Anticoccidial resistance in poultry: determination of ionophore sensitivity for *Eimeria acervulina* and *Eimeria maxima* isolated from broiler chicken farms in Tizi Ouzou province (Algeria)

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Abstract

This study aimed to determine the resistance of coccidia to ionophores used in broiler farms in Tizi-Ouzou province, Algeria. Droppings were collected and recovered *Eimeria* oocyst isolates were analysed by morphometry to determine their composition, and then inoculated by peros into chicks of the Arbor Acres strain, reared on the ground. Four of six groups of chicks were treated to test the sensitivity of oocysts to four anticoccidial agents added to their growth feeds [(robenidine (33 ppm), monensin (120 ppm), narasin-nicarbazin (80 ppm) and salinomycin (60 ppm)], while the other two groups were controls. The results revealed the presence of total resistance to monensin and robenidine, and partial resistance to salinomycin and the narasin-nicarbazin combination. The lack of sensitivity to monensin and robenidine was unsurprising, given their inappropriate and unreasonable use for years as the only anticoccidial compounds. The appearance of

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partial resistance to narasin-nicarbazin and salinomycin suggests the development of cross-resistance in the *Eimeria* population. The possibility of a relatively uniform composition of *Eimeria* species collected in these farms indicates that *Eimeria acervulina* and *Eimeria maxima* develop resistance more quickly to these ionophores. Finally, a control strategy must be rigorously developed by considering other molecules that are alternatives to anticoccidials.

Key words: *anticoccidial; coccidiosis; ionophore; lesion score; resistance*

Introduction

Avian coccidiosis is a common widespread disease associated with considerable economic losses to poultry farmers worldwide (Raman et al., 2011; Haritova et al., 2013). It can affect birds raised in any production systems and for any production purposes. The parasites causing coccidiosis in poultry include a wide range of single-celled protozoans of the genus *Eimeria* (Varenina et al., 2017). Seven species of *Eimeria* (*E. acervulina, E. brunetti, E. maxima, E. mitis, E. necatrix, E. praecox and E. tenella*) are known to affect chickens. Each of the seven species occurs in a single host species or a group of closely related hosts, and they invade the lining of the intestine or ceca, causing death or reduced productivity in poultry. Infection by coccidian parasites in sufficient numbers produces clinical manifestations of the disease (Conway and McKenzie, 2007). Methods for avian coccidiosis prevention and treatment has been studied and developed in recent years, and includes good husbandry practices and the use of anticoccidial drugs. However, extensive use of anticoccidials has resulted in the development of resistance (Gussem, 2007).

In the study area, anticoccidial control is purely preventive by using anticoccidial agents in food. The lack of knowledge of the factors of appearance and maintenance of the parasite in these farms has led to the emergence of resistance (Kostadinović et al., 2016). In this study, *Eimeria* oocysts were isolated from poultry farms with an average population of 2000 chicks, located in the Tizi-Ouzou province (Algeria). These parasitic forms were tested for their sensitivity to two ionophores: (monensin, salinomycin), a synthetic product (robenidine) and a mixed product (narasin-nicarbazin).

Material and methods

Animals and breeding management

After building cleaning and disinfection, breeding areas were prepared to accommodate 270 day-old chicks. This mixed batch of the Arbor Acres strain was purchased in a private hatchery and raised on the ground until 30 days of age (study duration) and under sufficient lighting for 24 hours. Subjects were placed in groups for the first ten days at a rate of 50 chicks/m^2. At 10 days, the chickens were distributed according to weight so that the average weight of each group is the same. Each group consists of 4 replicates of 10 chickens and is fed on a standard starter and growth ration of non-medicated broiler poultry up to 20 days with water *ad libitum*. Control and supplemented feeds are manufactured in a private feed manufacturing unit.

Oocyst sporulation and species identification

After collecting chicken droppings, one part was analysed for composition and the other was mixed in a pooled sample from which *Eimeria* oocysts were

recovered using standard procedures (Ryley et al., 1976). Isolated *Eimeria* oocysts were sporulated and stored at 4°C until analysis. The nature of the *Eimeria* oocysts sampled was determined by morphometry in the laboratory (National High School of Veterinary Medicine, Algiers-Algeria) at a magnification of 1000×, based on the known average length and width of seven species of *Eimeria* (Long and Reid, 1982). Morphometry analysis revealed that the suspension showed a predominance of *Eimeria acervulina* oocysts over *E. maxima*.

