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Research Article

Detection of Multiple Anthelmintic Resistance of *Haemonchus contortus* and *Teladorsagia circumcincta* in Sheep and Goats of Northern Punjab, Pakistan

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Abstract

The present study was conducted to investigate the efficacy of albendazole, levamisole and ivermectin against gastrointestinal nematodes of sheep and goats. One hundred and sixty small ruminants comprised four breeds, sheep (Salt range and Pak Karakul) and goats (Jattal and Beetal) were selected for this experiment. These animals were artificially infected with nematodes (*Haemonchus contortus* and *Teladorsagia circumcincta*). FECs were done to confirm the presence of adult stages of *H. contortus* and *T. circumcincta*. The selected sheep and goat were divided into four groups. First group was treated with albendazole, second with levamisole, third with ivermectin and fourth kept as untreated to serve as control. Faecal samples were collected on day first prior to administration of treatment followed by day 14 post treatment. The efficacy of each anthelmintic was measured using the faecal egg count reduction test and egg hatch test. The percentage reduction in FECs (95% CI) for albendazole, levamisole and ivermectin for salt range sheep were 88%, 89% and 99%, for Pak karakul sheep, 91%, 90% and 97%, for Jattal goat 91%, 91% and 98%, and for Beetal goat 91%, 92% and 99%. All results showed the presence of multiple anthelmintic resistances in selected sheep and goats flocks. All flocks were found resistance against the albendazol and three were resistance against levamisole. The arithmetic faecal egg count reduction with albendazole, levamisole and ivermectin recorded for *H. contortus* and *T. circumcincta* indicated the resistance against albendazole and levamisole and susceptibility of these worms against ivermectin. The results revealed that a significant difference ($P<0.05$) of FECRT were found on pre-treatment and post-treatment with different anthelmintic drug as compared to control group in all the flocks. The results of egg hatch test indicated that LC_{50} were ranged from 0.138 µg/mL to 0.141 µg/mL for four breeds, which were more than 0.1ug/ml of albendazole. Result indicates that resistance was found in the eggs with albendazole. The egg hatch assay also confirmed the result of faecal egg count reduction test.

Keywords: *Sheep, Goat, Anthelmintic resistance, Albendazole, Levamisole, Ivermectin, Northern Punjab*

Pakistan'ın Kuzey Punjab Eyaletindeki Koyun ve Keçilerde *Haemonchus contortus* ve *Teladorsagia circumcincta*'nın Anthelmentiklere Karşı Direncinin Belirlenmesi

Özet

Bu çalışma albendazol, levamizol ve ivermektinin koyun ve keçilerdeki gastrointestinal nematodlara karşı etkinliğini araştırmak amacıyla yürütüldü. Salt range ve Pak Karakul cinsi koyunlar ile Jattal ve Beetal cinsi keçilerinden oluşan toplam 160 adet küçük ruminant bu çalışmada kullanıldı. Bu hayvanlar suni olarak *Haemonchus contortus* ve *Teladorsagia circumcincta* nematodları ile enfekte edildi. *H. contortus* ve *T. circumcincta*'nın olgun evrelerini teyit etmek amacıyla dışkı yumurta sayımı yapıldı. Seçilen koyun ve keçiler 4 gruba ayrıldı. Birinci gruba albendazol, ikinci gruba levamizol, üçüncü gruba ivermektin uygulanırken dördüncü grup kontrol olarak tutularak herhangi bir uygulama yapılmadı. Uygulamalardan önce ve uygulamanın 14. gününde dışkı örnekleri toplandı. Herbir anthelmentiğin etkinliği dışkı yumurta sayımı azalma testi ve yumurta açılımı testi ile belirlendi. Albendazol, levamizol ve ivermektin için dışkı yumurta sayımında % azalma Salt range koyununda sırasıyla %88, %89 ve %99, Pak karakul koyununda %91, %90 ve %97, Jattal keçisinde %91, %91 ve %98, ve Beetal keçisinde %91, %92 ve %99 olarak tespit edildi. Çalışmanın sonuçları seçilen koyun ve keçi sürülerinde anthelmentik direncin mevcut olduğunu göstermiştir. Tüm sürüler albendazola karşı dirençli bulunurken üçü levamizole karşı dirençliydi. *H. contortus* ve *T. circumcincta* için albendazol, levamizol ve ivermektin ile tespit edilen aritmetik dışkı yumurta sayımı azalma albendazol ve levamizole karşı direnci gösterirken ivermektine karşı duyarlılığı ortaya koymuştur. Tüm sürülerde kontrol grubu ile karşılaştırıldığında uygulama öncesi ve sonrası arasında dışkı yumurta sayımı azalma testinde anlamlı derecede fark ($P<0.05$) belirlendi. Yumurta açılımı testinin sonuçları 4 cins için LC_{50} değerinin 0.138 µg/mL ile 0.141 µg/mL arasında, albendazol için 0.1ug/ml'den daha fazla, değiştiğini gösterdi. Bu sonuçlar albendazol için yumurtada direncin varlığını göstermektedir. Yumurta çıkma testi dışkı yumurta sayımı azalma testinin sonuçlarını destekler yöndedir.

