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PROTOZOOLOGY - ORIGINAL PAPER

Prevalence and molecular detection of *Eimeria* species in different types of poultry in Greece and associated risk factors

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Abstract

Coccidiosis is one of the most notable diseases in chickens having a high economic impact on the poultry industry worldwide. The present study is the first attempt to epidemiologically investigate *Eimeria* spp. distribution and associated risk factors under different housing and production systems in three major regions in Greece. Faecal samples were obtained from 42 operations (broilers, floor housed, free range and organic layers, backyard farms). A questionnaire was obtained from included operations to acquire additional information regarding farm management, location, production rate and diseases history. Positivity level was 85.7%. All seven *Eimeria* species were identified, and the most prevalent ones were *E. acervulina* (79.3%) and *E. tenella* (65.5%). Single-species and mixed infections were detected in 20.7% and 79.3% of the flocks, respectively. Flock size, type of outdoor area, production system and presence of respiratory disease proved significant risk factors. Flock size up to 10,000 birds correlated strongly ($p=0.02$) with higher *E. tenella* quantities. A very strong correlation ($p<0.001$) was found between the presence of respiratory disease and the average OPG level in broiler farms. Organic flocks showed higher prevalence of *E. tenella* ($p=0.023$), while presence of vegetation at the outdoor area correlated strongly with *E. brunetti* ($p<0.001$). Molecular analysis and correlation results in this survey give strong indications although more studies are needed to further understand the involvement of different *Eimeria* species in various husbandry, production and management systems, to gain more knowledge about the sustainable control of coccidia in poultry.

Keywords *Eimeria* · Prevalence · Chicken · Molecular diagnosis

Introduction

Coccidiosis is one of the most notable diseases in chickens and has a high economic impact on the poultry industry worldwide (Haug et al. 2008; Quiroz-Castañeda and Dantan-Gonzalez 2015). Blake et al. (2020) recently estimated that losses due to coccidiosis amount to more than USD 15 billion across the world, equalling USD 0.24 for each single chicken produced. This global cost has seemingly increased by USD 12 billion over the last decade, as Dalloul and Lillehoj (2006) had previously reported it USD 3 billion. Apicomplexan protozoan parasites of the genus *Eimeria* are the causative agent of avian coccidiosis. The infective agent was identified early (Railliet, 1913), and since then, it has been studied worldwide. Tyzzer et al. (1932) had shown that species of chicken *Eimeria* develop in different regions of the intestine. In chickens, there are seven recognized species of *Eimeria* that develop in certain locations within the intestine, each causing a different clinical manifestation (Williams et al. 2009), namely, *E. acervulina, E. mitis*, *E. brunetti*, *E. maxima*, *E. necatrix, E. praecox,* and *E. tenella*.

Chicken *Eimeria* species display varying degrees of pathogenicity. Depending on the respective species, infection dose and *Eimeria* species specific site of infection,

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coccidiosis can result in more or less severe disease. It can range from limited enteritis with malabsorption of nutrients and reduced growth rate (e.g. *E. praecox* and *E. mitis*), over inflammation of the intestinal wall with pinpoint haemorrhage and epithelial demolition (e.g. *E. brunetti, E. acervulina* and *E. maxima*), to complete villar destruction resulting in extensive haemorrhage, high morbidity and death (e.g. *E. necatrix* and *E. tenella*) (Iacob and Duma 2009; Morris et al. 2007; Williams et al. 2009). Mixed species infections are common (Callow 1984). In addition to direct *Eimeria*-related damages, co-infections with other pathogens are common and lead to aggravated disease and economic losses (Alnassan et al. 2013; Van Immerseel et al. 2004).

The genetic diversity of the *Eimeria* species and the lack of new anticoccidial drugs for decades have led to the development of widespread resistance for all the drugs approved for use in chickens (Chapman 1997; Peek and Landman 2011; Tan et al. 2017). Drug-resistant *Eimeria* strains can cause subclinical coccidiosis and low body weight gain and feed conversion ratio (Shirzad et al. 2011). Anticoccidial live vaccines have been used efficiently to prevent coccidiosis in the last decades (Marugan-Hernandez et al. 2016); however, they are expensive, and thus, they are not applied exhaustively on a global scale (Blake and Tomley 2014). Recent studies highlight the potential use of recombinant vaccines as an antimicrobial control measure (Clark et al. 2016; Kundu et al. 2017; Lin et al. 2017; Tian et al. 2017), but these tools are not commercially available yet. In addition to control programs based upon chemotherapy or vaccination, satisfactory control of coccidiosis in poultry requires strict attention to hygiene and sanitation, as well as biosecurity measures that limit human access to poultry facilities (Chapman 2018).

To efficiently control coccidiosis, it is important to understand the *Eimeria* species that are circulating and infecting the different types of poultry farming (i.e. backyard and commercial), as well as potential risk factors associated with the occurrence and burden of different *Eimeria* species. As far as we know, in Greece, there is no accurate data or previously published information about the prevalence of different *Eimeria* species in different types of poultry. The purpose of the present study was (1) to analyse the epidemiology of *Eimeria* species in Greece, using molecular methods, and (2) to investigate risk factors associated with the presence of *Eimeria* spp. for different housing systems and production types. This data will not only support the knowledge about occurrence and control options for *Eimeria* infections in poultry in Greece but also enhance our general knowledge about potential risk factors in poultry production under different husbandry conditions.

