Experimental Development of Resistance to Decoquinatone and Robenidine in a Multiple Drug-Resistant Strain of *Eimeria necatrix*

Yasuhide SAITOH, Hiroshi ITAGAKI, and Kiyoshi TSUNODA¹⁾

Department of Parasitology, School of Veterinary Medicine, Azabu University, Fuchinobe, Sagami-hara, Kanagawa 229, and ¹⁾National Institute of Animal Health, Yatabemachi, Tsukuba, Ibaraki 305, Japan

(Received 5 July 1984/Accepted 26 October 1985)

ABSTRACT. A strain of *Eimeria necatrix* isolated in Miyazaki Prefecture, Japan, was examined for its sensitivity to 13 anticoccidials. Nine out of the 13 drugs were not efficacious, but three quinolone derivatives and robenidine were efficacious at their recommended dose-levels. Minimal effective concentration levels of decoquinatone (one of the quinolone derivatives) and robenidine were approximately one-tenth of the recommended dose levels of manufacturers: 4 ppm for decoquinatone and 4.125 ppm for robenidine. Therefore, we examined the reduction in sensitivity of this strain to these drugs by serially passaging in chickens fed the ration containing suboptimal dose-levels of these anticoccidials. The strain deteriorated the sensitivity to minimal effective concentration levels of decoquinatone and robenidine after 50 and 10 passages, respectively. However, the strain was still highly sensitive to recommended-dose levels of decoquinatone and robenidine after 50 and 40 passages, respectively.—**KEY WORDS:** decoquinatone, drug-resistance, *Eimeria necatrix*, robenidine.

Jpn. J. Vet. Sci. 48(1): 69–74, 1986

When decoquinatone, a quinolone derivative, was first introduced into Japan in 1968, the level of dosage recommended by manufacturers was 40 ppm in rations for broilers. But about one year later, many decoquinatone-resistant strains of chicken coccidia appeared in many broiler farms, and the drug has not been used since then [8].

Robenidine, a guanidine derivative, was proposed for use at a dose-level of 66 ppm in rations for broilers when first introduced into Japan in 1970. But the drug gave an unpleasant smell to meat of broilers at this concentration. Therefore the drug has been used at one half of the initially proposed dose-level. The reduction of the concentration, however, allowed the development of resistance to this drug in a short period of time in the field [3, 4].

A strain of *Eimeria necatrix* isolated in Miyazaki Prefecture, Japan, was examined for its sensitivity to several anticoccidials. The strain was multiple drug-resistant, but its endogenous development was inhibited by

decoquinatone and robenidine with their approximately one-tenth of dose-levels recommended by manufacturers. It seemed likely that the strong anticoccidial activity of these drugs in the present experiments originated from the different mechanism of action of these drugs in the parasites between the present and the times when these drugs were introduced. If mechanism of action of these drugs was not varied between the present and the times when they were introduced, the efficacy of these drugs against the present strain would be reduced as rapidly as the results of experiments of *E. tenella* [1, 2, 4, 5, 6] and *E. maxima* [7] when the strain was passaged with suboptimal doses of these anticoccidials.

Therefore we examined the reduction in sensitivity of the present strain to these drugs by serially passaging in chickens fed the ration containing suboptimal dose-levels of these anticoccidials and tried to confirm the hypothesis.

MATERIALS AND METHODS

Coccidium: A strain of *Eimeria necatrix* isolated in Miyazaki Prefecture, Japan was used in the present experiments. A single oocyst of the Strain was isolated by the agar plate method [10] and cloned culture was established.

Chicken: Day-old hybrid cockerel chickens of White Leghorn type were purchased from a commercial hatchery and kept in wire-floored metal cages in an isolated poultry house until they were divided into groups. From five days of age the feces of the chickens were examined for oocysts every day by the flotation method with saturated NaCl solution to confirm whether the chicken used were free from spontaneous coccidial infections. In the experiments of sensitivity, chickens were divided into 32 groups of five birds at 11 days of age. In the experiments of serial passages, 10 to 21 days-old chickens that kept in the same way as mentioned above were divided into six to nine groups of five birds. After divided into groups, chickens were liberally fed water and rations which contained anticoccidials. Non-medicated control chickens were fed the basal ration.

Anticoccidials: The anticoccidials tested in the sensitivity experiments were those broadly used in Japan such as decoquinate, buquinolate, methyl benzoquate, amprolium-plus ethopabate, ethyl dimethialium nitrate, clopidol, robenidine, zoalene, nicarbazin, monensin, sulfamonomethoxine, sulfadimethoxine and sulfaquinoxaline. In the experiments of serial passages, decoquinate and robenidine were used. These anticoccidials were thoroughly mixed with the basal ration before administration. Administration of drugs was initiated 24 hours prior to inoculation of oocysts.

