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Prophylactic Efficacy of Diclazuril in Broilers Experimentally Infected with Three Field Isolates of *Eimeria tenella*

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ABSTRACT

The sensitivity profiles of three *Eimeria tenella* field isolates (collected from distantly located poultry farms in Faisalabad) against the anticoccidial drug diclazuril at the recommended dose level (1 ppm) were studied. A total of one hundred and fifty four days old chicks were reared and divided into 7 groups of 22 chicks each at the age of 12 days. The chicks were inoculated by oral route with 75,000 sporulated oocysts at the age of 14 days. The sensitivity or resistance was evaluated on the basis of weight gain, feed conversion ratio, lesion scores, faecal scores, oocyst scores, oocyst per gram of faeces, mortality and global index. On the basis of conclusive summary of the results for used parameters, all three isolates of *Eimeria tenella* included in present study showed varying levels of susceptibilities to diclazuril. Isolate 2 was the most susceptible followed by isolate 3 and 1, respectively. The partial resistance was observed only in isolate 1. This study shows that there is a trend for the loss of sensitivity in *Eimeria tenella* field isolates against diclazuril, however this problem can be managed by using the diclazuril in rotation and shuttle programmes.

Key Words: Coccidiosis; *Eimeria tenella*; Resistance; Anticoccidial; Diclazuril

INTRODUCTION

Coccidiosis is one of the most important poultry disease caused by intracellular protozoan parasite of the genus *Eimeria*. It is considered as one of the most expensive and common diseases of poultry. In Pakistan, economic losses due to coccidiosis are still un-known due to lack of indices; however it costs the world's commercial chicken producers at least US$ 1.5 billion annually (Stevens, 1998). Without the administration of anticoccidials in feed or in drinking water, economic broiler production is inconceivable, because still the control of coccidiosis chiefly depends upon prophylactic chemotherapy with anticoccidial drugs (McDougald & Reid, 1994). However, the development of resistance in coccidian parasites against commonly used anticoccidials is a great problem, which in due course, limits their use (Mathis & McDougald, 1982; McDougald *et al*., 1986 & 1987; Stallbaumer & Daisy, 1988; Chapman & Shirley, 1989; Rotibi *et al*., 1989; Bafundo & Jeffers, 1990).

A quote from Schnitzer and Grunberg (1957) aptly characterizes this problem: "Drug resistance has followed the development of chemotherapy like a faithful shadow". It is becoming increasingly difficult to develop anticoccidial active ingredients that can replace old products. Coccidiosis appears to be no exception and there is growing evidence that changes are occurring in the expected response to known coccidiostatic drugs. It is this phenomenon, which is now the subject for consideration.

In Pakistan, information is not available regarding the development of resistance in *Eimeria tenella* field isolates against diclazuril anticoccidial. Therefore the present study has been designed to investigate resistance to diclazuril in the field isolates of *E. tenella,* because rapid and accurate diagnosis of resistance could direct the more rationale use of drugs and help to improve the management of resistant parasites and prolong the life of many chemicals.

MATERIALS AND METHODS

Parasite. Three isolates of *E. tenella* were collected from the poultry farms, which were located distantly apart in Faisalabad district of Punjab-Pakistan. Coccidial oocysts were obtained from the caeca of infected chicks and were propagated in broiler chicks by giving oral infection. After obtaining sufficient amount of oocysts, they were sporulated by placing in 2.5% $K_2Cr_2O_7$ in the presence of suitable humidity and temperature. Sporulated oocysts were cleaned and counted by the McMaster technique (MAFF, 1986).

Standardization of *Eimeria tenella* doses for experimental infection. The infection dose was established after a dose titration study of three weeks. The effect of different doses 50,000, 75,000, 1, 00,000 and 150,000 was evaluated on the weight gain and mortality. The infection

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was given on 12th day of age. The birds were weighed on day of infection and again reweighed on 19th day of age (one week after inoculation of sporulated oocysts). Mortality was also observed during this period. A dose of 75,000 sporulated oocysts was found to give desired effects on weight gain and mortality for all the isolates with some variations among isolates. The required concentration of the sporulated oocysts (75,000 per mL) was maintained with phosphate buffered saline.

