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Prevalence of Eimeria species, detected by ITS1-PCR immobilized on FTA cards, in laying hens in six provinces in northeastern Algeria

--Manuscript Draft--

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Full Title:	Prevalence of Eimeria species, detected by ITS1-PCR immobilized on FTA cards, in laying hens in six provinces in northeastern Algeria
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Abstract:	Avian coccidiosis is an important and widely distributed disease that affects global agricultural economies through losses. In Algeria, there is limited epidemiological and ecological knowledge about this disease and this hinders implementation of control strategies. A recent study, in Algeria, demonstrated a high prevalence and diversity of Eimeria species in broiler chickens. However, very little is known in laying hens which are kept under different husbandry conditions. Samples were collected from 32 laying hen farms located in 6 northeastern Algerian provinces (Algiers, Batna, Bejaia, Bordj Bou Arréridj, Jijel, Mila). These included 22 pre-laying pullet farms, with hens aged between 11 and 17 weeks, and 10 breeding hen farms with older hens (over 20 weeks). FTA cards were used to capture DNA and internal transcribed Spacer 1 PCR (ITS1-PCR) was used to determine the prevalence and composition of Eimeria species in the chickens. This showed the presence of six species of Eimeria with a diverse prevalence range. E. necatrix (62.50%) was the most common species, followed by E. maxima (53.13%), E. tenella (31.25%), E. brunetti (18.75%), E. acervulina and E. mitis (both 0.25%). E. praecox (0%), was absent. All farms studied were infected and 2 or 3 different species of Eimeria were present at the same farm (62.5%) compared to single species infections (37.5%). The concentration of oocytes, per gram of faeces, was higher in caged hens compared to floor-raised hens. This study, taken alongside a previous study involving broiler farms, demonstrated that this parasite is a significant problem in Algeria.

Introduction

At an estimated cost of over £10 billion per year, Coccidiosis is a global parasitic disease affecting chickens (*Gallus gallus domesticus*) with economic consequences caused by production losses and associated control costs [1]. It is caused by the obligate apicomplexan protozoan parasites of the genus *Eimeria*. At least nine species of *Eimeria* are incriminated in poultry disease [2]–[5]. Seven of these that are pathogenic are known to be found in Algeria, namely: *E. acervulina, E. tenella, E. maxima, E. brunetti, E. mitis, E. necatrix*, and *E. praecox* [3]. They infect the cells of the digestive tract of the chicken, driving a life cycle that generates increasing numbers of coccidia. Given the generally close proximity of animals to each other in poultry farming, this presents a significant epidemiological challenge [4], [5]. As such, coccidiosis is one of the most important domestic diseases, and obstacles to success, affecting the global poultry industry, [6]–[8]. A current, additional, concern is the emergence of resistance of *Eimeria* species to anticoccidials [5], [9]. Thus, there is an important need for surveillance and epidemiological studies to understand distribution of *Eimeria* and the details of transmission on different types of poultry farms.

For surveillance, the identification of *Eimeria* species is commonly done by looking for oocysts in faeces or in the tissues of the digestive tract However, with the development of molecular methods, especially the use of ITS1-PCR [10]–[12] it has become a useful tool for identification [13], [14] and epidemiological use [3], [15]. FTA cards can be effectively used to immobilize and store DNA safely, as they bind DNA but denature proteins and viruses, and work well for detection of protozoan DNA [16].

The epidemiological situation of coccidiosis in Algeria is poorly understood. A recent previous study, on broiler farms, demonstrated that the prevalence of *Eimeria* in these farms was high (100% ~~infected~~) [3]. The important question of the importance of this parasite to different husbandry systems in Algeria, such as laying hen farms, has not, to our knowledge, been investigated. Using ITS1-PCR of DNA, immobilised on FTA cards, this study reports the diversity and high prevalence of *Eimeria* species collected from laying hen farms in north-east Algeria. The differences in composition of species between the broiler and laying hen farms are reported and we confirm the importance of this parasite in laying hen farms in Algeria.