Concentration of drugs: In the sensitivity experiments, each anticoccidials was initially administrated at its recommended dose-level in the field according to the reports of Imai

and Matuno [3] and Ryley and Bett [9]. When the strain developed resistance to the initial dose-level, the level was doubled in the following administration. But, when the drug was efficacious at the initial dose-level, as in the cases of quinolone and guanidine derivatives, the level was reduced in the following administration: decoquinate, a quinolone derivative was examined at levels of 8, 4 and 2 ppm whereas robenidine was examined at levels of 8.25, 4.125 and 2.0625 ppm. In the experiments of serial passages, minimal concentration level of each drug which completely inhibited oocyst production was determined from the results of sensitivity experiments. This level and one half were used as the concentration levels of the drug for serial passages: 4 and 2 ppm for decoquinate; and 4.125 and 2.0625 ppm for robenidine. The higher concentrations were used as an indicator of the degree of resistance of the present strain of *E. necatrix* to each of these drugs during the serial passages. The dose-levels of the drugs were increased when sufficient numbers of oocysts for the next passage had been harvested in a few successive passages.

Inoculation of oocysts: One day after the divided into groups, the chickens each received 2.5×10^4 and 1.0×10^4 oocysts in the crop via pipette in the experiments of sensitivity and serial passages, respectively. The oocysts in the inoculum had been sporulated within two months prior to inoculation. In the experiment of serial passages, oocysts for the next passage were harvested from cecal contents and feces, and preserved at 4°C till the subsequent inoculation after sporulation.

Evaluation of the drugs: All chickens were necropsied 9 days after inoculation of oocysts. Several criteria were employed for the evaluation of the drugs [10]; mortality rate, growth rate, gross pathological lesions in the intestine, propagation of the parasite in the intestine and oocyst production. But in the experiments of serial passages growth rate was not estimated.

RESULTS

Sensitivity of the strain to several anticoccidials: Amprolium-plus ethopabete, ethyl dimethialium nitrate, clopidol, zoalene, nicarbazin, monensin, sulfamonomethoxine, sulfadimethoxine and sulfaquinoxaline were neither efficacious when administered at the dose-levels recommended by manufacturers nor when these dose-levels were doubled. Quinolone derivatives and robenidine were efficacious at their initial dose-levels. Decoquinatate and robenidine were also efficacious at concentrations of 4 and 4.125 ppm, but not at those of 2 and 2.0625 ppm, respectively (Tables 1 and 2).

Development of resistance to decoquinatate and robenidine: The results are summarized in Table 3. The strain of *E. necatrix* was passaged 50 and 40 times at suboptimal dose-levels of decoquinatate and robenidine, respectively. Passage with decoquinatate at a level of 4 ppm did not produce sufficient number of oocysts for the following passages until the

50th passage. At the 51st passage, although a very small number of parasites completed their development in the intestinal tissues at a dose-level of 4 ppm, no oocysts harvested at this level sporulated. The strain did not propagate in the intestine at a dose-level of 8 ppm.

In the case of robenidine, the strain of *E. necatrix* was passaged at a dose-level of 2.0625 ppm until the 11th passage. Subsequent passages were performed at 4.125 and 8.25 ppm. Because at the 10th and 11th passages, the strain produced sufficient number of oocysts for the next passage, and the recovered oocysts were highly sporulated at a level of 4.125 ppm. Robenidine inhibited oocyst production at a dose-level of 8.25 ppm until the 41st passage.

The strain deteriorated the sensitivity to minimal effective concentration of levels of decoquinatate and robenidine at the start of the passage after 50 and 10 serial passages, respectively, at their suboptimal doses. But, the present strain still had high sensitivity to

Table 1. Sensitivity of the strain of *E. necatrix* to several anticoccidial drugs at their recommended concentrations

Anticoccidial drug	Concentration of drugs in ration (ppm)	Gross lesion in intestine	Proliferation of parasite in intestine	Oocyst production	Mortality rate	Body weight gain (%)
amprolium plus ethopabete	125+10	+++	+++	++	2/5	62.5
ethyl dimethialium nitrate	125	++	++	++	0/5	97.2
clopidol	125	++	+++	++	1/5	87.3
nicarbazin	125	++	+++	++	1/5	87.2
zoalene	125	++	+++	++	5/5	56.3
monensin	80	++	++	++	0/5	83.6
sulfamonomethoxine	1000	+++	+++	++	3/5	67.3
sulfadimethoxine	1000	+++	+++	++	2/5	68.3
sulfaquinoxaline	1000	+++	+++	++	3/5	69.0
robenidine	33	—	—	—	0/5	92.1
decoquinatate	40	—	—	—	0/5	98.2
buquinolate	80	—	—	—	0/5	100.0
methyl benzoquate	60	—	—	—	0/5	97.2
Non-medicated non-inoculated control		—	—	—	0/5	100.0
Non-medicated inoculated control		+++	+++	++	2/5	68.6

