



Evaluation of ionophore resistance in field isolates of *Eimeria tenella* from Jammu and Kashmir

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ABSTRACT

The present study was conducted to evaluate resistance against ionophores (salinomycin and maduramicin) in four field isolates (F) of *Eimeria tenella* collected from four distantly located poultry farms from Union territory of Jammu and Kashmir. The purity of isolates was ascertained by COCCIMORPH and ITS-1 gene amplification for *E. tenella*. For the drug sensitivity trial, Cobb strain, day-old male broiler chicks were used. At the age of 10 days, the birds were transferred *ad random* in 13 groups to battery cage system. All birds, except non infected non-medicated control were infected with 10⁵ sporulated oocysts each at two weeks. Prophylactic medication with ionophores commenced two days prior to infection in eight groups (two medicated groups for each of the four isolates). Another four groups served as infected and non-medicated control. Seven days post infection, all the surviving birds were sacrificed to study drug resistance by assessing the global index for individual ionophore compounds. The F-1 exhibited resistance against salinomycin and maduramicin whereas, the F-2 showed comparatively improved global index for both salinomycin and maduramicin thus indicating limited efficacy. The F-3 exhibited partial resistance against salinomycin and limited efficacy in case of maduramicin, and the F-4 was observed to be resistant against salinomycin whereas partially resistant against maduramicin. The present study provides evidence of resistance against maduramicin and salinomycin in field isolates of *E. tenella* from Union Territory of Jammu and Kashmir which may aid in management of poultry coccidiosis.

Keywords: Anticoccidial, *Eimeria tenella*, India, Ionophores, Resistance

Coccidiosis, caused by apicomplexan parasites of the genus *Eimeria*, is one of the most common diseases of poultry. Globally, coccidiosis cause an economic burden in the range of £771.5 to £13,012.54 millions annually pertaining to the cost involved in prevention, control and loss of birds from coccidiosis (Blake *et al.* 2020). Among various *Eimeria* spp. infecting poultry, *E. tenella* causing caecal coccidiosis is highly pathogenic and responsible for many outbreaks on the farms.

In poultry production, chemotherapeutic agents are the mainstay of protection against coccidiosis. The poultry industry scaled exponentially after the introduction of sulfa-medications, and later the breakthrough of effective drugs like ionophores. As a consequence of the prolonged use of anticoccidial medications, drug-resistant strains have evolved (Chapman 2014), reducing the effectiveness of these medications. The efficacy of a large number of anticoccidial drugs have been hampered in the past two

decades (Abbas *et al.* 2008, Zhan *et al.* 2013, Tan *et al.* 2017, Kraiesiki *et al.* 2021).

With an annual growth rate of about 8%, the Indian poultry industry is significant contributor to the animal production according to Economic Survey of India (www.indiabudget.gov.in). According to the 20th Livestock Census (<http://dadjf.gov.in>), the overall poultry population increased by 16.81 % from 729.21 million in 2012 to 851.81 million in 2019. Although almost all birds are raised with an anticoccidial medication as feed additive, coccidiosis continues to be a significant concern. India experiences yearly losses of around £445.9 million as a direct outcome of disease on poultry and its management (Blake *et al.* 2020). In India, partial to complete anticoccidial resistance against salinomycin, amprolium, maduramicin, clopidol, (Yadav and Gupta 2001, Metri *et al.* 2012) have been reported in several states of the country. There is scarcity of information on resistance against ionophores in *E. tenella* in North India. Although, *in vitro* methods are available, Anticoccidial Sensitivity Test (AST) using *in vivo* model in battery cages are generally preferred by scientific community (Jenkins *et al.* 2014) This study, therefore aims to assess the status of resistance to commonly

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used ionophores in the field isolates of *E. tenella* from Union Territory of Jammu and Kashmir.

MATERIALS AND METHODS

Ethical statement: All experiments involving the use of birds in this study were carried out in strict compliance with the recommendations and guidelines of the Institutional Animal Ethics Committee of Faculty of Veterinary Sciences and Animal Husbandry, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, vide no. NPI/IAEC-18/2018 under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. All efforts were made to reduce the pain and suffering of animals.