Bird's management. One hundred and fifty four days-old broiler chicks were purchased from local hatchery. Chicks were reared under standard management practices. All the chicks were kept on broiler starter ration up to 2 weeks of age and later on fed on broiler finisher ration. The birds were reared on the sawdust litter and the moisture level of the litter was kept 20-30%. The litter was replaced with clean litter after two weeks of age. The feed and water were provided *ad libitum* to all the birds. The temperature was maintained at 85-90^{0}F during the first week of age and was reduced by 5^{0}F on weekly basis. Lighting was provided for 24 h through out the experimental period. All the birds were vaccinated for Newcastle disease on 5th day of age, for Infectious bursal disease on 14th day of age and for Hydropericardium syndrome on 18th day of age.

Drugs. Diclazuril (1 ppm) was added in the feed at 12 days of age (2 days before inoculation of birds with *E. tenella* sporulated oocysts) and continued up to 7 days post-inoculation of sporulated oocysts.

Experimental design. A total of 154 chicks were divided into 7 groups of 22 chicks each at the age of 12 days. The chicks of all the groups except group 7 were inoculated with 75,000 sporulated oocysts of *E. tenella*. The chicks of groups 1–3 were medicated with diclazuril and infected with isolate 1, isolate 2 and isolate 3, respectively. The groups 4, 5 and 6 served as infected-non-medicated for isolate 1, isolate 2 and isolate 3, respectively. The group 7 was kept as non-infected non-medicated control (NNC).

Evaluation of sensitivity/resistance. Seventeen chicks from each group were weighed individually on day of inoculation (14th day of age) and then reweighed on 7th day post inoculation (21st day of age). The percentage body weight gain between these days was recorded.

Five chicks from each group were sacrificed for post mortem examination at 7th day of post inoculation (21st day of age). Caecal lesions were scored by the lesion scoring technique described by Johnson and Reid (1970).

An oocyst index of (0 to 5) was determined by microscopical examination of scrapings from the caeca of chicks sacrificed for lesion scoring at 7th day of post inoculation (Hilbrich, 1978).

Mortality was recorded through out the experimental period and the exact cause of mortality was confirmed by postmortem examination.

The drug resistance of each isolate of *E. tenella* against diclazuril anticoccidial drug was also determined by calculating the Global Index (Stephan *et al.*, 1997) by using the formula: $GI = \%WG_{NNC} - [(F_M - F_{NNC}) \times 10] - (OI_M - OI_{INC}) - [(LS_M - LS_{INC}) \times 2] - (\%mortality/2)$. Where GI is the global index, WG the weight gain, F the feed conversion ratio, OI the oocyst index, LS the lesion score, M the medicated group, NNC the non-infected non-medicated control group and INC is the infected non-medicated control group. Furthermore, the GI for each test group was calculated as percentage of the GI for the NNC. The following five categories were used for testing resistance to anticoccidials:

1. Very good efficacy ≥90% GI_{NNC}.
2. Good efficacy 80–89% GI_{NNC}.
3. Limited efficacy 70–79% GI_{NNC}.
4. Partially resistant 50–69% GI_{NNC}.
5. Resistant <50% GI_{NNC}.

Statistical analysis. Data obtained on various parameters were analyzed by analysis of variance and the mean values were compared by Tukeys test. The results were recorded as mean (SEM) and the differences among group means were considered significant at $P<0.05$.

RESULTS

The results of different parameters used to detect susceptibility and/or resistance among different isolates of *Eimeria tenella* against diclazuril collected from Faisalabad are presented in Tables I-VIII. It is evident from Table I that the weight gain in the medicated groups was higher ($P<0.05$) compared with infected non-medicated groups for all the three isolates. This indicated that to the extent of weight gain, all the isolates were susceptible to diclazuril.

The results of FCR (Table II) revealed the similar pattern as that of weight gain among different isolates of *Eimeria tenella*. Although a statistical comparison could not be made due to group feeding of birds, however the FCR values of medicated groups were numerically lower compared with infected non-medicated groups.

Data in Table III indicate non-significant anticoccidial effect of diclazuril in isolate 1 and 3 of *Eimeria tenella*. The lesion scores calculated for these two groups were similar ($P>0.05$) with their respective infected non-medicated groups. However, isolate 2 had significant difference ($P<0.05$) in lesion scores between medicated and infected non-medicated groups. The results of faecal scoring corresponded with the results of lesion scoring. The faecal scores (Table IV) were significantly lower ($P<0.05$) in the infected medicated groups compared with infected non-medicated groups through out the experiment except day 6 post inoculation for all the three isolates of *Eimeria tenella*. On day 6 post inoculation, the faecal scores calculated for isolate 1 and 3 were similar ($P>0.05$) with their respective infected non-medicated groups.