Table 2. Sensitivity of the strain of *E. necatrix* to several drugs at their doubled recommended concentrations and to decoquinate and robenidine at their subrecommended concentrations

Anticoccidial drug	Concentration of drugs in ration (ppm)	Gross lesion in intestine	Proliferation of parasite in intestine	Oocyst production	Mortality rate	Body weight gain (%)
amprolium plus ethopabete ethyl	250+20	+++	+++	++	2/5	67.5
dimethialium nitrate	250	++	++	+	0/5	97.8
clopidol	250	++	++	+	0/5	92.4
nicarbazin	250	++	++	+	0/5	53.1
zoalene	250	+++	++	++	1/5	72.5
monensin	160	++	++	+	0/5	83.6
sulfamonomethoxine	2000	+++	+++	++	1/5	69.0
sulfadimethoxine	2000	+++	+++	++	2/5	68.3
sulfaquinoxaline	2000	+++	+++	++	1/5	68.3
robenidine	8.25	—	—	—	0/5	92.3
	4.125	—	—	—	0/5	89.7
	2.0635	++	+++	+	0/5	78.7
decoquinate	8	—	—	—	0/5	90.4
	4	—	—	—	0/5	88.6
	2	++	++	+	0/5	80.3
Non-medicated non-inoculated control		—	—	—	0/5	100.0
Non-medicated inoculated control		+++	+++	++	2/5	68.7

Table 3. Sensitivity of the strain of *E. necatrix* to decoquinate and robenidine at the 51st and 41st passages, respectively

Anticoccidial drug	Concentration of drugs in ration (ppm)	Gross lesion in intestine	Proliferation of parasite in intestine	Oocyst production	Mortality rate
decoquinate	2	++	++	++	1/5
	4	—	+	+	0/5
	8	—	—	—	0/5
robenidine	2.0625	+++	++	++	2/5
	4.125	++	++	++	0/5
	8.25	—	—	—	0/5
	16.5	—	—	—	0/5
Non-medicated non-inoculated control		—	—	—	0/5
Non-medicated inoculated control		+++	+++	++	2/5

the recommended dose-levels of decoquinate and robenidine after 50 and 40 serial passages, respectively, at their suboptimal doses.

DISCUSSION

Many reports have been published on the

occurrence of the resistance of strains of chicken coccidia to anticoccidial drugs. Norton and Joyner [7] reported that the Weybridge strain of *E. maxima* increased the resistance of 264 ppm of robenidine after 7 serial passages in chickens fed increasing concentrations of the drug. Chapman [2] reported that the Houghton strain of *E. tenella*

increased the resistance to robenidine by serial passages in chickens fed increasing concentrations of the drug. In his experiments with groups of 10 birds, *E. tenella* developed resistance to 66 ppm of robenidine after 16 and 6 passages when birds were inoculated with 0.2×10^6 and 2.0×10^6 oocysts, respectively. Furthermore it took 13–14 and 6 passages to develop resistance to 264 ppm of robenidine when two groups of 5 and 45 birds were used and each bird was given 2×10^6 oocysts. He concluded that the *E. tenella* strain had developed resistance to robenidine more rapidly when the larger number of chickens were inoculated with the larger number of oocysts.

In the cases of quinolone derivatives, the sensitivity to the drugs was greatly reduced within a short period of time and cross-resistance was detected between the derivatives. The same situations were reported in such cases as the strains of *E. tenella*, *E. brunetti*, *E. maxima* and *E. acervulina* to amquinolate by McManus *et al.* [6]; a strain of *E. tenella* to decoquinate by McLoughlin and Chute [5]; and the Houghton, Beltsville, Elberfeld, and Weybridge strains of *E. tenella* to methylbenzoate by Chapman [1].