***Eimeria tenella* field isolates:** Four field isolates (F) of *E. tenella* viz., F-1, F-2, F-3 and F-4, were collected from broiler poultry farms located distantly apart at Jammu (32.72 N; 74.857 E), Poonch (33.7669 N, 74.0924E), Udhampur (32.9160 N; 75.1416 E) and Srinagar (34.0837 N; 74.7973 E), respectively of UT of Jammu and Kashmir, North India. The poultry farms located across different agro-climatic zones of Jammu and Kashmir were selected for the study. The study area comprised sub-tropical zone (Jammu), intermediate agro-climatic zone (Udhampur) and high altitude temperate zone (Srinagar and Poonch).

The *E. tenella* field isolates were obtained from those poultry farms where natural outbreaks of caecal coccidiosis had occurred, despite prophylactic medication with ionophores. The caeca of the dead/slaughtered birds were collected, transported on ice to the laboratory, examined grossly and microscopically to identify the species of coccidia. The isolates were identified as *E. tenella* based on the presence of typical schizonts and gametocytes in fresh smears of caecal mucosa and the emergence of prominent caecal lesions followed by oocysts harvesting (Davies *et al.* 1963).

Confirmation of *Eimeria tenella* pure inoculum: Confirmation of each *E. tenella* field isolate was done through oocyst morphometry by COCCIMORPH software (Castanon *et al.* 2007) and amplification of ITS-1 gene by PCR for different species of *Eimeria* as described by Kumar *et al.* (2014). All the field isolates revealed a specific product of 278 bp (Fig. 1) and thus confirmed to be *E. tenella*.

Propagation of *Eimeria tenella* oocysts: *E. tenella* oocysts harvested from caeca were sporulated and then inoculated orally into susceptible chicken to provide

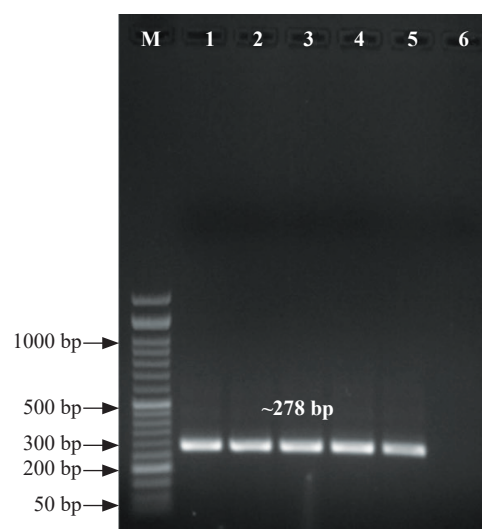


Fig. 1. Amplification of ITS gene by PCR. Lane M: 50 bp DNA ladder, Lane 1-4: Amplified ITS-1 gene of *E. tenella* of chicken (~278 bp), Lane 5: Positive control, Lane 6: No template control.

sufficient oocysts for drug sensitivity trial. Twenty, day-old, broiler chicks of Cobb strain were procured and reared under coccidia-free conditions. At 21 days of age, birds were divided into four groups (one for each isolate) of five birds each and inoculated with 1 ml suspension, containing 2×10^4 sporulated oocysts of a specific field isolate. The oocysts in the faeces were collected from 7th day post infection, purified using salt floatation method and finally stored as sporulated suspension in 2.5% potassium dichromate at 4°C till further use.

Anticoccidial drugs: Anticoccidial drugs salinomycin @ 60 ppm and maduramicin @ 5 ppm were incorporated in the standard chick mash by proper mixing.