Anahtar sözcükler: *Koyun, Keçi, Anthelmintik direnç, Albendazol, Levamizol, İvermektin, Kuzey Punjab*

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INTRODUCTION

The widespread prevalence of gastrointestinal nematodes (GINs) infections in tropical and sub-tropical areas has worried the production potential of livestock development by causing countless deaths and indirect economic losses [1]. About seventy percent nematode species have been isolated from small ruminants, with over thirty percent nematode species being isolated from the digestive system worldwide [2]. The most common ovine nematode genera include *Haemonchus contortus (H. contortus), Teladorsagia circumcincta (T. circumcincta)* and *Trichostrongylus* species [3].

Parasitism leads to lowered productivity of livestock [4]. Developing countries are much more affected due to very favorable conditions for helminths [5,6]. As a result small ruminant production in the tropics and elsewhere is greatly reduced due to diseases caused by helminths [1], and helminth infection is shown in up to 95 percent of small ruminants [1]. In Pakistan, prevalence of anthelmintic ineffectiveness, in sheep and goats, has been reported by several authors [8-11]. Recently, in Pakistan, one of the important factors of high prevalence of GINs infections in small ruminants might be due to the loss of anthelmintic activity [8,9,12-14]. Resistance to the major classes of anthelmintics (albendazol, levamisol and ivermectin) in all of the economically important nematodes of sheep and goats, in particular *H. contortus*, *T. circumcincta* and *Trichostrongylus colubriformis* [14] has been recorded in Canada [15], North America [16], Latin America [17], Europe [18], Australia [19], Asia [20], Pakistan [21] and even throughout the world [22]. Development of multiple resistance has been reported due to frequent treatments with one family and thus a change to alternative families of anthelmintics [23]. Anthelmintic resistance is becoming a main constraint in small ruminant production throughout the world [24] and has serious implications due to non-availability of new drugs.

Methodologies for the examination and detection of anthelmintic resistance (AR) from the field and in the laboratory such as controlled efficacy tests (CET, drench and slaughter), faecal egg count reduction tests (FECRT), egg hatch test (EHT) and larval development test (LDT). Standardization of general parasitological methods used in the detection of AR has been reported for sheep and goats [25]. General parasitological based techniques such as CET, FECRT, EHT and LDT have been used in the initial reporting and subsequent surveying of benzimidazole and Ivermectin resistance throughout the world in ruminants.

Therefore the present study has been designed to evaluate the development of resistance against commonly used anthelmintics in sheep and goat from Punjab, Pakistan.

