.6)	0.999
	Positive	192 (88.9)	24 (11.1)		82 (87.2)	12 (12.8)		55 (98.2)	1 (1.8)		55 (83.3)	11 (16.7)	
Intracellular													
Cryptosporidium spp.	*Negative*	395 (88)	54 (12)	0.012	147 (87)	22 (13)	0.014	124 (93.9)	8 (6.1)	0.999	124 (83.2)	24 (16.8)	0.077

n (%)								9)	1)		8)	2)	
	Positive	18 (69.2)	8 (30.8)		9 (60)	6 (40)		8 (100)	0		1 (33.3)	2 (66.7)	
E. bieneusi *n* (%)	*Negative*	388 (87.8)	54 (12.2)	0.06	140 (87)	21 (13)	0.055	123 (93.9)	8 (6.1)	0.99	125 (83.3)	25 (16.7)	0.172
	Positive	25 (75)	8 (24.2)		16 (69.6)	7 (30.4)		9 (100)	0		0	1 (100)	

Table 2. Multivariate analysis for HEV infection.

		Global population		**Large White**		**Iberian pig**	
Variable	**Condition**	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***	**OR (95% CI)**	***p***
Farming system	Intensive	1	**0.03**	–	–	1	**0.006**
	Extensive	1.89 (1.06–3.38)		–	–	3.52 (1.43–8.61)	–
G. duodenalis	*Negative*	1	0.317	1	0.693	1	0.327
	Positive	0.57 (0.19–1.69)		0.76 (0.2–2.89)		0.35 (0.04–2.79)	
Blastocystis sp.	*Negative*	1	0.734	1	0.172	1	0.59

	Positive	1.11 (0.6–2.04)		0.49 (0.18–1.36)		1.24 (0.54–2.84)	
B. coli	*Negative*	1	0.199	1	0.952	1	0.23
	Positive	0.66 (0.36–1.23)		0.97 (0.36–2.57)		0.6 (0.26–1.38)	
Cryptosporidium spp.	*Negative*	1	**0.037**	1	0.063	1	0.227
	Positive	3.14 (1.07–9.2)		4.1 (0.92–17.6)		2.97 (0.51–17.4)	
E. bieneusi	*Negative*	1	0.314	1	0.482	1	0.893
	Positive	1.72 (0.59–4.94)		1.61 (0.42–6.03)		1.17 (0.11–11.74)	