Materials and methods

Study design and sampling

Selection of poultry operations was based on the number of commercial farms in three major Greek regions. Particularly, the geographical location of poultry farms in Greece is concentrated in Epirus (in North-Western Greece), Central Macedonia and Central Greece (Hellenic Ministry of Rural Development & Food, www.minagric.gr). For the purpose of the study, sampling from both commercial operations (broiler and layer flocks) and backyard farms was conducted proportionately to their frequency. (Fig. 1)

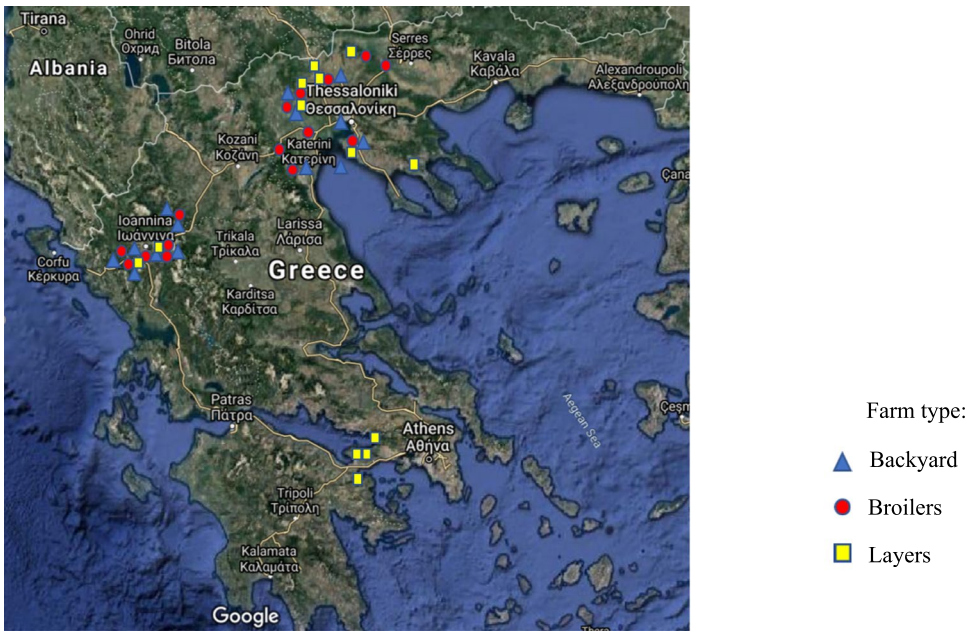

Fig. 1 Map of Greece reporting the geolocalization of backyard (blue triangle), broiler (red circle) and layer (yellow square) tested farms

The study was conducted on 42 poultry operations in Greece, between January 2016 and March 2017. Fifteen of them were raising broilers, fourteen were backyard farms, and thirteen were layer operations (conventional floor housed, free range and organic flocks) (Table 1). Broiler flocks sampled for this study were slow-growing chicken or commercial broiler breeds kept under conventional ($n=10$), free-range ($n=3$) or organic ($n=2$) production conditions, due to concurrent studies performed. In these flocks, slaughter age had a 75–100-day range, and animals were sampled twice, to record *Eimeria* occurrence (OPGs and *Eimeria* species). The first sampling was done at the age of 3–4 weeks, in the operation by collecting faeces from the litter and the second one at process by collecting the whole gut. As gut sampling relied on farm availability and our earlier notification to follow up the flocks at process, gut samples were collected only from six broiler farms in total, three located in Epirus and three located in Central Macedonia. Three out of six farms were employing conventional, two farms free-range and one farm organic production conditions. In layers and backyard flocks, faecal samples were collected once. On layer farms, sampling was performed at the age of 10–12 months, whereas in backyard flocks, according to the flock composition, chickens of different ages were sampled by the described pool sample technique.

Two hundred fifty-two faecal samples were collected from the litter of the chicken housing according to a W shape, in order to collect one fresh dropping every two to five paces according to the floor size (Fornace et al. 2013). Sampling was performed only indoors for all type of farms, and maximum three houses were sampled from all farms. A total of 18 samples were collected from each house, pooled in six samples of three subsamples, placed in labelled sterile bottles and stored in a cool box. From broiler flocks that were followed up to the slaughterhouse, the whole gut was extracted during evisceration and placed in labelled zipped plastic bags. All the samples were transferred immediately to the laboratory of Parasitology of the Veterinary Research Institute – Hellenic Agricultural Organization Demeter, (Thessaloniki, Greece), where they were stored at 4 °C for further parasitological examination. The total number of gastrointestinal tracts collected per broiler flock was calculated based on the flock size using SampSize (http://sampsize.sourceforge.net/iface) and OpenEpi (version 3.01, http://www.openepi.com/SampleSize/SSPropor.htm). According to the flock sizes available for our study, for conventional slow-growing flocks with capacities higher than 5000 birds, 19 guts were collected per flock, while for free-range and organic flocks with capacities higher than 3000 birds, 35 guts were collected per flock.

Data collection by questionnaire

Furthermore, a questionnaire was obtained from all included operations in order to acquire additional information for each farm. It included details about the farm management, location, production rate, disease history and poultry health (Table 2 and Supplemental Table 1), including anticoccidial measures, disinfection measures and perceived flock performance. Farms employing live vaccination protocols have been included to identify potential impacts on *Eimeria* species infection intensity. If applicable, coccidiostat use was classified as either rotation program or shuttle program; drugs applied were chemicals (nicarbazin) or ionophores (narasin, lasalocid, salinomycin).

Disease history was asking about perceived respiratory disease (sneezing, coughing, laboured breathing, nasal discharge, swollen heads) and perceived intestinal disease (diarrhoea), respectively.