MATERIAL and METHODS

Experimental Design

Sheep (Salt range and Pak Karakul) and goats (Jattal and Beetal) were selected for the obtaining *H. contortus* and *T. circumcincta* eggs from northern Punjab, Pakistan. The infective stage (L_3) larvae of both *H. contortus* and *T. circumcincta* were cultured and recovered through Baermann procedure [26]. Two thousand infective larvae (L_3) of *H. contortus* and five thousand *T. circumcincta* were given to the above selected goats and sheep breed. Four weeks post infection FECs was done to confirm the presence of adult stages of *H. contortus* and *T. circumcincta.*

Grouping of Animals and Treatment

Forty goats and sheep which experimentally infected with *H. contortus* and *T. circumcincta* herd were selected separately, aged between 3 to 4 months, to conduct efficacy trial. Selected goats and sheep were divided into four groups with ten animals each group, on the basis of their equal egg per gram (EPG). Group- I were treated with albendazole (Valbazen®, Pfizer Animal Health, Exton, PA 19341, USA) by oral drench, Group- II were treated with levamisole (Levasole®, Schering-Plough, Animal Health Corp., Union, NJ 07083), Group- III were treated with Ivermectin (Ivomec®, Merial Limited Iselin, NJ, USA) by injecting subcutaneous and Group- IV were serve as control (distilled water), respectively.

Faecal Egg Count (FEC)

Faecal samples were taken from experimental animals 0 and 14 days directly from rectum with a two finger procedure in label plastic bags by using disposable gloves. All samples were screened individually for nematode eggs (FEC) at 0 and 14 days using the McMaster technique as described by MAFF [26].

Faecal Egg Count Reduction Test (FECRT)

The formulae recommended by the WAAVP guidelines for detecting anthelmintic resistant nematodes of ruminants were used to calculate mean faecal egg count, percentage reduction and 95% confidence interval. The difference between the mean faecal egg counts recorded at the first and second visit were used to calculate the percentage efficacy of each anthelmintic treatment according to the formula:

$$\text{Efficacy} = \frac{\text{(Pre-treatment mean – Post-treatment mean)}}{\text{Pre-treatment mean}} \times 100$$

An efficacy of less than 90 percent and 95 percent upper confidence levels of less than 90 percent were taken as indicating the presence of anthelmintic resistant nematode.

Coproculture Analysis

Pooled faecal samples of all animals from each experimental group were used for coprocultures before (on day 0) and after treatment (on day 14). Baermann procedure was used to recover the larvae [26] and determine the relative composition of specific resistant nematode species. The identification of larvae (L_3) was carried out by following the keys and description given by MAFF [26].

Egg Hatch Assay (EHA)

The guidelines of WAAVP were used to carry out egg hatch assay for determining anthelmintic resistance [27].

Statistics

Data analysis by statistical package POST HOC TEST (Univariate analysis of variance) was performed. Fecal egg count and larval culture records were transformed before analysis [log10 (n+1)] to stabilize the variance. The data resulting from the study were analyzed for ANOVA. Data were also be analyzed by statistical package SPSS 16 were performed. Log probit analysis [28] was also done for EHA.

RESULTS

The present study results revealed post treatments mean FECs with different drug types in salt range sheep are 160±64.89, 140±51.53 and 15±7.63 for albendazol (ABZ), levamisol (LEV) and Ivermectin (IVM) respectively. Efficacy and confidence levels indicated presence of ABZ and LEV resistant nematodes in treated sheep. However, the nematodes were found susceptible to IVM *(Table 1)*. Coproculture revealed that *H. contortus* and *T. circumcincta* were resistant to ABZ and LEV. Nematode species were susceptible to IVM. The reduction in eggs per gram (EPG) of faeces (efficacy) was 87%, 87% and 99% respectively for *H. contortus* in salt range sheep treated with ABZ, LEV and IVM. The lower confidence interval was 69%, 72%, 95% and upper confidence interval was 95%, 94% and 100% respectively. The reduction in eggs per gram (EPG) of faeces (efficacy) was 88%, 92% and 99% respectively for *T. circumcincta* in salt range sheep treated with ABZ, LEV and IVM. The lower confidence interval was 73%, 81%, 93% and upper confidence interval was 95%, 96% and 100% respectively *(Table 2)*.

