Rapid Development of Drug-resistant Strains of *Eimeria tenella* to Diclazuril and Halofugenone in Laboratory*

LI An-xing[1], *XIE Ming-quan*[2], *CAI Jian-ping*[2], *WU Hui-xian*[2], *LI Xiang-hua*[1]
(1. School of Life Science, Zhongshan University, Guangzhou 510275, China
2. Veterinary Medicine Institute, Agricultural Science Academy of Guangdong Province, Guangzhou 510650)

Abstract: The development of resistance of the GS strain of *Eimeria tenella* to the anticoccidial drugs halofugenone and diclazuril has been studied. Resistance to halofugenone and diclazuril developed readily in experiments where a large number of coccidia were exposed to the drugs, either by increasing the number of oocysts in the inoculum or by increasing the number of birds in the group. When 100 birds were given 2×10^6 oocysts commenced from an initial concentration of 0.6×10^{-6} halofugenone or 0.125×10^{-6} diclazuril, resistance to halofugenone and diclazuril appeared after 6 and 5 passages respectively. The passaged lines did not recover their sensitivity to these 2 chemicals after 5 further passages in absence of the use of drug, suggesting that the resistance developed was stable. 7 anticoccidial drugs, salinomycin, monensin, maduramycin, lasalocid, zoalene, nicarbazin and clopidol were effective against halofugenone- and diclazuril-resistant line of *E. tenella*, suggesting that halofugenone and diclaruril did not exhibit cross-resistance with the above-mentioned compounds.

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Since Cuckler[1] first reported in 1955 that drug-resistant strain of coccidia could be conducted in experimental condition, the drug-resistance of Eimeria to furancillinum, sulfaquinoxaline, glycarbylamide, Nicarbazin, 2-chero-4-nitrobenzamide, decoquinate, halofugenone, robenidien and amprolium has been successfully developed in the laboratory. Meanwhile, it is a problem of considerable importance that drug-resistant strains of coccidia occurred widely in the fields[2]. However, mechanism of drug-resistance development of coccidia was amphibolous. The commonly accepted hypothesis about the development of drug-resistant strains of Eimeria is that populations of coccidia become resistant to anticoccidial compounds through the selection of spontaneous resistant mutants which are present, albeit in very small numbers, even in populations which have never been exposed to the drug. The drug serves only to select pre-existing mutants[2]. Study on this must induce experimentally resistance of coccidia to a certain anticoccidial drug. The drug-resistance of coccidia can be obtained by a traditional protocol in which oocysts of coccidia were passaged in chickens with progressively increasing level of anticoccidial in laboratory. In those expirements, the number of medicated chickens used per group was often less than ten, and sporulated oocysts of *E. tenella* used as passage did not exceed 1 $\times 10^5$.

However, that method is time-consuming in developing a drug-resistant line of coccidia and the results do not correlate with the rate at which resistance to a particular drug develops in the field[3]. In the field, especially at the high temperature and in the high humility environment in Guangdong Province of South China, a large crowd of fowls were often infected with high number of sporulated virulent oocysts in short time, and high level concentration of anticoccidial commpounds was used as common additive in feed. As a result, the problem of drug-resistant coccidia in chickens is very severe.

This paper presents a method by which large numbers (100) of birds were medicated with the same level of drug and inoculated with a high number of sporulated oocysts of *Eimeria tenella* to accelerate the development of drug-resistant strains.

1 Materials and methods

1.1 Chickens

AA broilers were obtained from a commercial hatchery, reared coccidia free, and fed with ad libitum. At 2 ~ 3 weeks of age, the birds were weighed and allocated to cages (10 birds/cage with randomisa-

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tion and birds in the cages of approximately equal weights) used as determining initial concentration of drugs. Subsequently, 100 two-week-old chicks per group were used for each passage.

1.2 Coccidial strain

Eimeria tenella, GS strain, chosen for study, descended from a single sporulated oocyst of a field isolate derived from Gansu province in Northwest China and was not known to be resistant to diclazurial, halofugenone, clopidol, salinomycin, madulamycin and monencin.

1.3 Isolation and purification of oocysts

The routine passage and purification of oocysts were performed by the procedures of Wagenback, Challey, and Burns[4].