Birds and experimental design: For the drug sensitivity trial, 169 Cobb strain, day-old male broiler chicks were purchased. At the age of 10 days, the birds were transferred *ad random* in 13 groups (Groups I-XIII) of 13 each to battery cage system and reared under coccidia-free environment. Birds of all the infected groups (Groups I-XII) were inoculated *per os* with 10^5 sporulated oocysts of *E. tenella* each at 2 weeks of age, whereas chicks of non-infected non-medicated control (NNC) group (Group XIII), common for all the groups, were inoculated with 1ml distilled water. The prophylactic medication with ionophores commenced 2 days (at the age of 12 days) prior to infection in a total of eight infected medicated (two medicated groups for each of the four isolates) groups

Table 1. Experimental design for studying resistance

Total no. of birds	Total no. of groups*	Nature of groups**	Ionophores used (at day12)	Field isolate	Place of collection	Dose of sporulated oocyst/bird (at day 14)
169	13	IMG	Salinomycin	F-1	Jammu	1,00,000
		IMG	Maduramicin	F-2	Poonch	
		INC		F-3	Udhampur	
		NNC***		F-4	Srinagar	

F, Field Isolate; *No. of birds in each group = 13; **IMG, Infected Medicated Group; INC, Infected Non-medicated Control; NNC, Non-infected Non-medicated Control; ***One NNC group for all four isolates.

(Groups I-IV medicated with salinomycin and Groups V-VIII medicated with maduramicin). Another four groups (Groups IX-XII) served as infected and non-medicated control (INC) i.e. one each for four isolates. Feed and water were supplied *ad lib.* during the study (Table 1).

All the birds were weighed individually on day of oocyst inoculation and 7 days after infection in order to calculate weight gain during the infection period. The weight gain of the NNC was used as the 100% value and the weight gain of the infected groups was expressed as a percentage of the weight gain of the NNC. Feed consumption was calculated by weighing feed just before oocyst inoculation and at termination of the study. Feed conversion ratio (FCR) was calculated by dividing total feed consumed by the total weight gain in a group. Mortality, if occurred during the study was recorded and necropsies were performed to ascertain the cause of the death. Seven days-post-infection, all the surviving birds were sacrificed to study various resistance parameters.

Resistance evaluation: Various parameters were recorded during drug resistance evaluation viz. weight gain (%), feed conversion (g/g), lesion scoring (Johnson and Reid 1970), semi-quantitative oocyst index (Hilbrich 1978) and mortality (%). Finally, as per the method of Stephan *et al.* (1997), global index (GI) was calculated for each ionophore using the formula:

$$GI = \%WGNNC - [(FM - FNNC) \times 10] - [OIM - OIINC] - [(LSM - LSINC) \times 2] - [\%Mortality/2]$$

Where GI is the global index, WG is the weight gain, F is the feed conversion ratio, OI is the oocyst index, LS is the lesion score, M is the medicated group, NNC is the non-infected non-medicated control group and INC is the infected non-medicated control group. The GI for each test group was calculated as percentage of the GI for the NNC. The five categories used for testing resistance to anticoccidials were delineated as very good efficacy with $GI \geq 90\%$; good efficacy ($GI \geq 80\%$), limited efficacy ($GI \geq 70\%$), partial resistance ($GI \geq 50\%$) and complete resistance ($GI \leq 50\%$).

Statistical analysis: To assess the comparative values of body weight gain, the lesion score and oocyst index of all the four isolates and different groups, two-way analysis of variance (ANOVA) test was applied. The data was considered to be significant at $P < 0.05$.

RESULTS AND DISCUSSION

Poultry coccidiosis remains among top ten veterinary diseases and the most economically significant parasite of poultry having major impact in South Asia with a global burden of £10.4 billion, annually (Blake *et al.* 2020, Attree *et al.* 2021). The poultry sector is a profitable venture under intensive management practices and as a livelihood support under backyard poultry for small holding farmers in Africa and South Asia including countries like India. Despite improvements in our understanding of immunology and genetics of coccidiosis in the past several decades, the

definitive solution towards vaccinological intervention is yet to be realized (Blake *et al.* 2017). In the wake of emergence of new strains or genetically diverse species, chemoprophylaxis through feed and water remains the main approach for coccidia control. Since the introduction of sulfonamides in the 1940s, anticoccidials have been utilised in broiler rations (Peek and Landmann 2011).

Parasite resistance has been delineated as the ability of parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limits of tolerance of the subject (Attree *et al.* 2021). Today, resistance has been reported against almost all anticoccidial drugs that have been introduced, from major broiler producing areas across the world demonstrating the extent of the problem (Abbas *et al.* 2008, Zhang *et al.* 2013, Tan *et al.* 2017, Kraiesiki *et al.* 2021).