Post treatments mean FECs with different drug types in Pak karakul sheep are 120±40.27, 130±42.29, 40±12.47 for ABZ, LEV and IVM, respectively. Efficacy and confidence levels indicated presence of ABZ and LEV resistant nematodes in treated sheep. However, the nematodes were found susceptible to IVM *(Table 1)*. The EPG of faeces (efficacy) was 88%, 87% and 96% respectively for *H. contortus* in Pak karakul sheep treated with ABZ, LEV and IVM. The lower confidence interval was 78%, 82%, 93% and upper confidence interval was 94%, 90% and 98%

Table 1. *Post treatments Mean faecal egg counts of different species of nematodes in different breeds of sheep and goat before and after treatment with anthelmintics*

Tablo 1. *Değişik nematodlara karşı anthelmentikler ile uygulama öncesi ve uygulama sonrası ortalama dışkı yumurta sayımı*

Breeds	Drug Types	EPG (Mean±SE) n=10	Efficacy (%)	Lower Confidence Level	Upper Confidence Level	Remarks
Salt Range Sheep	ABZ	160±64.89	88	71	95	Resistance
	LEV	140±51.53	89	76	95	Resistance
	IVM	15±7.63	99	97	100	Susceptible
	Control	1570±100	---	---	---	---
Pak Karakul sheep	ABZ	120±40.27	91	82	95	Resistance
	LEV	130±42.29	90	80	95	Resistance
	IVM	40±12.47	97	94	98	Susceptible
	Control	1310±44.59	---	---	---	---
Jattal Goats	ABZ	115±30.77	91	84	95	Resistance
	LEV	115±23.62	91	86	94	Suspected
	IVM	25±8.33	98	96	99	Susceptible
	Control	1285±38.76	---	---	---	---
BeetalGujrati Goats	ABZ	110±41.36	91	81	96	Resistance
	LEV	105±24.09	92	87	95	Resistance
	IVM	15±7.63	99	96	100	Susceptible
	Control	1345±29.29	---	---	---	---

ABZ (Albendazole), LEV (Levamisole), IVM (Ivermectin)

Table 2. *Post treatments mean FECs of nematodes with different drugs in sheep and goats*
Tablo 2. *Farklı ilaç uygulanan koyun ve keçilerde uygulama sonrası nematodarın ortalama dışkı yumurta sayıları*

Animal Breed	Drug Type	Nematode Species	Pre Treatment Mean EPG n=10	Post Treatment Mean EPG n=10	Efficacy (%)	Lower Confidence Level	Upper Confidence Level	Remarks
Salt Range Sheep	ABZ	*H. contortus*	705	90	87	69	95	Resistance
		T. circumcincta	585	70	88	73	95	Resistance
	LEV	*H. contortus*	705	95	87	72	94	Resistance
		T. circumcincta	560	45	92	81	96	Resistance
	IVM	*H. contortus*	735	10	99	95	100	Susceptible
		T. circumcincta	530	5	99	93	100	Susceptible
Pak Karakul Sheep	ABZ	*H. contortus*	725	85	88	78	94	Resistance
		T. circumcincta	575	35	94	84	98	Resistance
	LEV	*H. contortus*	705	95	87	82	90	Resistance
		T. circumcincta	570	35	94	89	97	Resistance
	IVM	*H. contortus*	715	30	96	93	98	Susceptible
		T. circumcincta	615	10	98	94	100	Susceptible
Jattal Goats	ABZ	*H. contortus*	675	75	89	82	93	Resistance
		T. circumcincta	550	40	93	83	97	Resistance
	LEV	*H. contortus*	715	80	89	84	92	Resistance
		T. circumcincta	565	35	94	87	97	Resistance
	IVM	*H. contortus*	690	20	97	93	99	Susceptible
		T. circumcincta	580	5	99	93	100	Susceptible
Beetal Goats	ABZ	*H. contortus*	660	85	87	75	93	Resistance
		T. circumcincta	615	25	96	86	99	Resistance
	LEV	*H. contortus*	715	80	89	83	92	Resistance
		T. circumcincta	570	25	96	89	98	Suspected
	IVM	*H. contortus*	700	10	99	94	100	Susceptible
		T. circumcincta	585	5	99	93	100	Susceptible

ABZ (Albendazole), LEV (Levamisole), IVM (Ivermecti

respectively. The reduction in EPG of faeces (efficacy) was 94%, 94% and 98% respectively for *T. circumcincta* in Pak karakul sheep treated with ABZ, LEV and IVM, respectively. The lower confidence interval was 84%, 89%, 94% and upper confidence interval was 98%, 97% and 100% respectively *(Table 2)*.

