Prevalence of *Eimeria* species, detected by ITS1-PCR, in boiler poultry farms located in seven provinces of northeastern Algeria

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Summary

Coccidiosis is an important global disease of chickens and can cause major economic losses for local poultry production in countries such as Algeria. Little is known about the extent of infection or diversity, of the causative agent *Eimeria spp.*, in Algeria. A priority, therefore, is to determine the prevalence and species composition to inform strategies on treatments and control measures. Samples were collected from 187 broiler farms, located in 7 Northeastern Algerian provinces (Jijel, Constantine, Skikda, Mila, Setif, Batna, Bordj bou-Arreridj), and ITS1-PCR was used to determine the prevalence and composition of *Eimeria* species in chickens. The survey revealed the presence of all seven species of *Eimeria* at different prevalences (*E. maxima* (69 %), *E. acervulina* (68.4%), *E. necatrix* (11.2%), *E. tenella* (8%), *E. praecox* (4.3 %), *E. mitis* (2.1 %), *E. brunetti* (2.1 %). Multiple infection, with up to 4 different *Eimeria* species present on a single farm, was the most frequent situation in our samples (51.9% mixed infections versus 47.6% single infections). All farms revealed infected samples and we conclude that this parasite is a significant problem in these provinces.

Keywords: Broiler; prevalence; *Eimeria*; ITS1-PCR; infections; Algeria.

Introduction

Coccidiosis is the most important parasitic disease of chickens (*Gallus gallus domesticus*). Impairing growth and suppressing the immune system, it leads to high mortality which has been estimated to cost more than US$3 billion annually in the poultry industry (Blake and Tomley, 2014). Caused by intestinal protozoan parasites, belonging to the Phylum Apicomplexa, the genus *Eimeria* manifests its disease by causing enteritis. It is found worldwide in all types of poultry production and the disease can take many clinical forms (McDougald and Reid, 1997).

It is generally accepted that seven species of *Eimeria* (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox* and *E. tenella*) parasitize chickens and different species have different degrees of pathogenicity (Shirley, 1986; Williams et al., 1996). *E. tenella, E. maxima* and *E. acervulina* are regarded as the most economically significant species (Shirley, 1986; Blake and Tomley, 2014). Co-infection of *Eimeria* species (Haug et al., 2008; Jenkins et al., 2008) is common in coccidiosis which contributes not only to pathogenicity but can also result in misleading diagnoses (Fatoba and Adeleke, 2018). Many factors, including the age and diet of the birds, the effectiveness of prophylactic anticoccidial drugs and intercurrent infections and stress, determine the degree of the clinical manifestation of coccidial infection (Williams et al., 1996). To our knowledge, only a few other studies have been conducted to ascertain the prevalence of infection in Algeria (Debbou-louknane et al., 2018). These previous studies have focused on using traditional parasitological identification of parasites to determine species identity. This is the first study to use a molecular approach (ITS1-PCR (Jenkins et al., 2006a; Jenkins et al., 2006b)) to determining the prevalence and species identity of each of the seven species of *Eimeria* spp. Conducted on 187 broiler farms across 7 provinces in northeastern Algeria, we show that this parasite could be a significant problem in this area of Algeria.

2. Materials and methods

2.1. Sample collection

The survey was carried out on 187 broiler farms located in seven north-eastern provinces of Algeria: Jijel (44 samples), Constantine (31 samples), Skikda (10 samples), Mila (17 samples), Setif (35 samples), Batna (42 samples), Bordj bou-Arreridj (8 samples) (Figure 1). Sampling was conducted over a period from June 2009 to March 2014. In all the selected flocks, samples were taken from the litter at

the frequency of one fresh individual chicken dropping per 100 birds (wet areas of the litter was avoided) and this, one day per week in the following weeks of birds' age: 3 or 4, 5 or 6, 8 or 9 as recommended previously (Williams, 2006).