Several reports on ionophore resistance are available worldwide until our study commenced but the development of resistance in one region cannot be used as a standard to infer ionophore resistance in another geographical area due to variations in local strains, species and different schedules of anticoccidial usage (Abbas *et al.* 2008). Ionophore antibiotics are extensively utilised by poultry farmers in India instead of synthetic compounds. Few investigations (Yadav and Gupta 2001, Metri *et al.* 2012) have revealed decrease of sensitivity to ionophore drugs from several states of India. The findings of these studies coupled with feedback of veterinary practitioners and farmers regarding coccidiosis outbreaks in broilers farms despite ionophore usage in poultry ration prompted to initiate present study to assess any resistance phenomena to commonly used ionophores in *Eimeria tenella* field isolates from Union Territory of Jammu and Kashmir in North India.

The selection of four field isolates from distant geographical location of UT of Jammu and Kashmir is in accordance of the guidelines of WAAVP which recommends use of more than one field isolate of *Eimeria* to evaluate anticoccidial drug sensitivity (Holdsworth *et al.* 2004).

The present study results showed resistance against both salinomycin and maduramicin in *E. tenella* F-1, whereas limited efficacy was recorded for F-2. F-3 exhibited partial resistance against salinomycin and limited efficacy against maduramicin. In case of F-4, resistance was recorded against salinomycin and partial resistance against maduramicin (Table 2).

The body weights in NNC birds showed a steady increase during the period of experiment, whereas in INC groups weight gain was significantly ($P < 0.05$) reduced as compared to NNC group (Table 2). Among the medicated groups, the average weight gain was highest in F-2 treated with maduramicin and lowest in F-1 treated with salinomycin as compared to NNC group. The average weight gain in both medicated groups was significantly ($P < 0.05$) higher from INC group.

Among the medicated groups, there was no significant difference regarding average weight gain in the birds

Table 2. Global indices and various parameters for its calculation with comparison of different *E. tenella* field isolates

Experimental group (Gr)		Average WG (g)	%WG	FCR (g/g)	Lesion score	Oocyst index	Mortality (%)	Global index (GI)	% GI
<i>Salinomycin</i>									
Gr I	F-1	197 ^{bF} ±13.07	71.05	4.91	3.07 ^{aA} ±0.19	3.53 ^{abA} ±0.38	46.15	32.55	29.52
Gr II	F-2	245 ^{aA} ±12.92	88.16	3.71	1.23 ^{aA} ±0.12	1.69 ^{cB} ±0.28	23.08	75.69	70.59
Gr II	F-3	227 ^{abBC} ±21.91	81.65	3.98	1.92 ^{bB} ±0.21	2.61 ^{bA} ±0.31	30.77	61.22	56.62
Gr IV	F-4	219 ^{bD} ±13.21	78.77	4.73	2.38 ^{bAB} ±0.28	2.92 ^{aAB} ±0.24	38.46	46.97	42.97
<i>Maduramicin</i>									
Gr V	F-1	230 ^{bF} ±13.32	82.73	4.73	1.76 ^{aB} ±0.24	2.30 ^{aB} ±0.24	38.46	53.63	48.64
Gr VI	F-2	249 ^{aA} ±7.09	89.60	3.57	1.46 ^{Ba} ±0.16	1.85 ^{Bb} ±0.70	15.38	81.76	76.25
Gr VII	F-3	241 ^{aB} ±10.21	86.69	3.64	1.23 ^{Bc} ±0.14	2.08 ^{abB} ±0.25	15.38	79.27	73.32
Gr VIII	F-4	235 ^{bDE} ±11.60	84.53	4.11	1.76 ^{Ab} ±0.24	2.15 ^{abB} ±0.19	30.77	64.78	59.26
<i>INC</i>									
Gr IX	F-1	143 ^{dG} ±13.49	51.43	5.19	3.28 ^{Aa} ±0.24	3.69 ^{Aa} ±0.31	46.15	110.25	100
Gr X	F-2	204 ^{eH} ±15.32	73.38	4.06	2.42 ^{bA} ±0.16	2.38 ^{bA} ±0.20	23.08	107.22	100
Gr XI	F-3	192 ^{cC} ±11.32	69.06	4.87	2.71 ^{bA} ±0.14	2.69 ^{bA} ±0.27	30.77	108.11	100
Gr XII	F-4	152 ^{dE} ±11.71	54.67	4.83	3.00 ^{abA} ±0.25	3.31 ^{abA} ±0.34	53.45	109.31	100
<i>NNC</i>									
Gr XIII	NNC	278 ^a ±16.08	100	3.31	---	---	---	---	---