Drug sensitivity tests

Table 1 explains the different groups tested during the 30 days of the study. Groups 1–4 received medicated feed from day 20, and Groups 5 and 6 (untreated controls) received non-medicated feed for the entire study. On day 22, chickens in Groups 1–5 were orally inoculated with 232.000 *E. acervulina* and 122.000 oocysts of *E. maxima*. Chickens in Group 6 (untreated, uninoculated controls) were not exposed to *Eimeria* oocysts.

Parameters studied

In the study, certain zootechnical and clinical parameters were retained:

- average weight was calculated by weighing all chickens individually at the age of 10 days, 22 days (day of the *Eimeria* oocyst test) and 30 days (end of the study);
- study duration was divided into three time intervals to calculate weight gain: days 10 to 22 (pre-*Eimeria* test), days 22 to 30 (post-*Eimeria* test), and days 10 to 30 (full study);
- the food consumption index was calculated by dividing the average quantity of food consumed by the average weight gain in each batch for days 22 to 30;
- intestinal lesion scores were observed on 20 chicks autopsied from each group on the last day of the study (day 30) using standard techniques (Johnson and Reid, 1970);
- faeces were collected between days 27 and 30 (5 to 8 days after inoculation) to quantify the number of *Eimeria* oocysts using a modified McMaster method (Taylor et al., 1995);
- daily mortality was calculated in all groups between days 22 and 30. Dead animals were weighed and autopsied to detect the presence of intestinal coccidiosis lesions.

All the experiments were carried out according to the guidelines of the Institutional Animal Care Committee of the Algerian Higher Education and Scientific Research (Agreement Number 45/DGLPAG/DVA.SDA.14).

Statistical analysis

All data were entered into a Microsoft Excel 2010 spreadsheet and analysis with the ANOVA test for one factor using XLSTAT software (version 2016.02.28451). Treated groups were compared to the untreated inoculated control (Group 5) and untreated control (Group 6) for statistical difference ($P<0.05$). Other statistical tests were used for multiple comparisons after the ANOVA test (Tukey test, REGWQ method and Dunnett test) for the following parameters: weight gain between D10 and D22, D22 and D30, D10 and D30, feed conversion index between D22 and D30 and injury scores. For the analysis of oocyst excretion, we used the Kruskal-Wallis test, though for mortality rates and cumulative mortality on day 30, we used the Chi-squared test. An exponential value was assigned to the mean values of all parameters to indicate a significant difference from the INT (infected untreated; Group 5) and NINT (non-uninfected untreated; Group 6) controls.

Resistance assessment

The intensive use of anticoccidial drugs has led to the development of resistance, which can be detected using different indices and criteria. In this study, resistance to several anticoccidial agents used against *Eimeria* isolates was investigated, and two approaches for evaluating anticoccidial efficacy were taken:

- In the first, lesion scores between untreated *Eimeria* groups and treated *Eimeria* groups were compared using a standard scale (McDougald et al., 1986). In this approach, referred to as Anticoccidial Sensitivity Profile 1 (ASP1), the percentage reduction is calculated as: 100% - (MSL of treated group / MSL of untreated *Eimeria* group × 100%). A percentage reduction indicates: 0-30% resistance, 31–49% reduced sensitivity or partial resistance, and ≥ 50% total sensitivity to the anticoccidial compound tested.
- In the second, referred to as ASP2, the anticoccidial index (ACI; Lan et al., 2017) was applied, and is calculated as: (% survival +% weight gain compared to uninfected control) - (mean lesion score × 10 + [mean number of oocysts / 10^6] × 0.4). Isolates were considered sensitive with a ACI > 160 index, reduced sensitivity or partial resistance when the ACI index was between 120–160, and total resistance when the ACI index <120.

Results

The results are presented in Table 2. No significant differences were observed before inoculation with Eimeria oocysts (day 22) between groups treated with robenidine (1) and salinomycin (2), monensin (3) vs the controls (5, 6), narasin (4) vs the controls (5, 6). However, a significant difference was observed between groups treated with robenidine (2) vs narasin (4), robenidine (2) vs the controls (5, 6), robenidine (2) vs monensin (4), salinomycin (1) vs narasin (4), salinomycin (1) vs the controls (5,6), salinomycin (1) vs monensin (3), and monensin (3) vs narasin (4).