Materials and methods

Sampling and sample preparation

Our survey was conducted between March 2015 and February 2016 on 32 intensive laying hen farms located in the following provinces: Algiers, Batna, Bejaia, Bordj Bou Arréridj, Jijel, Mila (Fig.1). The farms selected in the study include the following types: 22 pre-laying pullet farms (hens raised on the floor), with hens aged between 11 and 17 weeks, and 10 breeding hen farms (on the floor) over 20 weeks old.

The total number of birds on all farms studied was estimated at approximately 620,000 (14,000 to 30,000 pullets/farm). In all farms studied, the litter was composed of straw and wood shavings, and the feed distributed to the birds contained ionophore anticoccidials.

For every farm selected, fresh feces were collected directly from the litter at the rate of one drop per 100 birds, avoiding wet areas of the floor [17]. The collected faeces were mixed into a single pool/farm, and the oocysts were then separated and isolated using a standard method [18] as described previously [3]. The isolated non-sporulated oocysts of *Eimeria* spp. were then suspended in a PBS solution (pH 7.2). Repeated centrifugations of the parasite suspensions were carried out in order to obtain one enriched oocyst suspension/farm, (approximately 3,500 to 5,000 oocysts/mL for each suspension). The modified McMaster method was used to quantify the number of oocysts/mL of suspension [19] . Using sterile syringes, 0.4 ml of each enriched suspension of oocysts was individually applied onto Whatman FTA®cards (in the circle indicated on the card) and was identified by a corresponding farm number. The cards were stored at room temperature until use.

For the purpose of performing the PCR analysis, four 3 mm diameter discs were removed from each FTA®card (using a Harris Micro Punch) from the area where the prepared samples were applied (circle indicated on the card). These multiple samples were conducted to avoid spatial sampling bias on the FTA cards [20].

Each disc was washed twice in 200 µl of Whatman FTA purification reagent for 15 minutes each (according to the manufacturer's instructions.). Two 15-minute washes were then performed on each disc in 200 µl of 1 mM TE buffer (10 mM Tris-HCL pH 8.0; 1 mM Ethylenediaminetetraacetic acid pH 8.0); the discs were then transferred to PCR tubes and allowed to dry at room temperature for at least 90 minutes [21].

Identification of chicken *Eimeria* species by ITS-1 PCR

The genomic DNA obtained prepared discs from each FTA card sample was used as a template to PCR amplify the ITS-1 region (Internal transcribed spacer-1), which is specific for each species of chicken *Eimeria* [11] in order to identify the different species of *Eimeria* present in the different discs removed from the FTA® cards. Each disc was be subjected to seven separate PCR reactions corresponding to the seven species of *Eimeria* found previously in chickens in Algeria (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. tenella and E. praecox*).

The reaction volume (25 µl of master mix) containe either one FTA disc or 1 µl genomic DNA (DNA template) solution (positive control), 25 pmol of each primer (respective species-specific forward and reverse primer; Eurofins), 200 nM of each of the four-deoxynucleoside triphosphates (dNTPs), 20 mM Tris, pH 8.4, 50 mM KCl, 3.0 mM $MgCl_2$, one Unit of Taq DNA polymerase (Bioline) and 20 µl sterile water. A positive control (genomic DNA) and negative controls (1 µL of water instead of DNA or FTAdiscs) were run with each PCR reaction. DNA amplifications were carried out in a Stratagene Robocycler (UK) using the following cycling conditions: 1 cycle: 95 °C-7 min; 35 cycles: 95 °C-20 sec, 44–60 °C-30 sec, 72 C-1 min; 1 cycle: 72 °C-5 min [11], [12].

Statistical Analysis

The data were collected and calculated in Microsoft Excel 2019 (version 16.27). The chi-square tests as well as the correlation tests were carried out with the SPSS software (IBM SPSS Statistics, Version 24).