Faecal oocyst counts

Faecal samples were quantitatively examined, by a modified McMaster technique (Roepstorff and Nansen, 1998) with two counts per sample that were averaged. Results were expressed as oocyst content per gram of faeces (OPGs)

Table 1 Study design—location and number of sampled operations ($n=42$ total) and collected sample types

Farms per region (*n*)	Operation type (*n*)	Pooled samples collected per operation at the farm (*n*)	Total samples collected and tested per operation type (*n*)	Flocks sampled at process (*n*)	Intestinal tracts sampled in total during process (*n*)
Epirus (15)	Layers (2)	6	12		
	Broilers (6)	6	36	Broilers (3)	89
	Backyard (7)	6	42		
Central Macedonia (23)	Layers (7)	6	42		
	Broilers (9)	6	54	Broilers (3)	73
	Backyard (7)	6	42		
Central Greece (4)	Layers (4)	6	24		
Total (42)			252		162

Table 2 Summary of retrieved farm management data per operation type

Operation type (*n*)	No. of operations with respective farm size as birds per operation (*n*)				No. of sheds/houses per operation (range)	Production system (*n*)	Anticoccidial measures (*n*)
	1–50	51–300	301–10,000	> 10,000			
Layers (13)		6	4	3	Conventional floor-housed (1–3) Conventional free-range (1) Organic (1–10)	Conventional floor-housed (3) Conventional free-range (2) Organic (8)	Coccidiostat rotation (1) Coccidiostat shuttle program (0) Live vaccine (5) None (3) Other (4)
Broilers (15)			3	12	Conventional slow-growth (1–3) Conventional free-range (1–2) Organic (2–4)	Conventional slow-growth (10) Conventional free range (3) Organic (2)	Coccidiostat rotation (9) Coccidiostat shuttle (4) Live vaccine (1) Other (1)
Backyard (14)	14				1–3	Litter use (14)	Other, i.e. herbal remedies (3) None (11)

with a lower detection limit of 50 OPG. For every flock, six pooled samples were processed and 12 counts were performed (2 averaged counts per sample). From the positive faecal samples, oocysts were collected by a combined sedimentation and flotation method according to Daugschies et al. (2002). The purified oocysts were pooled back in one sample per farm for PCR analysis. All samples were afterwards transferred to a 2.5% potassium dichromate solution for sporulation at room temperature for up to 2 weeks and then stored at – 20 °C for further molecular analysis. For the six broiler farms (three in Central Macedonia and three in Epirus), where the whole gut was collected at the slaughterhouse during process, faecal samples were collected from the rectum or the lower part of the intestine and the same steps were followed in regards of McMaster examination, oocysts purification and storage.

DNA extraction

DNA was extracted from sporulated oocysts of *Eimeria* spp. using the QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions after initial sonication (6 min, pulse 0.5, amplitude 60%) using a Bandelin sonopuls HD70 (Bandelin electronics, Berlin, Germany) to disintegrate the *Eimeria* oocysts' wall. DNA concentrations were measured at 260 nm using a spectrophotometer (Eppendorf BioPhotometer, Hamburg, Germany). The extracted DNA was analysed directly by *Eimeria* spp.–specific polymerase chain reactions (PCR) or kept at – 20 °C until use.

Molecular analyses by PCR

Purified *Eimeria* spp. oocyst isolates from the different operations were identified to species level by multiple PCR assays (Schnitzler et al. 1998 and 1999) using species-specific primers (Table 3). The multiple PCRs were performed for each sample in a final volume of 25 µl using 1 U of Taq DNA polymerase (5 U/µl Promega, USA), 1 × PCR buffer (10X Promega, USA), 200 µM of each deoxyribonucleotide (Peqlab, Erlangen, Germany), 300 nM of each primer (MWG-Biotech, Ebersberg, Germany), 2 mM $MgCl_2$ (25 mM, Promega, USA), 30–50 ng template DNA and deionized DEPC-H_2O (Roth, Karlsruhe, Germany). The reaction in the PCR cycler (iCycler®, Bio-Rad, München, Germany) was programmed for initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation (45 s at 94 °C), annealing (40 s at 61 °C) and amplification (1 min at 72 °C) and a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis (Biometra, Göttingen, Germany) by loading 10 µl of PCR product onto 1.5% agarose gel (preqGold Universal Agarose, Peqlab, Erlangen, Germany). The gels were transferred to be stained in an aqueous ethidium bromide solution (0.5 µg/ml) for 15 min, and DNA bands were visualized under UV light (transilluminator; UV wavelength, 254 nm; TFX-20 M, Vilber Lourmat, France) and photographed by digital camera (CSE-0028, Cybertech, Berlin, Germany). Bands were categorized by intensity semiquantitatively as described before (Antiabong et al. 2016).

Statistical analysis

Statistical analysis of OPG counts and PCR findings and correlations with the questionnaire data was done using the PSPP statistical program (GNU PSPP 1.3.0). Following a Kolmogorov–Smirnov normality test that revealed all data were non-normally distributed, Kruskal–Wallis tests and bivariate correlations were used to determine if there

Table 3 List of PCR primers used for molecular study. *Ta* annealing temperature used