The results of oocyst scoring (Table V) differed with the results of lesion scoring. The oocyst scores were lower ($P<0.05$) in all the three isolates compared with their

Table I. Effect of diclazuril treatment on mean (n=17) weight gain (g) in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	Initial weight	Final weight	Weight gain	% Weight gain	% Weight gain of NNC
DIC 1	215.1176	442.2857	227.17^{bc}	106	80
DIC 2	213.1176	463.25	250.14^{b}	117	87
DIC 3	210.6471	456.3333	245.69^{b}	117	87
INC 1	224.5294	378.9167	154.39^{e}	69	52
INC 2	213.7647	387.3846	173.62^{de}	82	62
INC 3	210.1765	405.2857	195.11^{cd}	93	70
NNC	220.0588	509.8824	289.83^{a}	132	100
S.E.M.	–	–	13.41	–	–

Means sharing the same superscripts within a column do not differ (P<0.05); DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control; S.E.M: standard error of means

Table II. Effect of diclazuril treatment on feed conversion ratio (FCR) in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	Feed Consumption (g)	Final weight (g)	Feed conversion ratio (g/g)*
DIC 1	554.78	442.29	1.25
DIC 2	552.67	463.25	1.19
DIC 3	561.92	456.33	1.23
INC 1	536.2	378.92	1.42
INC 2	535.44	387.38	1.38
INC 3	534.19	405.29	1.32
NNC	580.85	509.88	1.14

*****Statistical analysis was not possible because of group feeding of chicks. DIC1: diclazuril amprolium medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non-medicated controls; NNC: non-infected non medicated control

Table III. Effect of diclazuril treatment on mean (n=5) lesion score (on 7th days post inoculation) in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	0	+1	+2	+3	+4	Mean
DIC 1	–	1	1	2	1	2.6^{ab}
DIC 2	–	3	2	–	–	1.4^{c}
DIC 3	–	1	3	1	–	2^{bc}
INC 1	–	–	-	3	2	3.4^{a}
INC 2	–	–	1	2	2	3.2^{a}
INC 3	–	–	2	1	2	3^{ab}
NNC	–	–	–	–	–	–
S.E.M.	–	–	–	–	–	0.37

Means sharing the same superscripts within a column do not differ (P<0.05); DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control; S.E.M: standard error of means; 0: no lesion; +1: mild lesions; +2: moderate lesions; +3: severe lesions; +4: very severe lesions

respective infected non-medicated groups. The results of oocyst counting (Table VI) corresponded with the results of oocyst scoring. There was also a significant reduction (P<0.05) in the oocyst counts in all the infected medicated groups compared with infected non-medicated groups from days 6 to 13 post inoculation. It is evident from the results (Table VI) that the peak oocyst counts were recorded in all the experimental chicks till day 7 post inoculation, which

Table IV. Effect of diclazuril treatment on mean (n=5) faecal score (3-7 days post inoculation) in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	3rd day	4th day	5th day	6th day	7th day	Mean
DIC 1	–	3.2^{bc}	3.4^{bc}	3^{abc}	–	3.2^{bc}
DIC 2	–	2.2^{d}	2.6^{c}	2^{c}	–	2.26^{d}
DIC 3	–	2.8^{cd}	3.2^{c}	2.6^{bc}	–	2.86^{cd}
INC 1	–	4.2^{a}	4.6^{a}	4^{a}	–	4.26^{a}
INC 2	–	4^{ab}	4.2^{ab}	3.8^{ab}	–	4^{ab}
INC 3	–	4^{ab}	4.2^{ab}	3.6^{ab}	–	3.93^{ab}
NNC	–	–	–	–	–	–
S.E.M.	–	0.28	0.32	0.40	–	0.30

Means sharing the same superscripts within a column do not differ (P<0.05); DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control; S.E.M: standard error of means