1.4 Determination of initial concentration of drugs

Eight groups of birds (10 birds/group) medicated respectively with 0.07×10^{-6}, 0.125×10^{-6}, 0.25×10^{-6}, 0.50×10^{-6} of diclazurial and 0.2×10^{-6}, 0.6×10^{-6}, 1.0×10^{-6}, 1.5×10^{-6} of halofugenone were inoculated with 5×10^{4} sporulated oocysts of *E. tenella*(GS) per bird. Seven days later the surviving birds were killed, the cecum removed, and lesion scores and the number of oocysts per one gram of feces were recorded.

1.5 Procedure of the selection of resistance

The parasites were passaged in medicated birds using the method as previously described[5]. Each group consisted of 100 birds and each bird was given a dose of 2×10^{6} oocysts for each passage. The experimental protocol for the development of resistance is given in Table 1. Results of the preliminary experiment indicated that large numbers of sporulated oocysts could be recovered from birds given 0.6×10^{-6} of halofugenone and 0.125×10^{-6} of diclazuril. Selection was therefore commenced at this concentration.

1.6 Evaluation of resistance

After 6 passages in medicated birds given 2×10^{6} sporulated oocysts of *E. tenella*(GS) with halofugenone and 5 passages with diclazuril, the sensitivity of passaged and parental lines of *E. tenella* to halofugenone and diclazuril at their recommended doses were evaluated. Inoculated and ininoculated unimedicated controls were included in the experiment. The effects of the drugs were assessed by POAA (percent of optimum anticoccidial activity), RLS (reduction of lesion scores) and ROP (relative oocyst production)[6].

Tab.1 Experimental protocol for the development of resistance to halofugenone and diclazuril in *E. tenella*[1)]

Passage No.	Mass fraction of drug/10^{-6}					
	halofugenone			diclazuril		
	0.6	2	5	0.125	0.5	1.5
1	61.50[a]/43.20[b]/47.5[c]			72.5/82.3/47.8		
2	33.3/24.1/125.0			43.5/-23.0/111.9		
3		42.5/31.2/58.1			40.5/13.2/91.5	
4			43.5/57.5/98.5			51.5/39.6/111.7
5			-3.5/31.1/125.5			-98.5/-6.9/192.7
6			-24.0/26.1/202.4			

1) a POAA; b RLS; c ROP, referred from Kong F. Y[6]

1.7 Stability of resistance or relaxed selection

Lines that had been passaged in birds given the highest level of diclazuril and halofugenone were passaged 5 more times in groups of 10 non-medicated chickens, each given 1×10^{6} oocysts. The effect of the drugs upon these lines was evaluated.

2 Results

2.1 The development of resistance to halofugenone and diclazuril

E. tenella(GS) rapidly became resistant to halofugenone (6 passages) and diclarudil (5 passages). With halofugenone, the passages commencing with a drug concentration of 0.6×10^{-6} raised it to 2×10^{-6} only through 3 passages. It took 2 passages to raise the drug level to 5×10^{-6}, and 1 further passage to stabilize the drug-resistance. Resistance to 5×10^{-6} of halofugenone, therefore, took totally 6 passages with groups of 100 birds, each given 2×10^{6} oocysts. Equivalent selection in the presence of diclazuril only required 5 passages for inducting resistant line of *E. tenella*(GS) to a high level of 1.5×10^{-6}. The concentrations of these 2 anticoccidial agents with which the resistance of *E. tenella*(GS) was induced in the experiment were above the commercially recommended levels. The results demonstrated that resistance of *E.*

tenella could readily appear to a high level of either halofugenone or diclazuril. Results are shown in Tab.1.

2.2 Effect of halofugenone and diclaruril upon chickens inoculated with drug-passage lines of *E. tenella*

Neither halofugenone nor diclazuril at its commercially recommended dose was effective, judged by comprehensive index including POAA, RLS and ROP against each corresponding resistant line. Their parental line, however, was highly susceptive to halofugenone and diclaruril at the recommended dose for commercial use. Results are presented in Table 2.

Tab.2 Effect of diclarurial and halofugenone upon chickens inoculated with 5×10^4 sporulated oocysts of drug-passaged lines of *E. tenella* and *E. tenella*(GS)

Evaluative parameter	1×10^{-6} diclarurial		3×10^{-6} halofugenone	
	Passaged lines	*E. tenella* (GS)	Passaged lines	*E. tenella* (GS)
POAA	-24.0	98.7	13.2	95.2
RLS	16.1	94.7	44.4	86.2
ROP	69.1	6.2	87.8	10.0

2.3 Relaxed selection

Results of relaxation of selection pressure are presented in Table 3. Neither halofugenone nor diclaruril was ineffective against each corresponding drug-resistant line through relaxed selection when judged by POAA, RLS and ROP. This result demonstrated that halofugenone- and diclaruril-resistance of *E. tenella* was stable. Halofugenone, however, was effective against diclazuril-resistant line and vice versa, indicating that there was no cross resistance between these two anticoccidial agents.