The total size of the chicken population present in all the selected farms was 1000580 animals distributed across 187 broiler flocks with each flock having a range of capacities from 4000 to 10000 chickens (the number of broilers varies from one farm to another). The birds were selected from different broiler-breeder flocks and commercial hatcheries located in these different provinces of the country. Broiler feeds in all the studied farms were the same (crumbled feed, containing corn, soybean meal, wheat bran and premix (starter and growth feeds containing an anticoccidial ionophore)). The litter used in all the selected flocks was also the same (composed of straw and wood shavings). In terms of husbandry, the age at slaughter for chickens on these farms was between the 50^{th} and 55^{th} days for an average weight of about 3.1 kg and the breeds of broilers used were: Cobb 500, Arbor Acre and Hubbard F15.

2.2. Oocyst preparation

For each broiler farm, the sampled faeces were mixed into a single pool (one pool/broiler farm; knowing that the weight of each pool of samples per farm ranges from 300 to 500 g, depending on the size of the sampled broiler facilities). Unsporulated oocysts of *Eimeria* oocysts were suspended in 2.5% potassium dichromate

(with a dose of 500 to 1200 oocysts/ml of potassium dichromate solution) and were stored at

4 °C until use.

2.3. Genomic DNA extraction from oocysts

Genomic DNA from purified sporulated oocysts was extracted using a phenol chloroform extraction process as described by Duncanson et al. (2001) and Bajnok et al. (2015) with modifications for small tissue samples (Dodd et al., 2014) and carried out at the laboratory GBBV (Laboratoire de Génétique Biochimie et Biotechnologies Végétales- University of Constantine 1-Algeria).

2.4. Identification of Eimeria species by PCR

Genomic DNA was transferred the University of Salford, Manchester UK and was used as a template to PCR amplify the ITS-1 region of specific *Eimeria* species (Jenkins et al., 2006a; Jenkins et al., 2006b)

in order to identify the different species of *Eimeria* present in the different pools of oocyst isolates. The reaction mixtures (25 μL of master mix) for each sample that contained 25 pmol forward (Eurofins; Forward primer, UK) and ITS1 reverse primer (Eurofins; Reverse primer, UK), 200 nM dNTP (Amersham, Piscataway, NJ), 20 mM Tris pH 8.4, 50 mM KCl, 3.0 mM MgCl2, and 1 U Taq polymerase ((Bioline-BIOTAQTMDNA Polymerase, UK). Add at the end 1 μL of the DNA of the sample (an amount of total *Eimeria* DNA equivalent to more than 4000 oocysts) to be identified in a reaction mixture (in the negative control, add 1 μL of water). DNA amplification was done in a thermal cycler (Stratagene Gradient RobocyclerTM96, UK). Reaction conditions were as follows: 1 cycle—95 C, 7 min; 35 cycles—95 C, 20 s, 44–60 C, 30 s, 72 C, 1 min; 1 cycle— 72 °C, 5 min (Jenkins et al. 2006a, b)

2.5. Statistical analysis

The data were collected and calculated in Microsoft Excel 2019 (version 16.27). Chi squared tests between the percentages found for the different types of infection were performed with RStudio environment version 1.2.5033 (RStudio Team, 2019).

3. Results

The prevalence of chicken Eimeria spp in all of our samples is in the order of 99.5%. The proportions of each *Eimeria* species present in the pooled samples differed between farms and between the 7 provinces (Table 1);however, *E. maxima* and *E. acervulina* were the most commonly found species, as follows (Table 2): *E. maxima* (69%), *E. acervulina* (68.4%), *E. necatrix* (11.2%), *E. tenella* (8%), *E. praecox* (4.3%), *E. mitis* (2.1 %), *E. brunetti* (2.1 %).

In an overview, the differences in the prevalences between the identified species were statistically significant ($p < 0.001$) (Table 2). Table 3 shows the status of mixed infections on single farms – these ranged from no mixtures to up to 4 species observed in the same sample.