Species		Primer sequence 5′-3′	Ta (°C)	PCR Product size (bp)	Reference
E. acervulina	ACER1	CTGCGAGGGAACGCTTAAT	61	303	(Su et al. 2003)
	ACER2	AACGAACGCAATAACACACG			
E. brunetti	BRUN1	AGCTTGGATTTTCGCTCAGA	61	395	(Su et al. 2003)
	BRUN2	CTTCCGTACGTCGGATTTGT			
E. maxima	MAX1	TTGTGGGGCATATTGTTGTG	61	151	(Su et al. 2003)
	MAX2	CAATGAGGCACCACATGTCT			
E. mitis	EMitF	TATTTCCTGTCGTCGTCTCGC	61	327	(Schnitzler et al. 1998; 1999)
	EMitR	GTATGCAAGAGAGAATCGGGA			
E. necatrix	NEC1	GTCACGTTTTTGCCTGGGTG	61	385	(Su et al. 2003)
	NEC2	ACAGACCGCTACACAACACG			
E. praecox	EPreF	CATCATCGGAATGGCTTTTTGA	61	391	(Schnitzler et al. 1998; 1999)
	EPreR	AATAAATAGCGCAAAATTAAGCA			
E. tenella	TEN1	CGCTGCTGGTTTTACAGGTT	61	463	(Su et al. 2003)
	TEN2	GCTGAAGCAAAGTTCCAAGC			

were any statistically relevant correlations between findings within the samples and the husbandry conditions on operations and flock size. The data was separated out between data on the individual production types (layer operations, broiler operations and backyard farms) and also analysed combined for all operation types. The influence of production type (conventional versus organic, as well as different conventional husbandry systems for both layer and broiler flocks, as listed in Table 2) on *Eimeria* species infection intensity and extensity was tested using a bivariate correlation. Statistical significance was assumed for p values < 0.05.

Results

Operation parameters

Farms selected included backyard farms, slow-growth, free-range or organic broilers, floor-housed conventional layer farms and free-range or organic layer farms. Backyard farms had up to 50 hens or chickens per flock. Seven out of 14 backyard farms (50%) had grass soil as outdoor area, while the rest 50% had bush land. Poultry from all 14 farms had access to the outdoor area for free grazing, whereas most of these farms (64.3%) did not perform any kind of disinfections. Only three out of 14 farms (21.4%) had taken some control measures against coccidiosis, through dietary supplements like oregano oil or garlic extract. In 35.7% of the backyard operations (five out of 14 farms), poor performance in terms of egg production was reported or observed, with two of these farms having intestinal disease or clinical disorders and one farm showing signs of respiratory disease, with nasal discharge and swollen heads.

Broiler flocks' samples had a flock size ranging from 4500 to 50,000 chickens per farm and were mainly (73.3%) multi-age farms with an average of 22,447.7 chickens and 2.8 sheds per farm. 80% of the farms' outdoor area was concrete floor, 13.3% was bush land and 6.6% grass soil. Access to the outdoor area in a well-fragmented area, especially designed for free grazing in some hours within the day, was given to 26.7% of these flocks; 46.6% reported intestinal disease or disorders. Sixty percent of the farms were applying rotation programs for the control of coccidiosis, and 26.7% had adopted a shuttle program. Only one farm was applying vaccination against *Eimeria* spp. with a live attenuated vaccine.

Layer farms sampled for this study were located in Central Macedonia (53.85%), Epirus (15.38%) and Central Greece (30.77%). Of these, 23.08% were single-age, floor-housed layers, with an average farm flock size of 11,000 hens and an average shed number of 1.66. Of the flocks examined, 15.38% were conventional free-range farms with single-age hens that had partial access to a fenced outdoor area. The average flock size was 3750 hens housed in one shed per farm. Organic layer farms were 61.54%, with an average flock size of 4375 hens per flock and 2.63 sheds per farm on average. Of the organic farms, 62.5% were multi-age ones. In total, 38.5% of the layer farms had bush land as surroundings, 53.85% had grass soil outdoor area and only one farm (7.7%) had concrete floor in the outdoor environment. All farms were applying disinfections on a regular basis, while 46.15% of them had also visitor books with daily records. Of the layer farms, 23.08% and 15.38% reported intestinal disorders or respiratory clinical signs, respectively. However, none of the farms reported significant losses in productivity, although in 53.85% of the farms, daily egg production and

performance were reported below the optimal age-related standards of the layer hybrids housed. Regarding coccidiosis control, 38.46% of the layer farms had performed vaccination against coccidiosis during rearing, 30.77% were adding essential oils or herbs with anticoccidial indications as dietary supplements, and 7.7% had used coccidiostats in the ratio during the rearing period. Of the layer farms, 23.07% did not apply any control measures against coccidiosis, or farmers were not aware of any treatments or vaccination against *Eimeria* spp. during rearing, as in most cases, hens were placed in the poultry houses as pullets.

Faecal oocyst counts

McMaster flotation results revealed the presence of *Eimeria* species oocysts in 36 out of 42 operations sampled, demonstrating a level of 85.7% positivity for the presence of *Eimeria* oocysts. Twelve out of 14 backyard flocks were found positive (85.7%), with an average mean OPG of 774.7. Broiler flocks demonstrated 80% positivity (12 out of 15 farms) during sampling at the age of 3–4 weeks, but with a significantly higher average mean OPG level of 33,502.2. Layers showed the highest positivity level (92.3%) with 12 out of 13 farms testing positive for the presence of *Eimeria* oocysts and 577.0 average mean OPG (Table 4). The highest mean OPG was recorded in broiler farm 2 (271,900) and the lowest mean OPG was observed in backyard farm 6 (6.66). Epirus showed the highest average mean OPG level of 29,840.6, followed by Central Macedonia (4,228.6) and Central Greece (300). A noteworthy result is the big difference observed in average mean OPG for broilers in Epirus (80,178.3) compared to broilers in Central Macedonia (10,164.2). As for layers, average mean OPG between conventional floor-housed and free-range or organic flocks was 521.7 and 588, respectively, with layer farms located in Central Macedonia showing a much higher average mean OPG (914.7) compared to layer farms located in Central Greece (384.4). Average OPG level ranked higher in broiler farms versus layer farms ($p < 0.001$).