Salinomycin

According to Table 2, average weight gain and consumption index did not differ significantly between day 22 and day 30 (infection period) and days from 10 to 30 (study duration). In addition, average weight gain was greater than the INT control and lower than the NINT control, while the consumption index was lower than the INT control and greater than the NINT control ($P<0.05$). Oocysts showed partial sensitivity to salinomycin, because in this group and as revealed by oocysts production which was about 3 times less, mortality was also lower than in the INT controls, and duodenal and jejunal lesions were observed (Table 3). With reference to Table 4, the use of RILS and ACI indicated the presence of partial sensitivity to salinomycin.

Robenidine

Table 2 showed that average weight gain and consumption index was less significant between day 22 and day 30 (infection period) and days from 10 to 30 (study duration). In addition, the average weight gain was slightly higher than the INT control and significantly lower than the NINT control, while the consumption index was lower than the INT control and higher than the NINT control ($P<0.05$). We also noticed a minor production of oocysts in chickens treated with robenidine when mortality was lower than in the INT controls, which suggested resistance to this

ionophore; and this could be explained by lesions with holes in the duodenum and jejunum, due likely to *E. acervulina* and *E. maxima* (Table 3). Table 4 indicates the presence of oocyst resistance to robenidine based on the calculation of RILS and ACI.

Monensin

Table 2 revealed that the average weight gain and the consumption index were slightly less between days 22 and 30 (infection period) and days from 10 to 30 (study duration). In addition, the average weight gain was slightly higher than the INT control and much lower than the NINT control, while the consumption index was lower than the INT control and higher than the NINT control (P<0.05). In broiler chickens treated with monensin, oocyst production and mortality were near that of INT controls, suggesting complete resistance to this ionophore (Table 3), while lesion scores did not differ significantly. Results of RILS and ACI in Table 4 supported the presence of complete resistance.

Table 1. Description of treatment groups in the anticoccidial sensitivity tests of *Eimeria acervulina* and *Eimeria maxima* isolated from poultry farms

Group number	Anticoccidial agent	Trade name	Concentration (ppm)	Inoculation test
1	Salinomycin	Sacox (Uvepharma)	60	Yes
2	Robenidine	Cycostat 66G (Alpharma)	33	Yes
3	Monensin	Coxidine (Uvepharma)	120	Yes
4	Narasin-Nicarbazin	Maxiban (Elanco)	80	Yes
5	none	/	/	Yes
6	none	/	/	No

Table 2. Sensitivity of *Eimeria acervulina* and *Eimeria maxima* oocysts isolated from broiler farms to 4 anticoccidial drugs measured by weight gain and feed conversion efficiency

Group number	Inoculation test	Weight gain (g)*			Consumption index*
		D10-D22	D22-D30	D10-D30	
1 (salinomycin)	Yes	366±4.5^{a}	378±8.3^{b}	744±6.5^{a}	1.8±0.04^{a}
2 (robenidine)	Yes	365±3.8^{a}	341±4.3^{c}	706±5.3^{b}	2.0±0.03^{b}
3 (monensin)	Yes	369±4.8^{b}	330±5.3^{d}	699±5.1^{c}	2.0±0.04^{c}
4 (narasin-nicarbazin)	Yes	373±6.3^{c}	492±19.2^{a}	865±19.3^{d}	1.3±0.02^{d}
5 (INT)	Yes	370±4.7b^{c}	289±4.8^{e}	659±6.0^{e}	2.3±0.04^{e}
6 (NINT)	No	371±5.2^{bc}	588±7.6^{f}	959±5.7^{f}	1.1±0.01^{f}

*Averages with same superscripts in a row do not differ (P<0.05) as indicated by ANOVA, Tukey test, REGWQ method and Dunnett test.