Table V. Effect of diclazuril treatment on mean (n=5) oocyst score (on 7th day post inoculation) in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	0	+1	+2	+3	+4	+5	Mean
DIC 1	–	1	4	–	–	–	1.8^{c}
DIC 2	–	4	1	–	–	–	1.2^{c}
DIC 3	–	3	1	1	–	–	1.6^{c}
INC 1	–	–	–	–	2	3	4.6^{a}
INC 2	–	–	–	2	2	1	3^{b}
INC 3	–	–	–	1	2	2	4.2^{a}
NNC	–	–	–	–	–	–	–
S.E.M.	–	–	–	–	–	–	0.35

Means sharing the same superscripts within a column do not differ (P<0.05); DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control; S.E.M: standard error of means; 0: no oocyst; +1: 1-10 oocyst /field; +2: 11-20 oocyst /field; +3: 21-50 oocyst /field; +4: 51-100 oocyst /field; +5: >100 oocyst /field

gradually decreased from day 8 post inoculation onwards. Comparison of the mean oocyst count indicated that all the isolates of *Eimeria tenella* were almost equally susceptible to diclazuril. This indicated that to the extent of oocyst scoring and oocyst counting, all the isolates were susceptible to diclazuril.

The percent survival (Table VII) was higher in infected medicated groups of all the isolates being highest in isolate 2 (95.46%) followed by isolate 3 (90.91%) and isolate 1 (86.37%). Lowest survival rate was recorded in infected non-medicated group.

The data (Table VIII) on the considered criteria i.e., global index, compared with negative and positive controls revealed that the three isolates of *Eimeria tenella* included in present study have varying levels of susceptibilities. Isolate 2 was the most susceptible followed by isolate 3 and 1, respectively.

DISCUSSION

The development of resistance in coccidian species against commonly used anticoccidials is a major constraint

Table VI. Effect of diclazuril treatment on mean (n=3) oocyst count (x 10^3 g^{-1}) in droppings of broiler chicks (on days 6, 7, 8, 9, 11 and 13 post inoculation) artificially infected with three field isolates of *Eimeria tenella*

Groups	6th day	7th day	8th day	9th day	11th day	13th day	Total
DIC 1	32.66^{c}	61^{c}	35.33^{d}	3.23^{c}	1.63^{c}	0.26^{c}	22.35^{d}
DIC 2	21^{d}	43.33^{d}	24.33^{f}	2.1^{c}	1.26^{d}	0.16^{c}	15.36^{e}
DIC 3	26.66^{cd}	52^{cd}	30^{e}	2.2^{c}	0.96^{d}	0^{d}	18.63^{e}
INC 1	70.33^{a}	123^{a}	62.33^{a}	12.53^{a}	3.36^{a}	1.63^{a}	45.53^{a}
INC 2	51.66^{b}	97^{b}	49.33^{c}	7.73^{b}	2.73^{b}	1^{b}	34.91^{c}
INC 3	65.66^{a}	114.33^{a}	57^{b}	10.4^{a}	3.13^{a}	1.03^{b}	41.92^{b}
NNC	–	–	–	–	–	–	–
S.E.M.	2.38	3.77	1.59	0.75	0.12	0.05	1.08

Means sharing the same superscripts within a column do not differ (P<0.05); DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control; S.E.M: standard error of means

Table VII. Effect of diclazuril treatment on mortality %age (n=22) in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	Mortality Days post inoculation					Total Mortality	Mortality %age	Survival %age
	3	4	5	6	7			
DIC 1	–	3	–	–	–	3	13.63	86.37
DIC 2	–	–	1	–	–	1	4.54	95.46
DIC 3	–	–	1	1	–	2	9.09	90.91
INC 1	–	3	2	–	–	5	22.72	77.28
INC 2	–	2	2	–	–	4	18.18	81.82
INC 3	–	2	1	–	–	3	13.63	86.37
NNC	–	–	–	–	–	0	0	100

DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control

Table VIII. Efficacy status of diclazuril in broiler chicks artificially infected with three field isolates of *Eimeria tenella*

Groups	Global index	%Global index of NNC	Efficacy status
DIC 1	76.39	69.25	1
DIC 2	91.53	82.98	3
DIC 3	88.06	79.83	2
INC 1	37.84	34.30	–
INC 2	50.41	45.70	–
INC 3	61.39	55.65	–
NNC	110.3	100	–

DIC 1: diclazuril medicated isolate 1; DIC 2: diclazuril medicated isolate 2; DIC 3: diclazuril medicated isolate 3; INC 1, 2, 3: infected non medicated controls; NNC: non infected non medicated control; (3): good efficacy; (2): limited efficacy; (1): partial resistance

among drug users in the poultry industry. Timely detection of the drug resistance gives the idea about the applications and limitations of the anticoccidials to be used. Chances of development of resistance are very high in the specific geographical location, because of using the same drug frequently.