F–Field Isolate, INC–Infected Non-medicated control, NNC–Non-infected Non-medicated control, WG–Weight gain and FCR–Feed Conversion Ratio. Different superscripts indicate significant difference ($P<0.05$) in average weight gain for between isolates under same medication (a,b,c,d) and within isolate under different medications (A,B,C,D,E,F,G,H). Different superscripts indicate significant difference ($P<0.05$) in lesion score for between isolates under same medication (a,b) and within isolate under different medications (A,B,C). Different superscripts indicate significant difference ($P<0.05$) in oocyst index for between isolates under same medication (a,b,c) and within isolate under different medications (A,B).

infected with different field isolates of *E. tenella*. In salinomycin treated group, the average weight gain in F-2 isolate was non-significantly ($P<0.05$) high as compared to other field isolates of *E. tenella*. Birds infected with F-1 and F-4 and treated with maduramicin showed a significant ($P<0.05$) decrease in the weight gain as compared to F-2 and F-3. The birds infected with caecal coccidiosis consumed less feed than the NNC group and best FCR was recorded in maduramicin treated F-2 group. The FCR values could not be analysed statistically because of group feeding of the birds. Among medicated birds, maduramicin treated groups exhibited better FCR as compared to salinomycin treatment.

Higher lesion score was recorded in INC groups as compared to treated groups. Among medicated groups, salinomycin treated birds showed comparatively higher lesion score in all four field isolates than the maduramicin treated groups. Lesion score of maduramicin treated F-1, F-3 and F-4 groups was significantly ($P<0.05$) lower than INC groups. Lesion score of F-1 birds treated with

salinomycin was comparable to respective isolate of INC group. The comparative lesion scores are given in Table 3.

Oocyst index in INC group birds was higher as compared to other groups. Birds of F-1 and F-4 of both medicated groups exhibited appreciably higher oocyst index as compared to the other isolates. Oocyst index of INC groups varied significantly ($P<0.05$) with maduramicin treated groups, whereas it was non-significant in salinomycin treated birds. Mortality was observed in medicated as well as INC groups following *E. tenella* infection and it was comparatively high in F-1, and F-2. Irrespective of the isolate, comparatively high mortality was observed in salinomycin medicated groups than maduramicin treated groups. No mortality was observed in NNC group.

The global index (GI) was calculated to assess any reduction in the efficacy of test ionophore compounds. The GI of the groups is given as percentage of the corresponding global index for the NNC. The F-1 exhibited resistance against salinomycin and maduramicin with per cent GI of 29.52 and 48.64, respectively. In F-2, 70.59 and 76.25

Table 3. Lesion score in broiler birds following *Eimeria tenella* infection with field isolates

Experimental group	Isolate			
	F-1	F-2	F-3	F-4
Salinomycin	3.07 ^{aA} ±0.19	1.23 ^{aA} ±0.12	1.92 ^{bB} ±0.21	2.38 ^{bAB} ±0.28
Maduramicin	1.76 ^{aB} ±0.24	1.46 ^{bA} ±0.16	1.23 ^{bC} ±0.14	1.76 ^{aB} ±0.24
INC	3.28 ^{aA} ±0.24	2.42 ^{bA} ±0.16	2.71 ^{bA} ±0.14	3.00 ^{abA} ±0.25

Different superscripts indicate significant difference ($p<0.05$) for between isolates under same medication (a,b) and within isolate under different medications (A,B,C); F-1: Field Isolate-Jammu; F-2: Field Isolate-Poonch; F-3: Field Isolate-Udhampur; F-4: Field Isolate-Srinagar; INC: Infected Non-medicated Control.