Table 3. Sensitivity of *Eimeria acervulina* and *Eimeria maxima* oocysts isolated from broiler farms to four anticoccidial drugs, measured by intestinal lesion score, oocyst excretion and mortality

Group number	Inoculation test	Intestinal lesion score*		Oocyst *	
		Duodenum	Jejunum	Excretion (10^4 opg/d)	Mortality (%)
1 (salinomycin)	Yes	2.0 ± 0.6^{ab}	2.2 ± 0.7^{a}	21 ± 3.5^{a}	20 ± 1.3^{a}
2 (robenidine)	Yes	2.5 ± 0.7^{bc}	2.4 ± 1.0^{a}	39 ± 8.5^{b}	25 ± 1.2^{b}
3 (monensin)	Yes	3.1 ± 0.8^{cd}	3.2 ± 0.8^{b}	49 ± 7.9^{c}	30 ± 1.3^{c}
4 (narasin-nicarbazin)	Yes	1.7 ± 0.6^{a}	1.9 ± 0.7^{a}	19 ± 7.5^{d}	20 ± 1.1^{a}
5 (INT)	Yes	3.3 ± 0.7^{d}	3.4 ± 0.5^{b}	57 ± 8.4^{e}	30 ± 1.4^{c}
6 (NINT)	/	/	/	/	5 ± 0.5^{d}

opg/d, oocysts per gram of faeces per day; *Means with the same superscript in a row do not differ (P <0.05), as indicated by ANOVA, Tukey's test, REGWQ method and Dunnett test; Kruskal-Wallis test was for used for lesion score and oocyst excretion; Chi-square test used to determine mortality rates.

Table 4. Anticoccidial Susceptibility Profiles (ASP) as indicated by Reduction in the Intestinal Lesion Score (RILS) and Anticoccidial Index (ACI) of *Eimeria acervulina* and *Eimeria maxima* oocysts isolated from broiler chicks

Group number	E. acervulina		E. maxima		ACI	ASP2**
	RILS	ASP1*	RILS	ASP1*		
1 (salinomycin)	39.4	SR	35.3	SR	123	PR
2 (robenidine)	24.2	R	29.4	R	108	R
3 (monensin)	6.1	R	5.9	R	94	R
4 (narasin-nicarbazin)	48.5	SR	44.1	SR	148	PR

* ASP1, anticoccidial sensitivity profile using RILS as a criterion. R, resistant; RS, reduced sensitivity; S, sensitive; ** ASP2, anticoccidial sensitivity profile using ACI as a criterion. R, resistant; PR, partially resistant; S, sensitive

Narasin-Nicarbazin

The results shown in Table 2 demonstrated that average weight gain and consumption index were suitable between days 22 and 30 (infection period) and days from 10 to 30 (study duration). In addition, the average weight gain and ICA in broilers fed with food medicated with the combination narasin-nicarbazin showed a significant improvement (P<0.05) compared to the INT controls. The production of oocysts recorded in broilers treated with narasin-nicarbazin was clearly lower, while mortality was less than in the INT controls, which suggesting partial sensitivity to this product (Table 3). Partial sensitivity observed could be explained by the weak duodenal and jejunal lesions found in poultry. Table 4 showed incomplete sensitivity to narasin-nicarbazin revealed by RILS and ACI.

Discussion

Coccidiosis is considered one of the most significant protozoan parasitic diseases of poultry, and costs the world's commercial chicken producers at least USD 1.5 billion per year (2013).

Despite advances in immunology, biotechnological and genetic methods, prophylactic chemotherapy with anticoccidial drugs is still widely used for control. Unfortunately, the coccidia have readily developed resistance to these chemicals, severely limiting their long-term effectiveness in preventing the disease (Bino Sundar et al., 2017). Intensive chicken farming depends on specific prophylaxis of coccidiosis with in-feed anticoccidial drugs and live vaccines. Drug resistant *Eimeria* strains are responsible for subclinical coccidiosis and, subsequently, for impaired economic performance, including body weight gain and feed conversion ratio (Shirzad et al., 2011).