Results

The prevalence of *Eimeria* species found in samples collected from laying hen farms, listed in descending order, showed that *E. necatrix* (~~62.50~~%) was the most common species, followed by *E. maxima* (~~53.13~~%), *E. tenella* (~~31.25~~%), *E. brunetti* (~~18.75~~%), *E. acervulina* and *E. mitis* (both ~~0.25~~%). The species *E. praecox* was not found at any of the farms (0%). The difference between the prevalence of the 6 identified *Eimeria* species is statistically significant within these samples of farms ($p < 0.01$) (Table 1).

The proportion of each species recorded in infected farms in each of the different locations was not significantly different. The only exception was *E. maxima* where the proportion of infected farms differed between regions ($p<0.001$). For this species of parasite, the most affected regions were the wilayas (Algerian term for regions) of Béjaïa, Bordj-Bou-Arreridj and Mila, while, on the other hand, the proportions were lower in Batna and Jijel and absent in the wilaya of Algiers. When comparing the different farm types (Caged hens vs Floor-raised hens) there was no significant difference (Table 1). It should be noted that there was no statistical correlation found between age and number of oocysts found in each sample with the infection rates in any of the species of *Eimeria*.

The presence of multiple infections on each farm was also investigated (Table 2). Single infections were seen on 37.5% of farms whilst double (46.9%) and triple infections (15.6%) were recorded. Differences in combinations of *Eimeria* species were significantly different between these three categories of infection ($p < 0.01$). In the singly infected farms *E. maxima* (18.8%) was the most frequent ($p < 0.01$), followed by *E. necatrix* (9.4%), *E. brunetti* (6.3%) and *E. mitis* (3.13%) (Table 2). In doubly infected farms, *E. necatrix* + *E. tenella* (18.8%) were the most frequent ($p < 0.01$), followed by *E. necatrix* + *E. maxima* (12.5%), *E. maxima* + *E. Brunetti* (6.3%) and finally 3 combinations with the same low percentage of 3.13% which were E. maxima plus E. tenella, E. acervulina plus E. tenella and E. acervulina plus E. necatrix. In a small number of farms, triple infections were observed: *E. necatrix* + *E. maxima* + *E. brunetti* (6.3%); *E. necatrix* + *E. maxima* + *E. mitis* (6.3%); *E. necatrix* + *E. maxima* + *E. tenella* (3.13%) (Table 2). In terms of differences in farm management, the concentration of oocysts, per gram of faeces, was significantly higher in caged hens compared to Floor-raised hens ($p<0.05$) (Table 3).

Discussion

To our knowledge, no study has been previously been carried out on the prevalence of *Eimeria* species in laying hen farms in Algeria. The present study made use of ITS1-PCR and storage of DNA on FTA cards to identify the diversity of *Eimeria* species in Algerian laying hens. It showed that100% of the laying hen farms had infection with *Eimeria* species This result also concurred with a recent survey of broiler farms carried out in Algeria [3] which also demonstrated 100% of farms with an infection. This suggests that coccidiosis is widely distributed across different types of poultry farms in different regions of the country. Several factors can favor infections with coccidiosis in Algeria such as environmental conditions, with the higher temperatures and the humidity found in deep litter favorable to the sporulation of oocysts. Although these data are collected from Algeria, these conditions, and possibly associated higher disease frequency, may be found in many areas with intensive management systems, associated poor management and lack of hygienic conditions prevail [2], [3], [22]–[24]. Interestingly, analysis of fecal oocyst concentration, in this study, indicated that the concentration of oocytes in faeces was higher in caged hens compared with floor-raised hens. This is in

contrast to a study conducted in South Korea [9] who found no difference between the two types of poultry management systems. Our observations suggested that high oocyst concentrations were associated with a higher density of animals kept in a confined space. Perhaps the conditions were more intensive than those observed in the Korean study for the caged animals. In Algeria, future layers and breeding stock receive only one anticoccidial in the feed for several years (continuous program) which can foster anticoccidial drug resistance in chicken coccidia; in other studies around the world, resistance can appear after a few months or even a few years of consumption of anticoccidials [5], [9]. However, the excretion of oocysts is a poor indicator for evaluating drug resistance of poultry *Eimeria* spp. against anticoccidials.