Table 4. Efficacy of ionophores against different *E. tenella* isolates

Isolate	Ionophore efficacy status	
	Salinomycin	Maduramicin
F-1	●	●
F-2	▲	▲
F-3	○	▲
F-4	●	

where Resistance (●), Partial Resistance (○) and Limited efficacy (▲). F - Field Isolate.

of %GI was recorded for salinomycin and maduramicin, respectively, indicating limited efficacy. The F-3 exhibited partial resistance (%GI: 56.62) against salinomycin and limited efficacy (%GI: 73.32) in case of maduramicin. In F-4 isolate, resistance was recorded against salinomycin and partial resistance against maduramicin with %GI of 42.97 and 59.26, respectively. For a better understanding, the resistance experiment with *E. tenella* field isolates is summarized in Table 4, in which the efficacy of the individual anticoccidial drug has been provided. Approaches used to detect resistance or virulence factors in several bacteria are not available for *Eimeria* spp. Therefore, *in vivo* experiments are the method of choice to estimate the sensitivity profile of *Eimeria* spp. to various anticoccidials, although, the cost and time involved in such procedures remain the limiting factors (Peek and Landman 2011). The determination of resistance/efficacy in *E. tenella* field isolates by means of the global index of each individual ionophore was evaluated in our study as has been adopted in recent past in other studies (Abbas *et al.* 2008, Tan *et al.* 2017).

Limited efficacy of ionophore against *E. tenella* F-2 may be attributed to the fact that these drugs have not been used in the area for a long time. The isolate F-2 was procured from Poonch, a remote area of UT of Jammu and Kashmir and feedback from poultry farmers and feed manufacturers revealed that other drugs like sulphaquinoxaline and amprolium are still in use for coccidiosis prophylaxis/control program and usage of ionophore has gained traction in the recent years, and only during last five years, ionophores are being used as anticoccidials. According to Tan *et al.* (2017), environmental selection pressures and history of drug usage in various geographical places may vary and strains of *E. tenella* that are resistant in one area may be susceptible in another. Another reason might be that the ionophores specific mode of action makes resistance to this class of anticoccidials difficult to develop (Peek and Landman 2011).

Coccidiosis prophylaxis in the study area is mostly reliant on the anticoccidials used by the feed mills. Some feed manufacturers, on the other hand, do not disclose the anticoccidial agent or quantity utilised and may be using same drugs for longer durations or spurious quality drugs. Sometimes, if mild form of the diseases occurs in the broiler flocks, the farmers without consulting veterinarians,

themselves do the treatment in poultry farms by employing anticoccidial medications in the drinking water as well as feed premix. In such practices, the drug is likely to be overused for longer duration. Furthermore, poultry farmers lack mixers necessary for adequate anticoccidial mixing in the feed and may result in incorrect dosing of anticoccidials.

From the results of the present study, it is concluded that in Union Territory of Jammu and Kashmir (North India), resistance/partial resistance to the ionophores (salinomycin, maduramicin) has developed against *E. tenella* field isolates. It is recommended that use of these ionophores should either be restricted or if possible be discontinued and awareness programmes among poultry farmers for adoption of rotation and shuttle programmes of anticoccidials usage should be strengthened. Moreover, significance of proper feed mixers, deleterious effects of over/under dosing coupled with rejection of spurious drugs should be prioritized in the region. It is evident with several studies that restoration of sensitivity towards anticoccidials is time and resource-intensive process. It is plausible to promote strategic assessment of resistance status *vis-à-vis* species and strain diversity in a geographical domain for informed decision making under chemotherapeutic management. Also, the available vaccines face impediment due to species and strain variations in different regions as well. This suggests that coccidiosis management will remain combinatory approach of chemotherapy and vaccination.

The present study evaluated four field isolates of *Eimeria tenella* for ionophore sensitivity (Salinomycin and Maduramicin) in *in vivo* trial. The study revealed variable degree of resistance in the filed isolates of Union Territory of Jammu and Kashmir, based on assessment of global index for the treated groups. The evidence suggests that there is need of updated strategic management of coccidiosis in the study area.

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