Drug-resistance and economic impact of infection caused by *Eimeria* spp. was described recently in Romanian broiler farms using monensin, salinomycin, narasin, nicarbazin, robenidine, losalocid, and diclazuril (Györke et al., 2011; 2012). The results presented here revealed complete resistance of *Eimeria* isolates to monensin and robenidine, and the emergence of this resistance was due likely to unreasoned heavy use of anticoccidials. According to Abbas et al. (2011), development of drug resistance in *Eimeria* is commonly due to the unreasonable intensive use of anticoccidials drugs for avian coccidiosis control. Evidence of resistance was elucidated using the anticoccidial sensitivity profile (ASP) based on reduction in lesion score (RILS) or the anticoccidial index (ACI). Indeed, these two parameters showed total resistance to monensin and robenidine in *E. acervulina* and *E. maxima* recovered from poultry farms in the Tizi Ouzou area. The scoring of intestinal lesions in the upper and middle intestine was useful in determining *E. acervulina* and *E. maxima* resistance to monensin and robenidine, as supported by weak ASP1 for both species (*E. acervulina* 6.1, *E. maxima* 5.9) and (*E. acervulina* 24.2, *E. maxima* 29.4). These findings were consistent with the ASP2 index based on ACI.

In addition, our results were in accordance with those reported by several authors who observed the development of monensin-resistant *Eimeria* after continued use of this ionophore in experimental (Kheirabadi et al., 2014) or field studies (Peek and Landman, 2006; Chang et al., 2016). Djemai et al. (2016) demonstrated complete resistance to monensin and narasin based on lesion scores and the anticoccidial index.

These authors reported that a lack of sensitivity to monensin is not surprising since it is used as the sole anticoccidial agent, and they suggested that resistance to monoether (narasin) and polyether (lasalocid) ionophores could be attributed to cross-resistance developed in the *Eimeria* population. Stephan et al. (1997) attested that there were also multiple resistances in monensin, halofuginone, nicarbazin, robenidine, diclazuril and toltrazuril. Other studies have also highlighted the continued decline in the effectiveness of ionophores used in coccidiosis control. For example, monensin and other ionophores have been found to have little or no efficacy (Mathis and McDougald, 1982; Agatha et al., 2018).

On the other hand, daily weight gains in the group treated with robenidine were significantly improved compared to the untreated infected batch. Lesions due to *E. acervulina* were significantly reduced by all anticoccidials. For *E. maxima*, monensin, salinomycin, lasalocid and especially robenidine significantly reduced lesions.

Robenidine reduced oocyst excretion by *E. maxima* to a level below the detection limit (Naciri et al., 2003). Kaewthamasorn et al. (2015) demonstrated the efficacy of salinomycin, robenidine and decoquinate against coccidia in a densely populated area of chicken farms in Thailand.

The partial resistance observed in groups treated with salinomycin and

the combination narasin-nicarbazin is reasonable compared to the other two treated groups. Indeed, the anticoccidial sensitivity profile based on use of RILS and ACI respectively showed a partial resistance of *Eimeria* isolate to salinomycin and the narasin-nicarbazin combination (*E. acervulina* ASP1 = 39.4, *E. maxima* ASP1 = 35.3; ASP2 = 123) (*E. acervulina* ASP1 = 48.5, *E. maxima* ASP1 = 44.1; ASP2 = 148). We also observed that the production of oocysts was nearly identical between the two groups, indicating partial resistance despite a reduction in oocyst production compared to the untreated inoculated control.

Oocyst production in groups receiving narasin-nicarbazin or salinomycin indicated difficulties in completely preventing parasite development, even when performance showed partial resistance to an anticoccidial. We hypothesized that incomplete efficiency of ionophores could be advantageous, since it allows sufficient parasitic development to induce protective immunity.

Our results were in accordance with those reported by Gerhold (2010), on the identification and control of *Eimeria* species-associated coccidiosis in northern bobwhites. These authors demonstrated very good to excellent performance with narasin + nicarbazine, sulfadimethoxine + ormetoprine, clopidol, decoquinate, diclazuril (1 and 2 ppm), lasalocid, robenidine and zoalene (150 ppm).

In addition, Bafundo and Jeffers (1990) illustrated the potential of *Eimeria acervulina* to develop resistance to NIC and to potentiated NIC combinations (e.g., NAR + NIC). These authors reported that the same trend was unlikely for *Eimeria tenella*. They recommended limiting the use of potentiated NIC combinations such as NAR + NIC to starter feeds to minimize resistance development risks. Bafundo et al. (2008) stated that the majority of coccidian strains evaluated retained their sensitivity to NIC, and that it is reasonable to assume that the loss of activity associated with the combination NAR + NIC is associated with a reduction in effectiveness of the ionophore part of the combination.