Post treatments mean FECs with different drug types in Jattal Goats are 115±30.77, 115±23.62, 25±8.33 for ABZ, LEV and IVM, respectively. Efficacy and confidence levels indicated presence of ABZ resistant nematodes in treated goats. However, the nematodes were found susceptible to LEV and IVM *(Table 1)*. The reduction in EPG of faeces was 89%, 89% and 97% respectively for *H. contortus* in Jattal goats treated with ABZ, LEV and IVM. The lower confidence interval was 82%, 84%, 93% and upper confidence interval was 93%, 92% and 99% respectively. The reduction in EPG of faeces was 93%, 94% and 99% respectively for *T. circumcincta* in Jattal Goats treated with ABZ, LEV and IVM. The lower confidence interval was 83%, 87%, 93% and upper confidence interval was 97%, 97% and 100% respectively *(Table 2)*.

Post treatments mean FECs with different drug types in Beetal goats are 110±41.36, 105±24.09, 15±7.63 for ABZ, LEV and IVM, respectively. Efficacy and confidence levels indicated presence of ABZ and LEV resistant nematodes in treated goats. However, the nematodes were found susceptible to IVM *(Table 1)*. The reduction in EPG of faeces was 87%, 89% and 99% respectively for *H. contortus* in Beetal Goats treated with ABZ, LEV and IVM. The lower confidence interval was 75%, 83%, 94% and upper confidence interval was 93%, 92% and 100%, respectively. The reduction in EPG of faeces was 96%, 96% and 99% respectively for *T. circumcincta* in Beetal Goats treated with ABZ, LEV and IVM. The lower confidence

Table 3. *Percent eggs hatched at different concentrations of albendazol in sheep and goats*
Tablo 3. *Koyun ve keçilerde farklı albendazol konsantrasyonları için yumurtadan çıkma yüzdeleri*

Animal Breed	Conc. Albendazol µg/mL	Conc. Albendazol ng/mL	Log (Albendazol)	% age Hatch	Probit Hatching	Regression	LC_{50} µg/mL
Salt Range Sheep	0.001	1	0	97	6.88	7.1971	0.138
	0.05	50	1.699	82	5.915	5.336048	
	0.1	100	2	54	5.1	5.0063	
	0.3	300	2.4771	28	4.417	4.483661	
	1	1000	3	18	4.084	3.9109	
	2	2000	3.301	3	3.119	3.581152	
Pak Karakul Sheep	0.001	1	0	96.3333	6.774	7.1294	0.138
	0.05	50	1.699	81	5.877	5.253737	
	0.1	100	2	51	5.025	4.9214	
	0.3	300	2.4771	26.6667	4.375	4.394658	
	1	1000	3	16	4.005	3.8174	
	2	2000	3.301	2	2.946	3.485063	
Jattal Goats	0.001	1	0	96	6.75	7.1708	0.141
	0.05	50	1.699	84	5.994	5.242639	
	0.1	100	2	51.6667	5.041	4.901	
	0.3	300	2.4771	23	4.261	4.359515	
	1	1000	3	17	4.045	3.7661	
	2	2000	3.301	1.3333	2.773	3.424461	
Beetal Goats	0.001	1	0	95.3333	6.674	7.0455	0.141
	0.05	50	1.699	81	5.877	5.236777	
	0.1	100	2	51.6667	5.041	4.9163	
	0.3	300	2.4771	27.6667	4.405	4.408357	
	1	1000	3	15.6667	3.989	3.8517	
	2	2000	3.301	2.3333	3.004	3.531223	

interval was 86%, 89%, 93% and upper confidence interval was 99%, 98% and 100%, respectively *(Table 2)*.

Results of EHT revealed that all the four flocks found positive for resistance against ABZ had LC_{50} values more than 0.1 µg/mL. The LC_{50} value for salt range sheep was 0.138 µg/mL, Pak Karakul sheep 0.138 µg/mL, Jattal goats 0.141 µg/mL and Beetal goats 0.141 µg/mL which confirmed the results of FECRT *(Table 3)*.