Molecular analyses by PCR

Sufficiently high numbers of oocysts for purification and DNA extraction were present in samples from 33 out of the sampled 42 farms (78.6%). PCR-positive findings for any *Eimeria* spp. were obtained from DNA extracts out of 29 farms. All seven *Eimeria* species were identified over all 29 operations: *E. tenella, E. acervulina, E. maxima, E. brunetti, E. necatrix, E. mitis* and *E. praecox*. The distribution of *Eimeria* species in poultry farms is presented in Table 5. Overall, the most prevalent species were *E. acervulina* (23/29; 79.3%), followed by *E. tenella* (19/29; 65.5%), *E. brunetti* (8/29; 27.6%) *and E. maxima* (7/29; 24.1%), while the less abundant were *E. necatrix* (4/29; 13.8%), *E. mitis* (3/29; 10.3%) and *E. praecox* (3/29; 10.3%).

In broiler farms, the most prevalent species were equally *E. tenella* and *E. acervulina* (13/15; 86.7%), in layer flocks *E. brunetti* and *E. necatrix* (4/7; 57.1%) equally, while in backyard flocks *E. acervulina* represented the most frequently found species (8/11; 72.7). In backyard flocks, no *E. brunetti* was detected. *E. praecox* and *E. mitis* were absent in layer flocks. *E. praecox* was found in broilers only on two operations at the second sampling during slaughter, while it could not be detected on any operation at the age of 3–4 weeks when the first sampling was performed.

E. tenella and *E. acervulina* were significantly more prevalent in broilers compared to layers ($p = 0.01$ and $p = 0.04$, respectively). On layer farms, *E. brunetti* and *E. necatrix* were more prevalent than on broiler farms ($p = 0.019$ and $p = 0.02$, respectively). The two latter species had also a strong correlation with each other ($r = 0.76$, $p = 0.001$) for layer flocks, indicating that they are commonly occurring together.

Table 4 Average OPG counts per type of poultry, region and type of coccidiosis control. Farms where no oocysts were detected were not included in the OPG average calculations

	McMaster average OPG				Type of coccidiosis control				
	Average for all regions	Epirus	Central Macedonia	Central Greece	None	Rotation	Shuttle	Vaccination	Other
Backyard	774.7	1,476	273.8		258.9				2,322.2
Broilers	33,502.2	80,178.3	10,164.2			10,587	103,920	5,570	
Slow-growing	43,049.3	155,850	10,820.5			12,613.9	103,920		
Free-range	4,506.7	4,506.7				4,506.7			
Organic	5,570		5,570					5,570	
Layers	577	76.7	928	300	320.7	996.7		249.3	1239.4
Floor-housed indoor	521.7		996.7	46.7		996.7		46.7	
Free-range	376.7		376.7		456.7				296.7
Organic	640.9	76.7	1273.4	384.4	252.7			300	1710.8

Table 5 Occurrence of different *Eimeria* spp. by operation based on different *Eimeria* spp.–specific PCR data. (+, positive finding; −, negative finding)

Broiler farms	*E. tenella*	*E. acervulina*	*E. maxima*	*E. brunetti*	*E. necatrix*	*E. praecox*	*E. mitis*	Number of species
1	+	+	+	-	-	+	-	4
2	+	+	+	-	-	-	-	3
3	+	+	-	-	-	-	-	2
4	+	+	-	-	-	-	-	2
5	+	+	-	-	-	-	-	2
6	+	-	+	-	-	-	-	2
7	+	+	-	-	-	-	+	3
8	+	+	+	-	-	-	-	3
9	+	+	+	+	-	+	+	6
10	+	+	-	-	-	-	-	2
11	+	+	-	-	-	-	-	2
12	+	+	-	-	-	-	-	2
13	-	+	-	-	-	-	+	2
14	+	+	-	-	-	-	-	2
Backyard farms								
1	-	-	-	-	-	-	-	0
2	+	+	-	+	-	-	-	3
3	-	-	-	+	-	-	-	1
4	-	-	-	-	-	-	-	0
5	-	+	-	-	-	-	-	1
6	+	+	+	-	-	-	-	3
7	+	+	-	-	-	-	-	2
8	+	+	-	-	-	+	-	3
9	-	+	-	-	-	-	-	1
10	+	+	+	+	-	-	-	4
11	-	+	-	-	-	-	-	1
12	-	+	-	-	-	-	-	1
Layer farms								
1	+	-	-	-	-	-	-	1
2	-	-	-	+	+	-	-	2
3	-	-	-	-	-	-	-	0
4	-	-	-	+	+	-	-	2
5	-	-	-	+	+	-	-	2
6	-	+	-	-	-	-	-	1
7	-	+	-	+	+	-	-	3

The number of *Eimeria* spp. recovered per operation is listed in Table 5. Infection with only one species was detected in backyard and layer chickens only. Mixed infections with two or more species were found in 23 (79.3%) flocks. Out of these co-infections, thirteen flocks were infected by two *Eimeria* species, seven flocks by three species, two flocks by four species and only one by six *Eimeria* species. On average, largest *Eimeria* spp. variety was seen in broiler flocks (Table 5).

Regional differences There were some regional differences observed in animal husbandry parameters and *Eimeria* occurrence. For both *E. tenella* ($p = 0.003$) and *E. acervulina* ($p = 0.029$), higher prevalence rates were observed in Epirus compared to Central Macedonia. In Central Greece, *E. brunetti* ($p = 0.019$) and *E. necatrix* ($p = 0.001$) were significantly more prevalent than in Epirus. No statistically significant differences were observed between Central Macedonia and Central Greece.