According to Arabkhazaeli et al. (2013), the results of sensitivity tests indicated that none of field isolates was completely sensitive to the selected anticoccidial agents. All isolates showed reduced sensitivity / partial resistance to salinomycin.

The recent findings of Gerhold et al. (2011) in North America, for example, demonstrated that excellent to good efficacy was found for decoquinate (30 ppm), robenidine (33 ppm) while moderate to high resistance was found using salinomycin (60 ppm). Additionally, salinomycin was found to be partially resistant in the Middle East (Arabkhazaeli et al., 2013).

In this study, the susceptibility of isolates to anticoccidials was assessed using two different approaches (RILS and ACI), though other parameters could also be considered. According to the scientific literature, several indices are used to evaluate efficacy of anticoccidial drugs, based on several factors, such as weight gain, feed conversion ratio, lesion score, oocyst output, survival rate, etc. Understanding these factors is fundamental to evaluating the widespread development of resistance using various indices (Optimum Anticoccidial Activity, Global index...) (Bino Sundar et al., 2017). In this study, use of an index is preferred because of the unchallenging task of measuring group weight in OAA, and therefore this index can be proposed as the single means for evaluating drug resistance to allow for the comparability of studies (Arabkhazaeli et al., 2013).

The results provided by experiments are insufficient to show the true sensitivity profile of *Eimeria* isolates

present in faeces collected from the field. This can be explained by the low diversity of oocyst species isolated and determined using only morphometry, due to the possibility of the presence of other species. Therefore, PCR remains safest and most sensitive way to demonstrate the composition of isolates from the field. However, most authors analysing resistance to ionophores have found a low diversity of *Eimeria* species in the faeces, with *E. acervulina* and *E. maxima* or *E. tenella* usually present (Daugschies et al., 1998; Peek and Landman, 2003; Jenkins et al., 2010).

According to Djamai et al. (2016), the results suggested several possibilities. One is that *E. maxima, E. tenella* and particularly *E. acervulina* could more easily develop resistance to anticoccidial drugs compared to other *Eimeria* species infecting chickens. Another is that under field conditions, *Eimeria* species have a selective advantage (for example, resilience, drying, invasion and faster development at intestinal sites invaded by other *Eimeria* species, higher fertility), which leads to an increase in the number of broods. The relative abundance of different *Eimeria* species is likely based on multiple factors which are yet to be fully understood.

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Otpornost na antikokcidike u peradi: određivanje osjetljivosti na jonofor za *Eimeria acervulina* i *Eimeria maxima* izoliranih na farmama tovnih pilića u provinciji Tizi Ouzou (Alžir)

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Cilj je studije bio odrediti otpornost kokcidija na jonofore rabljene na farmama tovnih pilića u provinciji Tizi-Ouzou. Prikupljen je izmet i pronađeni izolati *Eimeria* oociste su analizirane morfometrijom da bi se odredio njihov sastav te su njima *per os* cijepljeni pilići Arbor Acres soja, uzgojeni na zemlji. Četiri od šest skupina tih pilića podvrgnuto je ispitivanjem osjetljivosti oocista na četiri antikokcidika dodanih njihovoj hrani [(robenidin (33 ppm), monenzin (120 ppm), narazin-nikarbazin (80 ppm) i salinomicin (60 ppm)], druge dvije skupine bile su kontrolne. Rezultati studije otkrili su prisutnost potpune otpornosti na monenzin i robenidin, kao i djelomičnu otpornost na salinomicin i kombinaciju narazin-nikarbazin. Izostanak osjetljivosti na monenzin i robenidin nije bio iznenađujući obzirom na njihovu neprikladnu i nerazumnu dugogodišnju uporabu kao jedine antikokcidne tvari. Pojava djelomične otpornosti na narazin-nikarbazin i salinomicin ukazuje na postojanje razvoja unakrsne otpornosti u populaciji *Eimeria*. Mogućnost relativno ujednačenog sastava *Eimeria* vrste prikupljene na ovim farmama upućuje na to da *Eimeria acervulina* i *Eimeria maxima* brže razvijaju otpornost na rabljene jonofore. Zaključno, potrebno je rigorozno razviti strategiju kontrole razmatrajući druge molekule koje su alternativne antikokcidicima.

Ključne riječi: *antikokcidici, kokcidioza, jonofor, bodovanje lezija, otpornost*