DISCUSSION

Faecal egg count reduction (efficacy in percent) and confidence levels indicated that the presence of ABZ, LEV and IVM resistant nematodes in treated sheep and goats. Mostly, the nematodes were found susceptible to IVM. The results revealed that a significant difference ($P<0.05$) of FECRT were found on pre-treatment and post-treatment with different anthelmintic drug as compared to control group. These results are inconformity with that of Sheferaw *et al.*[29] suggested that it is safe to use these drugs because they all have safe efficacy. The efficacy values are for albendazole, tetramisole and ivermectin were 99.34%, 97.77% and 98.30%, respectively. Moreover, Gill [20] and Uppal *et al.*[30], verified similar findings in India but with low efficacy.

Copro-culture revealed that *H. contortus* and *T. circumcincta* were resistant to ABZ and LEV. Nematode species were susceptible to IVM. These results also similar with the other studies and are especially for the ABZ [23,31,32]. Explanation for the effectiveness of potent agent IVM among all sheep and goats might be introduction of susceptible parasite population which replaced the resistant parasites by presenting the susceptible status to the nematode parasite population within the host body. These results are inconformity with previous studies [23,31]. Moreover, similar findings were verified in India [30].

According to our coproculture analysis results, in sheep and goat which have shown *Haemonchus contortus* to have the greatest propensity for deworming resistance to ABZ. These findings are in accordance with Chandrawathani *et*

al.[33]. Moreover, sheep and goats are constantly at the high risk to acquire the nematodes infection, as a result farmers continue using drugs without the basic information of their dosage and administration hence resulting in a stern multiple anthelmintic resistances as indicated in our Salt range breed results. According to Coles and Roush [27], the optimal recommendation is to use anthelmintic different families one by one according to the demand of the host. Overall, the supporting fact regarding drug resistance against heterogeneous nematodes population within and outside the body of host in Northern region points towards existing poor farm management husbandry practices, illiteracy of farmer and lack of basic knowledge about drug, nematodes infection and administration to host. Similar trend for efficacy of ABZ and LEV in sheep and goat was also reported [22]. Basically emergence of AR is a result of many factors such as genetic, biological or operational.

Egg hatch test (EHT) was performed for confirmation of ABZ resistance detected by FECRT. Results of EHT revealed that all the four flocks found positive for resistance against ABZ had LC_{50} values more than 0.1 µg/mL. The LC_{50} values ranged from 0.138 µg/mL to 0.141 µg/mL, which confirmed the results of FECRT. The egg hatch assay was carried out using the WAAVP guidelines for determining anthelmintic resistance [34] with modifications that allowed the testing of these natural compounds. EHT was performed for confirmation of results of FECRT and found 100% correct. Logarithmic concentration (LC_{50}) value was calculated for the eggs by log probit analysis [28]. Eggs having LC_{50} value in excess of 0.1 µg anthelmintic per mL was indicative of anthelmintic resistance against ABZ [35].

The present study results revealed that, percent reduction in EPG of faeces was resistant with ABZ in all breeds of sheep and goat against *H. contortus* and *T. circumcincta* infection which are in agreements with Gill [20] who observed resistance to ABZ and LEV against sheep nematodes. These findings are against previous studies that reported no resistance against ABZ in sheep [36,37]. Sargison [38] reported multiple anthelmintic resistances in sheep and goat. Marian *et al*.[39] used egg hatch test for benzimidazole resistance and compared it with FECR test and declared resistance with two tests. The LD_{50} was higher than 0.1mg/ml thiabendazole indicating resistance, which in agreement to the present study. The economic significance of helminths throughout the World [40] necessitates immediate attention to launching an effective control program. Chemical control is the main part of all helminth control programs [22]. The other control programs includes an integrated approach based on the use of plants with modern anthelmintics, Mass education awareness program and most importantly pasture management (clean and alternated pasture).

The present study indicates the presence of anthelmintic resistance against nematode infections in native sheep and goats of Northern Punjab. Therefore it is recommended that the proper anthelmintic dose with 3-4 months interval and rotation of anthelmintics for minimized the nematode infection and enhance goat and sheep productivity.

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