Correlation of Eimeria oocyst counts and species identity with questionnaire data

For both broilers and layers, some *Eimeria* species and OPGs have been found to be significantly linked with the respective production or housing system parameters assessed by the questionnaire (Table 6).

Broilers In broilers, *E. tenella* was more prevalent in organic flocks or flocks with partial outdoor area access compared to flocks that were housed only indoors on litter ($p = 0.023$). Smaller flocks of up to 10,000 birds per flock had significantly ($p = 0.02$) higher *E. tenella* quantities, as compared to larger sized flocks. *Eimeria* species and oocyst quantities (per semiquantitative PCR, as described by Antiabong et al.2016)), also correlated with the type of the outdoor area; presence of soil and vegetation were correlated with higher oocyst quantities of *E. tenella* ($p = 0.06$), *E. brunetti* ($p < 0.001$), and *E. praecox* ($p = 0.07$), respectively. The presence of neighbouring broiler farms correlated moderately with lower quantities of *E. maxima* ($p = 0.012$) and *E. praecox* ($p = 0.021$). Per producers' performance and productivity evaluation for their flocks, *E. tenella* quantity correlated with perceived, however not quantified, economic losses ($p = 0.034$). Additionally, a very strong correlation ($p < 0.001$) was found between the presence of respiratory disease and the average OPG level in broiler farms. The type of on-site coccidiosis control measures on broiler operations influenced both *E. tenella* ($p = 0.034$) and *E. brunetti* ($p = 0.063$) quantities. Vaccination, which was defined as the maximum control level in our study's questionnaire (Supplemental Table 1), seems more likely to reduce the quantities of *E. tenella* and *E. brunetti* when compared to anticoccidial drug rotation or shuttle programs.

Table 6 Pearson correlation test results for broiler and layer farms sampled, with the respective correlation coefficient *r* and correlation strength, as interpreted by Sachs 1992. Only statistically significant correlations are shown ($p < 0.05$)

Test parameter 1	Test parameter 2	Correlation coefficient r	*p* value	Correlation strength
Broilers				
Outdoor area	*E. tenella* quantity	0.69	0.006	Moderate
Outdoor area	*E. brunetti* quantity	0.81	< 0.001	Strong
Outdoor area	*E. praecox* quantity	0.68	0.007	Moderate
Flock size	*E. tenella* quantity	− 0.74	0.002	Strong
Flock size	Type of coccidiosis control	− 0.72	0.002	Strong
Production System	*E. tenella* quantity	0.8	0.001	Strong
Respiratory diseases	Average OPG	0.97	< 0.001	Very strong
Type of coccidiosis control	*E. tenella* quantity	0.57	0.034	Moderate
Type of coccidiosis control	Flock size	− 0.72	0.002	Strong
Type of coccidiosis control	*E. brunetti* quantity	0.51	0.063	Moderate
Neighbouring farms	*E. maxima* quantity	− 0.65	0.012	Moderate
Neighbouring farms	*E. praecox* quantity	− 0.61	0.021	Moderate
Production System	Type of coccidiosis control	0.68	0.005	Moderate
E. tenella quantity	Production losses observed	0.57	0.034	Moderate
Layers				
Disinfection	*E. tenella* quantity	− 0.6	0.019	Moderate
Neighbouring farms	*E. acervulina* quantity	0.53	0.04	Moderate
Intestinal disorders	Average OPG	0.41	0.026	Weak
Respiratory diseases	*E. acervulina* quantity	0.58	0.024	Moderate
E. tenella quantity	Production losses observed	0.9	< 0.001	Strong
E. tenella quantity	*E. maxima* quantity	0.9	< 0.001	Strong
E. maxima quantity	Production losses observed	1	< 0.001	Very strong
E. brunetti quantity	*E. necatrix* quantity	0.76	0.001	Strong
Flock size	*E. brunetti* quantity	0.69	0.005	Moderate
Flock size	*E. necatrix* quantity	0.88	< 0.001	Strong
Production System	*E. brunetti* quantity	− 0.6	0.018	Moderate
Production System	*E. necatrix* quantity	− 0.74	0.002	Strong
E. maxima quantity	Production losses observed	1	< 0.001	Very strong
Flock size	Disinfection	0.63	< 0.001	Moderate
Production system	Disinfection	− 0.44	0.013	Moderate
Feeding system	*E. necatrix* quantity	0.69	0.004	Moderate

Layers For layers, *E. necatrix* was more prevalent in conventional floor housed flocks ($p=0.03$), free-range flocks ($p=0.046$) and organic flocks ($p=0.015$), compared to backyard flocks. Floor-housed layers were also more likely to be infected with *E. brunetti* ($p=0.018$) and *E. necatrix* ($p=0.02$) than free-range and organic layers, respectively. Larger layer flock size showed a positive correlation with the quantity of *E. brunetti* ($p=0.05$) and *E. necatrix* ($p<0.001$) oocysts excreted, respectively. The presence of neighbouring farms was linked with higher presence of *E. acervulina* ($p=0.04$). Absence of a routine disinfection program correlated with higher *E. tenella* oocyst quantities ($p=0.019$). Both *E. tenella* ($p<0.001$) and *E. maxima* ($p<0.001$) presence were found to be associated with observed production losses. Interestingly, respiratory diseases on layer operations correlated with higher oocyst excretion of *E. acervulina* ($p=0.024$). Intestinal disorders observed on layer farms displayed a weak but statistically significant correlation with the average *Eimeria* OPGs ($p=0.026$). Ground feeding of layers was linked with higher oocyst excretion of *E. necatrix* ($p=0.017$). From a farm management perspective, floor-housed layers and large size flocks were more likely to employ regular disinfection measures compared to free-range, organic ($p=0.013$) and smaller sized flocks ($p<0.001$).