Tab.3 Effects of diclarurial and halofugenone upon chickens inoculated with 5×10^4 sporulated oocysts of drug-passaged lines added 5 more times in absence of drug

Drug	$\frac{w}{10^{-6}}$	Relaxed drug-resistant lines	
		diclarurial (1.5×10^{-6})	halofugenone (7×10^{-6})
diclarurial	1	-31.5[a]/32.4[b]/81.1[c]	87.2/56.3/11.7
halofugenone	3	101.7/78.2/2.3	5.2/18.4/22.3

a POAA; b RLS; c ROP, referred from KONG F.Y[6].

2.4 Response of the halofugenone and diclazuril resistant lines of *E. tenella* to other anticoccidial drugs

The response of the halofugenone or diclaruril-line to the following anticoccidial drugs were investigated; Salinomycin (60), Monensin (120), Maduramycin (5), Lasalocid (90), Zoalene (125), Nicarbazin (125) and Clopidol (250). Groups of 10 birds were inoculated with 5×10^4 sporulated oocysts of 2 resistant lines of *E. tenella*(GS). All these drugs effectively controlled the infection of birds given halofugenone- and diclazuril-resistant line when judged by POAA, RLS, ROP, indicating that the above anticoccidial drugs showed no cross-resistance with halofugenone and diclaruril. The results are shown in Table 4.

Tab.4 Responce of the halofugenone and diclaruzial resistant lines of *E. tenella*(GS) to other anticoccidial drugs

Drug	$\frac{w}{10^{-6}}$	halofugenone	diclazurial
Salinomycin	60	152.26[a]/93.74[b]/6.65[c]	111.66/77.15/12.94
Monensin	120	112.55/65.02/34.55	74.18/47.23/20.18
Maduramycin	5	235.09/87.67/12.54	154.10/62.63/11.23
Lasalocid	90	111.31/55.00/4.59	65.05/33.52/7.09
Zoalene	125	89.33/97.08/67.50	23.55/58.32/13.17
Nicarbazin	125	321.01/76.55/33.25	88.77/92.66/10.01
Clopidol	250	63.66/33.55/12.11	55.11/77.56/24.14

a POAA; b RLS; c ROP, referred from Kong F.Y[6]

3 Discussion

Resistance to diclazuril and halofugenone developed rapidly after passages in large number of chickens given a high number of *E. tenella*(GS) oocysts. But Chapman[5] inducted a partial resistant line to diclazuril after 10 passages of Houghton strains of *E. tenella* and *E. acervulina* and to halohugenone after 8 passages.

The number of experimental birds in each group used by Chapman was ten and the dose of inoculated coccidia was 1×10^5.

Weppelma[7] have shown that resistance to glycarbylamide and amquinolate can be selected, by using 1×10^6 of oocysts in inoculum and 90 chickens in passage, to the commercially recommended level of drug after only 3 passages, but were unsuccessful in developing resistance to monensin and McLoughlin Chute[8] found that resistance to buquinolate, decoquinate and methyl benzoquate had already developed by the 6th and 4th passage respectively, whereas resistance to clopidol was not consistently present until the 17th passage[8]. In an earlier study McLoughlin Gardiner[9] showed that reduction in sensitivity to amprolium was not apparent until 15 passages in the presence of drug. Our results indicated that by using large numbers of oocysts and chickens, the resistance of *E. tenella* to chemical compounds could be rapidly induct-

ed.

Resistant lines to diclazuril and halofugenone did not recover their drug-susceptibility after passaging further more in absence of drugs. Resistance to some anticoccidial drugs, especially chemical compounds, once developed, may render their completely ineffective, and its susceptibility is difficult to recover even in absence of those drugs[7]. This result was somewhat different from that of Chapman[10] who reported that monencin-resistant line was able to restore its sensitivity by passaging 5 more times in nonmedicated chickens. Our results are identical with that of Weppelman, suggesting that chemical compound-resistant lines reproduced as fast as their sensitive-lines, but resistant lines to polyether antibiotic anticoccidial drugs reproduced more slowly than their sensitive lines.