Numerous anticoccidial drugs have been introduced since the end of the 1940s and sooner or later resistance to all these anticoccidial drugs developed in *Eimeria* species (Chapman, 1993 & 1997). A number of researchers (Kawazoe & Fabio, 1993; Haberkorn, 1994; Stephan *et al.,* 1997) have reported the development of resistance/loss of sensitivity in *Eimeria* isolates to the anticoccidial drug diclazuril used in the present studies. Therefore, resistance against commonly used anticoccidials is a universal issue. Although, a number of reports are available regarding the loss of sensitivity in *Eimeria* field isolates from neighboring countries of Pakistan like China (Zeng & Hu, 1996; Zhou *et al.,* 2000; Li *et al.,* 2004) and India (Panda *et al.,* 1973; Gill & Bajwa, 1979; Rana, 1993; Yadav & Gupta, 2001), this is the first ever study conducted in Pakistan.

Different formulae have been used by different workers to find out the efficacy/resistance of anticoccidial drugs. The older formulae or indices used for determining efficacy or resistance were: performance index (Morehouse & Baron, 1970) and anticoccidial index (Jeffers & Challey, 1973; Jeffers, 1974; Ramadan *et al.,* 1997), where no importance was given to feed conversion ratio, which is also considered to be the important parameters in determining resistance/sensitivity to any anticoccidial drug. Therefore, in the present studies, the newly devised method of Stephan *et al.* (1997) was used for calculating the global index to detect resistance to anticoccidials in *Eimeria* spp. In this formula, performance parameters of weight gain and feed conversion ratio and as well as the pathological parameters of lesion scores, oocyst scores and mortality have been given their due importance. In the recent past, many workers (Stephan *et al.,* 1997; Daugschies *et al.,* 1998; Yadav & Gupta, 2001) have correlated the resistance results with the practical field conditions with great success by using global index.

The high sensitivity of *Eimeria tenella* field isolates (except isolate 1) for diclazuril is consistent with the previous reports (Vanparijs *et al.,* 1989; McDougald *et al.,* 1990; Peeters *et al.,* 1994; Matsuno *et al*., 1996; Conway *et al*., 2001; Chapman *et al*., 2004). Even in some countries like China, diclazuril is being used as a standard anticoccidial drug to evaluate the anticoccidial activity of other products against *Eimeria tenella* (Du & Hu, 2004). The sensitivity of *Eimeria* isolates to diclazuril is most likely due to its unique mode of action (Maes *et al.,* 1988). Diclazuril is a nucleotide analogue. Up to now, very few anticoccidials of this type have been developed. Diclazuril is lethal against all endogenous developmental stages of *Eimeria tenella* (both sexual & asexual stages) and therefore, oocyst shedding is completely prevented. However, like that of development of partial resistance in isolate 1 in the present studies, reports of resistance to diclazuril in *Eimeria* isolates in the field conditions have also been documented (Kawazoe & Fabio, 1994; Peeters *et al.,* 1994; Peek & Landman, 2003). Environmental selection pressures in different geographical locations, as well as the histories of drug use, may differ with each other, therefore strains resistant in one area may be sensitive in other area (Martin *et al.,* 1997). Therefore, it is also recommended to use more than one field isolates of the *Eimeria* species, because only one isolate of *Eimeria* species is insufficient to

give conclusive summary about the resistance in field conditions. In the similar way, the three *E. tenella* field isolates respond differently against diclazuril.

Diclazuril in shuttle programs is highly efficacious against different *Eimeria* spp. in comparison with other chemical anticoccidials and ionophores (Conway *et al*., 2001). In the similar way, the rotation of diclazuril with the vaccine contained anticoccidial drug sensitive strain has been reported to enhance the sensitivity of *Eimeria* isolates from 25% to 100% (Mathis & Broussard, 2006). Therefore, in case of diclazuril, it is recommended to use this anticoccidial in continuous alternation with other anticoccidials to prolong its activity life span.

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