Overall, flock size, as well as type of outdoor area, was found to impact *Eimeria* species presence and OPG significantly stronger on broiler farms compared to layer farms ($p<0.001$).

Discussion

Epidemiological studies on the prevalence of *Eimeria* species are useful tools for the prevention and control of coccidiosis (Gyorke et al. 2013). Traditionally, *Eimeria* species have been identified using morphometry of the sporulated oocysts (McDougald et al. 1997) as well as intestinal lesion evaluation during necropsy (Johnson and Reid 1970). Molecular techniques can overcome morphometry limitations (Andrews and Chilton 1999; Gasser 1999) and do not require euthanasia of animals for diagnostic necropsies. This study appears to be the first epidemiological survey to be conducted in a wider area of Greece and in different types of poultry farming. For the current study, we used species-specific primers in multiple conventional PCRs to determine which *Eimeria* species are circulating in Greek poultry farms under different production conditions. Caged layers were not included in the study design, as *Eimeria* spp. and coccidiosis are believed to be a less prominent problem in hens housed in battery cages (Lunden et al. 2000). Indeed, after indicative faecal sampling in caged layers with both presence and absence of automatic manure belt, no *Eimeria* oocyst excretion was detected, which is in contrast with reports by Soares et al.2004. Coproscopy revealed the presence of *Eimeria* species in 36 out of 42 operations sampled, demonstrating a level of 85.7% positivity for the presence of *Eimeria* oocysts, similar to what was reported in other countries worldwide such as Romania, France, Jordan, India, Argentina and Turkey where prevalence ranged between 70 and 96% (Al-Natour et al. 2002; Gyorke et al. 2013; Karaer et al. 2012; Kumar et al. 2015; McDougald et al. 1997; Williams et al. 1996). We detected less *Eimeria*-positive findings by PCR than by McMaster analysis. In samples from McMaster positive and PCR negative flocks, we detected a low OPG count. Our samples may not have contained enough oocysts to be detected in the small faecal volume subjected to DNA extraction (Haug et al. 2007; Gyorke et al. 2013). Though we employed an ultrasound disintegration of oocysts before using the DNA extraction kit, our DNA extraction protocol may have a limited sensitivity as Haug et al. (2007) described reduced efficiency of their method of oocyst cracking by grinding if oocyst concentration was low, leading to reduced amounts of extracted parasite DNA.

In our study, 38.5% of the layer farms sampled had performed vaccination against coccidiosis during rearing, while 7.7% had used in-feed coccidiostats during the rearing period. Interestingly, 80% of the layer farms that performed vaccination had established organic production settings, where certain limitations for the use of anticoccidial drugs apply, based on the EU organic regulation (EC No 834/2007). For 23.1% of the layer farms, owners did not apply any control measures against coccidiosis and farmers were not aware of any treatments or vaccination against *Eimeria* spp. on a previous rearing operation. These findings are not in accordance with coccidiosis management in the floor-housed layer flocks in the USA, where the majority (83%) of the flocks used a coccidiostat-based anticoccidial program versus only 12% that used vaccination (Soares et al. 2004). In broilers, 60% of the farms were applying rotation programs for the control of coccidiosis. Generally, our observed OPG levels were significantly higher than described for some other European countries; e.g. a study in Norway revealed a median OPG of less than 14,000 OPG for indoor floor pen housed broilers (Haug et al. 2008). While Kaboudi et al. (2016) found a weak correlation between diarrhoea and *Eimeria* presence, our study could not establish a significant correlation between intestinal disease (per questionnaire) and high oocyst quantities on Greek broiler operations. Potentially, the actual *Eimeria* species present and their virulence may determine the clinical signs to a higher degree than the total OPG number observed, especially since the OPG numbers are prone to variation over time based on the *Eimeria* life cycle. This may explain the lacking consistency in findings from different studies.

Eimeria oocysts are considered omnipresent, spreading rapidly in the poultry house environment and showing resistance to various disinfection practices (Hadipour et al. 2011; López-Osorio et al. 2020). In our study, outdoor access obviously contributed in favour of *Eimeria* species circulation in both broiler and layer production, fostering faecal-oral transmission, particularly for *E. tenella* ($p = 0.06$), *E. brunetti* ($p < 0.001$) and *E. praecox* ($p = 0.07$). One notable result was that we did not find any *E. brunetti* in backyard flocks. This is in accordance with Godwin and Morgan (2015), who also reported *E. brunetti* as the least common species in backyard flocks. There are reports about persistence of cattle *Eimeria* oocysts on pastures over different seasons (Lassen et al. 2013, 2014). Any unpaved outdoor premises are hard to disinfect, which increases the outdoor area contamination level (Meroz and Samberg 1995). Hence, disinfection is an important risk management factor in coccidiosis control. In our study, floor-housed layers had a more regular disinfection protocol compared to free-range or even organic flocks. In the European Union, organic poultry producers are allowed to use selected approved cleansers, disinfectants and sanitizers only (EC 834/2007), which obviously limits the application of efficient anticoccidial disinfectants (e.g. cresol-based products). On backyard farms, coccidiosis control measures were not common. Only three out of the 14 backyard farms (21.4%) were attempting some control measures through alternative dietary supplements like oregano essential oil or garlic extract, and there was no clear impact of these measures on the observed *Eimeria* OPG count. Oregano (*Origanum vulgare* subsp. *hirtum*) is used as a bioactive compound that is thought to act as a natural growth enhancer with potent anticoccidial properties and has been studied for several years. Garlic (*Allium sativum*) metabolites have been reported to display potential performance-enhancing, antioxidant and anticoccidial activities (Burt et al 2013; Partheniadis et al. 2019; Sheoran et al. 2017). In vivo studies performed on herbal formulas based on garlic and wild thyme have previously failed to control *E. tenella* burden and pathology (Pop et al. 2019), highlighting the necessity for further research into dietary supplements use. In total, five layer farms reported poor performance, including one farm that used oregano oil, coinciding with high *Eimeria* OPG counts and PCR-based identification of up to four *Eimeria* species in four out of these five farms (80%).