Resistance to halofugenone and diclazuril rose rapidly as a high dose of sporulated oocysts was serially passaged with increasing concentration of drugs in large number of chickens (over 100 birds) given a high number of organisms. Chapman[10] presented a similar report in which resistance to robenidine developed more readily in experiment using larger numbers of birds with higher numbers of oocysts in the inocuberm, suggesting that the speed of development of drug-resistant Eimeria strain is in correlation with the numbers of animals used in passages and oocysts in the inocuberm.

Diclazuril and halofugenone did not show cross-resistance to 9 anticoccidial drugs used in the experiment. This result was in agreement with that of Chapman[5], in whose article diclazuril was effective against lines of *E. tenella* resistant to amprolium, arprinoid, clopidol, dinitolmide, halofuginone, methylbenzoquate, monensin and robenlidine and the results supported that drugs with different mode of action shows no existence of cross-resistance.

References:

[1] CUCKLER A C, MALANGA C M. Studies on drug resistence in coccidia[J]. J Parasitol, 1955, 41: 302 – 311.

[2] CHAPMAN H D. Biochemical, genetic and applied aspects of drug resistance in *Eimeria parasites* of the fowl [J]. Avian Pathol, 1997, 26: 221 – 244.

[3] RYLEY J F, BETTS M J. Chemotherapy of chicken coccidiosis[J]. Advances in Pharmacology and Chemotherapy, 1973, 11: 221 – 293.

[4] WAGENBACK G E, CHALLEY J R, BURNS W C. A methods for purifying coccidial oocysts employing chlorox and sulfuric aciddichromate solution [J]. J Parasitol, 1966, 52: 1222 – 1223.

[5] CHAPMAN H D. *Eimeria tenalla*, *E. acervulina*, and *E. maxima*: studies on the development of resistance to diclarzuril and other anticoccidial drugs in chicken[J]. Parasitology, 1989, 99: 189 – 193.

[6] KONG F Y. Survey of sensitivity to five anticoccidial drugs in 15 isolates of *Eimeria tenella* [J]. Acta Agri Uni Pekinensis, 1994, 20(3): 302 – 307.

[7] WEPPELMAN R M, BATTAGLIA J A, WANG C C. *Eimeria tenella*: the selection and frequency of drug resistant mutants[J]. Exp Parasitol, 1977, 42: 56 – 66.

[8] McLOUGHLIN D K. Efficacy of nequinate against thirteen strains of *Eimeria tenella* and the development of a nequinate resistant strain[J]. Avian Diseases, 1973, 17: 717 – 721.

[9] McLOUGHLIN D K, GARDINER J L. Drug resistance in *Eimeria tenella*. Ⅵ. The experimental development of an amprolium-resistant strain[J]. J Parasitology, 1968, 54: 582 – 584.

[10] CHAPMAN H D. *Eimeria tenalla*: experimental studies on the development of resistance to monensin in the chicken[J]. Parasitology, 1984, 89: 9 – 16.

柔嫩艾美耳球虫对氯嗪苯乙氰和常山酮抗性快速诱导

李安兴，谢明权，蔡建平，吴惠贤，廖翔华

（中山大学生命科学学院，广东 广州 510275）

摘 要：诱导了柔嫩艾美耳球虫（*Eimeria tenella*）甘肃株对两种药物-氯嗪苯乙氰和常山酮的抗药性. 增加实验动物数和球虫的接种剂量可加快柔嫩艾美耳球虫对氯嗪苯乙氰和常山酮抗药性的产生. 每组动物用 100 只 2 ~ 3 周龄 AA 肉鸡，每只鸡接种 2×10^6 个孢子化卵囊，分别从氯嗪苯乙氰的质量比为 0.125×10^{-6}和常山酮的质量比为 0.6×10^{-6}的起始浓度开始，逐渐增加药物浓度，结果表明诱导柔嫩艾美耳球虫对 w(氯嗪苯乙氰)1.5×10^{-6}产生抗药性仅需传 5 代，对 w(常山酮)5.0×10^{-6}产生抗药性需 6 代. 传代株在没有药物的情况下传 5 代后仍不能恢复对氯嗪苯乙氰和常山酮的敏感性，这一结果表明人工诱导对氯嗪苯乙氰和常山酮的抗药性是稳定的. 实验检测了 7 种药物：盐霉素、莫能霉素、马度拉霉素、拉萨洛菌素、球痢灵、尼卡巴嗪和克球粉对传代株的敏感性，结果显示这 7 种药物和氯嗪苯乙氰及常山酮无交叉抗药性.

关键词：柔嫩艾美耳球虫（*Eimeria tenella*）；抗药性；氯嗪苯乙氰；常山酮