It is difficult to compare the results of these reports with those of the present experiments because the experiments were performed under different conditions, such as the number of oocysts in inoculum and the number of chickens used in each passage. But, it may be said that the coccidia in chickens easily developed the resistance to these anticoccidials under laboratory conditions. In the cases of quinolone derivatives, a tendency to easily developed the resistance to these drugs was also supported by the biochemical experiments of the drugs. Wang [11, 12] stated that the mechanism of action of quinolone coccidiostats is in inhibiting the cytochrome-mediated electron transport in mitochondria of coccidia, and activity of the cytochrome from quinolone resistant *E. tenella* was more 100

times less sensitive than quinolone sensitive one. He also stated that the resistance to quinolones could be under non-Mendelian genetic control in mitochondria of the parasite, and mitochondrial basis for activity of these drugs could account for the potential for rapid drug resistance, because there are many mitochondria per cell and thus more opportunities for mutation.

However, when estimated for the degrees of deterioration of sensitivity to decoquinate and robenidine by comparing the number of passages in the present experiments with those of *E. tenella* [1, 2, 4, 5, 6] and *E. maxima* [7], the present strain of *E. necatrix* showed lesser degrees of deterioration than *E. tenella* and *E. maxima*.

The results seem to suggested the different mechanism of action of decoquinate and robenidine between the present strain of *E. necatrix* and coccidia of chicken that were initially medicated with these drugs, respectively, when they were introduced.

REFERENCES

1. Chapman, H. D. 1975. *Eimeria tenella* in chickens: development of resistance of quinolone anticoccidial drugs. *Parasitology* 71: 41–49.
2. Chapman, H. D. 1976. *Eimeria tenella*: experimental studies on the development of resistance to robenidine. *Parasitology* 73: 265–273.
3. Imai, K., and Matsuno, T. 1978. Recent anticoccidial drugs. *Farumashia* 14: 676–680 (in Japanese).
4. McDougald, L. R. 1982. Chemotherapy of coccidiosis. pp. 373–427, In the biology of the coccidia (Long, P. L., ed.) University Park Press, Baltimore.
5. McLoughlin, D. K., and Chute, M. B. 1971. Efficacy of decoquinate against eleven strains of *Eimeria tenella* and development of a decoquinate resistant strain. *Avian Dis.* 15: 342–345.
6. McManus, E. C., Campbell, W. C., and Cuckler, A. C. 1968. Development of resistance to quinolone coccidiostat under field and laboratory conditions. *J. Parasit.* 54: 1190–1193.
7. Norton, C. C., and Joyner, L. P. 1975. The development of drug-resistant strains of *Eimeria maxima* in the laboratory. *Parasitology* 71: 153–165.

8. Oikawa, H., Kawaguchi, H., Nakamoto, K., and Tsunoda, K. 1975. Field survey on coccidial infection in broilers in Japan-Results obtained in autumn and winter and summarized in 1973. *Jpn. J. Vet. Sci.* 37: 271-279.
9. Ryley, J. F., and Bett, M. J. 1973. Chemotherapy of chicken coccidiosis. *Adv. Pharmacol. Chemother.* 11: 221-289.
10. Tsunoda, K., and Ishii, T. 1971. Technical Manual of Avian Coccidia. *Keibyo Kenkyu-kai Tokyo*, pp. 30 (in Japanese).
11. Wang, C. C. 1975. Studies of the mitochondria from *Eimeria tenella* and inhibition of the electron transport by quinolone coccidiostats. *Biochimica et Biophysica Acta*, 396: 210-219.
12. Wang, C. C. 1976. Inhibition of the respiration of *Eimeria tenella* by quinolone coccidiostats. *Biochemical Pharmacology*. 25: 434-449.

要 約

Eimeria necatrix 多剤耐性株のデイコキネイトおよびロベニジン耐性化試験: 齊藤康秀・板垣 博・角田清¹⁾ (麻布大学獣医学部寄生虫学教室, ¹⁾農林水産省家畜衛生試験場・現住所, 国際協力事業団)——宮崎県で採取した鶏コクシジウム *E. necatrix* に対する抗コクシジウム剤の効果を13薬剤を用いて検討した。13薬剤のうち、キノリン誘導体の3剤およびロベニジンを除いた9剤には感受性の低下がみられ、それらの常用量では無効であった。キノリン誘導体のデイコキネイトおよびロベニジンについては、投薬量を減じてさらに検討した。結果、両剤の最少有効量はそれぞれ4および4.125 ppM (飼料添加)であった。このような *E. necatrix* 株をデイコキネイトおよびロベニジンのサブオプティマル・ドースを投与した鶏で継代し、継代にともなう感受性の変化をそれぞれ観察した。その結果、本 *E. necatrix* 株は、デイコキネイトで50回およびロベニジンで10回継代することによってそれぞれの薬剤の継代開始時の最少有効量に対して感受性を低下させた。しかしながら、デイコキネイトで50回およびロベニジンで40回継代した後でもそれぞれの薬剤の常用量に対しては、依然として強い感受性を保持していた。