On broiler chicken farms, all typical *Eimeria* species were found except for *E. necatrix,* while studies from other European countries like Sweden, France and former Czechoslovakia showed the presence of all seven species (Kucera 1990; Williams et al. 1996). In our observations, the most prevalent species in broilers were *E. acervulina* and *E. tenella*, similar to findings in Romania and Norway (Gyorke et al. 2013, Haug et al. 2008). Mixed species infections were common and found in 79.3% of the flocks. This is in accordance with previously reported studies from Europe, Africa, Asia and America (Aarthi et al. 2010; Gadelhaq et al. 2015; Gyorke et al. 2013; Huang et al. 2017; Kaboudi et al. 2016, Jenkins et al. 2017).

Interestingly, average OPG levels were higher in broiler farms than in layer farms. This may be due to the young age of broilers, as younger birds are more susceptible while older birds like layers are more likely to be protected by immunity resulting from previous pathogen contact. Moreover, in broilers like *E. acervulina* and *E. tenella* are prevailing, as species of a particularly high reproductive potential (Williams 2001), which results in particularly high OPG.

In our study, smaller sized broiler flocks were more likely to exhibit higher *E. tenella* burdens, which is attributed to a better hygienic management on larger operations. Particularly in broilers, *E. tenella* was most common in organic flocks and flocks with partial outdoor area access that is often related to poorer sanitation. Anyway, consumers nowadays show increased interest in organic poultry production and the number of organic farms has increased significantly in the EU (Agency for the Development and Promotion of Organic Farming 2019). Although literature about prevalence of *Eimeria* species in organic poultry farming is still limited, the need for sustainable alternative control methods against coccidiosis compatible with organic production is considered high.

E. tenella quantity correlates with losses in performance and productivity in broilers and layers, while *E. maxima* primarily alters productivity in layers. *E. tenella* is highly pathogenic and specifically infects epithelial cells of the caecal crypts of Lieberkühn, resulting, along with *E. maxima*, in the induction of a range of pro- and anti-inflammatory cytokines including interleukin IL-6, IL-17A, IL-10 and interferon IFN-γ (Hong et al. 2006; Laurent et al. 2001; MacDonald et al. 2017). Infection may also result in haemorrhagic lesions of varying severity that affect performance parameters for both broilers and layers. A very strong correlation has also been found between the presence or history of respiratory disease signs and the average OPG level in broiler farms, while for layers, respiratory disease history correlated with a stronger presence of *E. acervulina*. According to Chapman (2014), this is not a surprising finding since interactions with co-infecting pathogens via immunosuppression and thus mutual aggravation of clinical signs are commonly seen.

In broilers, anticoccidial drug rotation and shuttle programs seemed more efficient for *E. brunetti* and *E. tenella* control than the adoption of anticoccidial vaccination. Introduction of attenuated live vaccine strains may have confounded these results and thus findings have to be interpreted with caution. However, overall *Eimeria* excretion was

lower in vaccinated (averaging at 1,136 OPG) versus drug treated flocks (averaging at 33,121 OPG). Medicated flocks have been compared before with vaccinated flocks, observing higher peak oocyst shedding in drug treated birds as compared to vaccinated flocks (Snyder et al. 2021).

E. tenella and *E. acervulina* were more prevalent in Epirus (North-western Greece) than in other examined regions, whereas *E. brunetti* and *E. necatrix* were more frequently found in Central Greece. This can be explained by the main type of poultry production in the respective region. Epirus is the region where 45% of Greek broiler farms are located (Andreopoulou et al. 2019), with *E. acervulina* and *E. tenella* being the dominant species in broilers according to our PCR results. Central Greece has a particularly high number of layer farms with 50% of Greek layer farms being located in this prefecture, and consequently, *E. brunetti* and *E. necatrix* were more frequently found (Koutoulis 2012). In Central Macedonia, intestinal disorders were more frequently recorded than in other prefectures, which is in line with more *Eimeria* co-infections seen in this region. Apart from these correlations, farm management practices, level of biosecurity and type of coccidiosis control programs play an important role that should be further investigated. Intestinal disorders were more frequently observed in organic layer flocks when compared to floor housed and free-range flocks. This is most probably attributed to the regulatory limitations regarding the use of antimicrobials and anticoccidial feed additives in organic farms. However, apart from coccidiosis, other unfavourable factors may as well contribute to reduced productivity and higher frequency of disease under such circumstances.

In conclusion, the present study demonstrates a high prevalence of chicken *Eimeria* in Greece that may vary under different production systems. Circulation of all seven *Eimeria* species in both broiler and layer flocks and across the country is obvious from our data. The current study appears to be the first attempt to investigate *Eimeria* species distribution within Greece and associated risk factors in relation with type of farm, flock size, general management practices, disease history and farm location. Due to limited owner consent and thus lacking availability, we did not take samples from intensively reared broilers and a more even farm distribution could not be achieved. Therefore, the molecular analysis of *Eimeria* species and correlation results should be further evaluated to increase understanding of the presence, transmission and relevance of different *Eimeria* species in different systems of husbandry, production and management, in order to develop sustainable control of coccidia in poultry production in general and in Greece in particular.

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