E. maxima was the most prevalent species (in 42 cases; 22.5%) ($p < 0.001$) when only one *Eimeria* species was found in the sample pools, followed respectively ($p < 0.001$) by *E. acervulina* (38 cases; 20.3%), *E. tenella* (5 cases; 2.7%), *E. necatrix* (3 cases; 1.6%), *E. praecox* (one case; 0.53%) (Table 3). The prevalence of single infections was 47.6% compared with 51.9% for mixed infections (41,2 %, 7.5% and 3.2% respectively for double, triple and quadruple infections) There was a significant

difference between the prevalences of single, double, triple and quadruple infections (P <0.001) (Table 3).

Mixed infections (double, triple or quadruple infections) were found in 97 (52.15%) flocks. Numerically, the most prevalent combinations were *E. acervulina* + *E. maxima* (60/187; 32.1%), followed respectively by *E. acervulina* + *E. maxima* + *E. necatrix* (6 /187; 3.2%), *E. maxima* + *E. necatrix* (5 /187; 2.7%), *E. acervulina* + *E. necatrix* (4 /187; 2.1%) and *E. acervulina* + *E. maxima* + *E. tenella* (4 /187; 2.1%), *E acervulina* + *E. maxima* + *praecox* (3 /187; 1.6%) (Table 3).

Double infections *E. acervulina* + *E. tenella, E. acervulina* + *E. mitis* and *E. maxima* + *E. tenella*, each have a prevalence of 1.1%, while all quadruple infections each have a frequency of the order of 0.5% (Table 3).

4. Discussion

In this study, the prevalence of *Eimeria* spp was measured using molecular approaches (ITS1-PCR) and found to be very high (99.5%) across all farms. which is probably due to the poor hygienic conditions and / or poor control of rearing techniques, in particular, the high stocking density. Generally, in Algeria, the stocking density of broilers is greater than 10 / m^2 around the seventh week which may be a factor accounting for the high prevalence. Few other studies exist on *Eimeria* prevalence in Algerian broilers, to our knowledge. Debbou-louknane et al., (2018) found a much lower prevalence of 93/147 (63.3%) of litter samples or 78/109 (71.6%) chicken intestinal contents with an overall prevalence rate of 54.3% farms infected. This study was conducted in the Bejaia province (adjoining Jijel, Setif and Bordj bou-Arreridj) and it involved parasitological examination and identification of *Eimeria* species using traditional characteristics such as size, shape and pathology. They found *E. acervulina* and *E. tenella* to be the most prevalent species but did not detect *E. necatrix* or *E. praecox*. Our study is the first to use molecular tools and, while its tempting to speculate that the 100% infection rate observed in this study indicates that molecular tools may be a more sensitive, robust comparative studies would be needed to establish this. However, other molecular studies have also reported high prevalences, for example Györke et al. (2013), who obtained two prevalences: 100% and 91%, obtained respectively by flotation

of oocysts and PCR. However, this latter study was conducted in Romania – a significant geographical distance from Algeria – and may not be directly comparable.

Our study confirms the presence of all seven species of *Eimeria* spp in chickens, in the field, in Algeria; with *E. maxima* (69%), *E. acervulina* (68.4%) being the most frequent species. This has also been found by numerous other studies in other locations. Moraes et al. (2015) obtained the same results (*E. maxima* was 63.7%; *E. acervulina* was 63.3%) in a study conducted in Brazil on 251 broiler farms. Jeffers (1974) confirms that these two species of *Eimeria* are ubiquitous and their occurrence is largely unaffected by the anticoccidial medication employed. However, *E. acervulina* is the most prevalent species in France (Williams et al. 1996), in the United Kingdom (Williams, 2006) and in Norway (Haug et al. 2008). This is probably due to the very high reproductive potential of this species (Williams, 2001).