A previous study demonstrated the presence of 7 species of *Eimeria* on broiler farms in Algeria (*E. necatrix, E. maxima, E. tenella, E. brunetti, E. acervuline, E. mitis*, and *E. praecox*) [3] using the ITS1-PCR detection system. In this study, despite using the same detection system on pooled faecal matter from approximately 620000 birds, *E. praecox* was not detected.

When looking at the prevalence different *Eimeria* species, differences were seen between the laying hen farms, reported here, and the broiler farms. In this study *E. necatrix* (62.50%) was the most predominant species compared to the two other abundant species *E. maxima* (53.13%) and *E. tenella* (31.25%). These results are in agreement with those found in studies, in Asia, [8], which showed that *E. necatrix* (27.7%) was the most predominant species, compared to *E. maxima*, *E. tenella* (22.2%) and *E. acervulina* (19.4%) and [25] which showed , *E. necatrix*, *E. brunetti*, *E. maxima* in all samples. However, in our other study on the broiler farms [3], *E. maxima* (69%) followed by *E. acervulina* (68.4) with *E. necatrix* much lower (11.2%). The lower prevalence of *E. necatrix* has been reported in several regions of the world such as Asia [26]–[28], Europe [29]–[32] and in Africa [3]. It is not clear whether these differences indicate anything significant, such as poultry farm type, geographical location, age of birds or timing of collection, but they maybe just due to a perpetual change in the population dynamics of *Eimeria* species infecting poultry [9]. In the present study hens were aged (over 6 weeks) and according to some reports [33], *E. necatrix* often affects older birds.

In this study, double and even triple infections were detected on the same farms. The most frequent situation was co-infections of two species (62.5%) which compared with 37.5% for singly infected farms.) This situation was als reported for the broiler farm study in Algeria [3] suggesting that this is commonplace, at least in Algeria.

In summary, this study on laying hen farms, taken together with our previous study on broiler farms, shows that infection with *Eimeria* species is commonplace on poultry farms in Algeria and that coccidiosis is an important problem. Furthermore, an implication, based on both studies where birds are fed rations containing anticoccidials, is that resistance to anticoccidials is rife on poultry farms in Algeria. Further studies are required to fully establish this but there may be a concern that extensive use of these drugs may be driving the evolution of resistance. It raises the question as to whether withdrawal of anticoccidials would either exacerbate disease or enable return to a situation where these drugs were effective when needed. Farmers may be being driven towards the use of high-cost drugs which have little efficacy. The role of multispecies infection on farms in Algeria is also highlighted and raises the question as to whether exposure to a pool of different species might exacerbate transmission and hinder development of natural flock immunity. It raises the interesting possibility of complex "ecosystems" of species driving different epidemiological scenarios depending on external conditions. Overall, the study demonstrates the need to consider the role of coccidiosis in the delivery of poultry farming in Algeria [5].

Conclusion

The present study made use of ITS1-PCR and storage of DNA on FTA cards to identify the diversity of Eimeria species in Algerian laying hens shows that avian coccidiosis poses a real problem in poultry farming of laying hens, and other works are necessary to identify this problem, especially with the appearance of resistance to anticoccidials.