In this study *E. acervulina* and *E. maxima* were recovered as single species from 44 (23.5%) and 36 (19.3%) of the farms sampled, respectively; this observation corroborates other studies (Jeffers, 1974; Haug et al., 2008; Györke et al., 2013; Moraeset al 2015), who have also shown that *E. acervulina* and/or *E. maxima* also dominate single infections in broilers.

Interestingly, the prevalence of *E necatrix* (11.2 %: third place in the species ranking in this study) was higher than that observed in previous European studies (Warren et al. 1966, Hodgson et al., 1969; Williams et al., 1996; Williams et al., 1999) which suggests that this species of *Eimeria* seems to be generally uncommon (or of low prevalence) in Europe. Similarly, McDonald and Shirley (2009) attest that *E. necatrix* is rare in North America. However, studies carried out in Africa, the Middle East and Asia show that *E. necatrix* is one of the most frequent species (4-30%) reported in broilers (Lobago et al., 2005; Aarthi et al., 2010; Shirzad et al., 2011; Awais et al., 2012). Our studies are consistent with this.

Contrary to what is reported in this study (it ranks fourth with a relatively low prevalence 8%), *E. tenella* is one of the predominant species in broilers (Lee et al., 2010; Al-Natour et al., 2012; Awais et al., 2012; Györke et al., 2013; Moraes et al 2015) and also has a very high reproductive potential (Williams, 2001).

The species less frequently detected in our survey were: *E. praecox* (4.3%), *E. mitis* (2.1%), *E. brunetti* (2.1%). Jeffers (1974) observed a prevalence of around 2.3%, of *E. brunetti* in litter from the major broiler-producing regions of the USA. However, according to several authors (Lobago et al., 2005;

Aarthi et al., 2010; Lee et al., 2010), *E. brunetti* is one of the most frequent species in broiler chickens. On 18 samples from broiler farms, Aarthi et al. (2010) obtained the following results: *E. necatrix* (100%), *E. brunetti* (83.33%), *E. tenella* (83.33%), *E. maxima* (77.77%), *E. acervulina* (55.55%), *E. praecox* (16.66%) and *E. mitis* (11.11%).

E. mitis and *E. praecox* are generally underestimated and underdiagnosed species, due to the less identifiable lesion manifestations (McDougald and Reid, 1997). However, these two species have been frequently detected in certain surveys, for example those carried out by Kučera (1990) in Czechoslovakia (50% and 31.25% respectively for *E. mitis* and *E. praecox*), Williams et al. (1996) in France (82% and 45% respectively for *E. mitis* and *E. praecox*), Williams (2006) in the United Kingdom and McDougald et al. (1997) in Argentina (67% and 51% respectively for *E. mitis* and *E. praecox*).

Multiple infections with different *Eimeria* species affecting chickens were the most frequent situation in our samples (51.9% mixed infections versus 47.6% single infections). This conclusion is also generally reached in previous studies (Williams et al., 1996; Haug et al., 2008; Györke et al.,2013). Double infection *E. acervulina*+ *E. maxima* predominates mixed infections with a prevalence of around 60%, while *E. acervulina* dominates single infections (50% of single infections).

Despite the presence of highly pathogenic species, including *E. tenella*, *E. brunetti* and *E. necatrix*, we have not found severe episodes of clinical coccidiosis across the 187 farms we have studied. This observation of subclinical infections is probably the result of the addition of ionophore coccidiostats in the food (three ionophores are used in the surveyed farms: monensin, salinomycin, lasalocid). However, there are concerns that resistance to these coccidiostats may be developing (Djemai et al., 2016). The high background levels of parasite prevalence provide a worryingly large potential baseline on which resistance can evolve. There is little current data concerning subclinical coccidiosis in this region and the baseline data reported here offer the opportunity to be followed up with future studies to investigate the possible progression of resistance.