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Table 1: Prevalence of *Eimeria* spp. in 32 farms collected from laying hen farms using species specific PCR

	***Eimeria* species: positive number (Percentage%) / Standard deviation**								
Parameters	***E. necatrix***	***E. maxima***	***E. tenella***	***E. brunetti***	***E. acervulina***	***E. mitis***	***E. praecox***	**Total of samples**	**P. value**
Total	20 (62.50) /0.492	17 (53.13) /0.507	10 (31.25) /0.471	6 (18.75) /0.397	2 (0.25) /0.246	2 (0.25) /0.246	0 (0.00) /0.00	32 (100%)	<0,01*
Location	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	
Béjaïa	1/3 (33.33)	3/3 (100)	0/3 (0)	1/3 (33.33)	0/3 (0)	0/3 (0)	0/3 (0)	3/3 (100)	
Bordj-Bou-Arreridj	3/5 (60)	5/5 (100)	1/5 (20)	0/5 (0)	0/5 (0)	1/5 (20)	0/5 (0)	5/5 (100)	
Mila	3/6 (50)	6/6 (100)	2/6 (33.33)	0/6 (0)	0/6 (0)	0/6 (0)	0/6 (0)	6/6 (100)	
Batna	5/7 (71)	2/7 (29)	4/7 (57)	2/7 (29)	0/7 (0)	0/7 (0)	0/7 (0)	7/7 (100)	
Jijel	3/4 (75)	1/4 (25)	2/4 (50)	1/4 (25)	1/4 (25)	0/4 (0)	0/4 (0)	4/4 (100)	
Algiers	5/7 (71.43)	0/7 (0)	1/7 (14.29)	2/7 (28.57)	1/7 (14.29)	1/7 (14.29)	0/7 (0)	7/7 (100)	
P. value	0.814	<0.001**	0.364	0.557	0.470	0.591	N		
Total	20/32 (62.5)	17/32 (53.13)	10/32 (31.25)	6/32 (18.75)	2/32 (6.25)	2/32 (6.25)	0/32 (0)	32 /32	
Type of poultry	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	P/T (%)	
Caged hens	6/10 (60)	3/10 (30)	1/10 (10)	3/10 (30)	1/10 (10)	1/10 (10)	0/10 (0)		
Floor-raised	14/22 (63.64)	14/22 (63.64)	9/22 (41)	3/22 (13.64)	1/22 (4.55)	1/22 (4.55)	0/22 (0)		
P. value	0.844	0.077	0.08	0.272	0.555	0.555	N		
Total	20/32 (62.5)	17/32 (53.13)	10/32 (31.25)	6/32 (18.75)	2/32 (6.25)	2/32 (6.25)	0/32 (0)		

P/T : Number of positive farms/ Total number of farms
* Significant difference
N: none

Table 2: Co-infection rates in 32 samples DNA analysed by PCR

Infection status	**Identified species**	**Number of samples (% C.I)**
Single infection (a)	*E. maxima*	6 (18.8±4.3)
	E. necatrix	3 (9.4±3.2)
	E. brunetti	2 (6.3±2.7)
	E. mitis	1 (3.13±1.9)
Total		12 (37.5± 5.3)
P value		<0.01*
Co-infections		
Double (b)	*E. necatrix* + *E. tenella*	6 (18.8±4.3)
	E. necatrix + *E. maxima*	4 (12.5±3.7)
	E. maxima + *E. brunetti*	2 (6.3±2.7)
	E. maxima + *E. tenella*	1 (3.13±1.9)
	E. acervulina + *E. tenella*	1 (3.13±1.9)
	E. acervulina + *E. necatrix*	1 (3.13±1.9)
Total		15 (46.9±5.5)
P value		<0.01*
Triple (c)	*E. necatrix* + *E. maxima*+ *E. brunetti*	2 (6.3±2.7)
	E. necatrix + *E. maxima* +*E. mitis*	2 (6.3±2.7)

	E. necatrix + *E. maxima*+ *E. tenella*	1 (3.13±1.9)
Total		5 (15.6±4.0)
P value		>0,05
No infection	*E. praecox*	1 (3.13±1.9)
P value (a) (b) (c) (d)		<0.01*

C.I.: 95% confidence interval
* Significant difference

Table 3: Influence of the type of poultry on the average concentration of fecal oocysts

Types of poultry	**Number of samples**	**Average of fecal oocyst concentration/g**
Caged hens	10	14228,9
Floor-raised	22	13115,1
P value		<0.05*

* Significant difference

Geographical location of region study, Algeria

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