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Table 1. Number of samples and prevalence of *Eimeria* species by region

	Number of samples	*E.acervulina*	*E.maxima*	*E.tenella*	*E.mitis*	*E.necatrix*	*E.brunetti*	*E. praecox*
Jijel	44	27 (61.4%)	34 (77. 3%)	9 (20.5%)	3 (6.8%)	8 (18.2%)	1 (2.3%)	4 (9.1%)
Constantine	31	25 (80.6%)	14 (45.2%)	1 (3.2%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Skikda	10	9 (90%)	0 (0.0%)	1 (10%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Mila	17	8 (47.1%)	13 (76.5%)	2 (11.8%)	0 (0.0%)	1 (5.9%)	0 (0.0%)	1 (5.9%)
Setif	35	19 (54.3%)	29 (82.9%)	2 (5.7%)	0 (0.0%)	1 (2.9%)	0 (0.0%)	0 (0.0%)
Batna	42	32 (76.2%)	31 (73.8%)	0 (0.0%)	1 (2.4%)	11 (26.2%)	2 (4.8%)	0 (0.0%)
Bordj bou-Arreridj	8	8 (100%)	8 (100%)	0 (0.0%)	0 (0.0%)	1 (12.5%)	1 (12.5%)	3 (37.5%)

Table 2: Prevalence of *Eimeria* species in the 187 broiler farm samples

Eimeria species	Number	Percentage (%) ± Standard deviation
E. maxima	129	69.0±0.464
E. acervulina	128	68.4±0.466
E. necatrix	21	11.2±0.317
E. tenella	15	8.0 ±0.272
E. praecox	8	4.3±0.203
E. mitis	4	2.1±0.145
E. brunetti	4	2.1±0.145
Total of samples	187	100%
P value		<0,001*

* : Significant difference between the prevalence of different *Eimeria* species.

Table 3. Co-infection rates in 187 samples DNA analysed by PCR.

Infection status	Identified species	Number of samples (%± C.I)
Single infection	*E. maxima*	42 (22.5±5.9)
	E. acervulina	38 (20.3±5.8)
	E. tenella	5 (2.7±2.34)
	E. necatrix	3 (1.6±1.44)
	E. praecox	1 (0.53±1.82)
	Total	89 (47.6±7,17)
	P value	<0.001*
Double infection	*E. acervulina+ E. maxima*	60 (32.1± 6,61)
	E. maxima+ E. necatrix	5 (2.7±2.34)
	E. acervulina+ E. necatrix	4 (2.1±2.03)
	E. acervulina + E. tenella	2 (1.1±1.44)
	E. acervulina + E. mitis	2 (1.1±1.44)
	E. maxima+ E. tenella	2 (1.1±1.44)
	E. acervulina+ E. praecox	1 (0.5±1.05)
	E. acervulina+ E. brunetti	1 (0.5±1.05)
	Total	77 (41.2±6.98)
	P value	<0.001*
Triple infection	*E. acervulina+ E. maxima+E. necatrix*	6 (3.2±2.52)
	E. acervulina+ E. maxima+ E. tenella	4 (2.1±2.03)
	E. acervulina+ E. maxima+ E. praecox	3 (1.6±1.8)
	E. acervulina+ E. maxima+ E. brunetti	1 (0.5±1.05)
	Total	14 (7.5±3.79)
	P value	0.175

Quadruple infection	*E. acervulina* + *E. maxima*+ *E. tenella*+ *E. necatrix*	1 (0.5±1.05)
	E. acervulina+ *E. maxima*+ *E. tenella*+ *E. mitis*	1 (0.5±1.05)
	E. acervulina+ *E. maxima*+ *E. necatrix*+ *E. brunetti*	1 (0.5±1.05)
	E. acervulina+ *E. maxima*+ *E. necatrix*+*E. praecox*	1 (0.5±1.05)
	E. acervulina +*E. maxima*+ *E. mitis*+ *E. praecox*	1 (0.5±1.05)
	E. acervulina+ *E. maxima*+ *E. brunetti*+ *E. praecox*	1 (0.5±1.05)
	Total	6 (3.2±2.52)
	P value	1

C.I: 95% confidence interval.