DETERMINATION OF MULTIPLE ANTHELMINTIC RESISTANCE AGAINST *HAEMONCHUS CONTORTUS* IN SHEEP IN AZAD JAMMU AND KASHMIR

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ABSTRACT

The present study was designed to investigate the development of multiple AR in *H. contortusin vivo* and *in vitro* using Fecal Egg Count Reduction Test (FECRT) and Egg Hatch Assay (EHA). Fecal samples (n=300) were collected and screened for *H. contortus* eggs through McMaster technique.40 sheep positive for *H. contortus* having EPG of more than 500 were divided into four groups of 10 animals each. Four anthelmintics were used including; Albendazole (10 mg/kg BW), Levamisole (7.5 mg/kg BW), Ivermectin (0.2 mg/kg BW) and Oxfendazole (4.5 mg/kg BW). The FECRT was done at 0, 7, 14 and 21 days post treatment. The results of FECRT and EHA revealed that the mean efficacy of Albendazole and Oxfandazole was less than 95% and higher than 0.1µg/ml respectively as described by World Association for the Advancement of Veterinary Parasitology (WAAVP) which confirms the presence of AR of *H. contortus*against tested anthelmintics.

Keywords: Anthelmintic Resistance, FECRT, EHA, Albendazole, Oxfandazole.

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INTRODUCTION

Haemonchus contortus sucks blood which eventually decrease red blood cells, white blood cells, hemoglobin body weight and wool growth as it is present in abomasum of them (Borges *et al*. 2020). After its presence an infected animal shows multiple signs, mostly in those hosts or animals which have regular infections or in young the animals. The main medical signs identified due to infection of are the reason of haematophagic behaviour of *H. contortus.* These parasites becomethe reason of an animal's death as they cause anemia. It is perceived that in 24 hours 0.05 ml of blood had sucked by every worm (Shen *et al*. 2019). In case of severe infection as in some cases due to heavy blood loss caused by sucking leads towards inappetance and loss of weight and at last condition of anemia occurs because animals get nutritional stress (Durate *et al*. 2019). *H. contortus* parasite has key economic importance and infections caused by them are identified all over Pakistan.

A drop-in effectiveness of drugs against parasite's population due to genetics termed as anthelmintic resistance (AR) (Stuchlikova *et al*. 2018). In the world domestic animals have been treated with anthelmintics to cure infection of helminth (Shen *et al*. 2019). A lot of nematodes which relates to the field of veterinary sciences have some qualities which can be used to develop anthelmintic resistance. Geneticaly they are gifted with a gene which can fight against anthelmintics action and this particular gene guarantees the movement of its qualities of resistance through the movement of host (Mohanraj *et al*. 2017). A bunch of information have been collected regarding species which spread helminthes through numerous studies. Those studies also identified its area of abundance, different techniques to detect it, numerous drugs to control, measure and different recommendation for its control (Wang *et al*. 2017).

Not only in Pakistan but also all over the world, GINs' resistance regarding anthelmintic has been increase at high level counter to anthelmintic chemotherapeutic individuals (Papadopoulos *et al*. 2012; Chaudhry *et al*. 2015). The only trustworthy method to control GINs is Chemotherapy however it is restricted with only three classes which belongs to anthelmintics. Those three classes are benzimidazoles, imidazothiazoles and macrocyclic lactones. Moreover, globally it is proved that nematodes of goats have developed resistance against these drugs (Saeed *et al*. 2010). The movement of resistant strains and its frequency is improves directly after improvement in the movement of its host (Muchiut *et al*. 2018). Many Scientist have quoted anthelmintic

presence as failure of goats and sheep farm in Pakistan (Saeed *et al*. 2010). Presence of *H. contortus* in small animals like goats and sheep in Pakistan with high rate is only due to anthelmintic activity (Ali *et al*. 2019).

After assessing all the details this study was planned to identify multiple anthelmintic resistance against *H. contortus* in sheep in Mirpur Azad Kashmir. The main objectives of this study were to diagnose haemonchosis in Mirpur Azad Jammu and Kashmir and assess the presence of anthelmintic resistance in *H. contortus* against commonly available anthelmintics.

MATERIALS AND METHODS

Study Area: The study was done in district Mirpur Azad Jammu and Kashmir (AJK). Mirpur Azad Kashmir and its surroundings were considered for the selection of animals from population of sheep. District Mirpur located at highest area of southern Azad Kashmir having elevation of 459m (1509 ft) from sea level. The latitude and longitude of Mirpur AJK is 33.1480° N, 73.7537° E respectively.

Sampling: Fecal samples around 300 in numbers were collected from rectum of animals regardless of breed, age and sex.The weight of each samples was maintained upto 5 gram and samples were kept at 4°C. The bags in which samples were preserved were zip lock and each sample was given a tag number.

Examination of fecal samples: The samples of fecal were then brought into laboratory and examined for the purpose to check the existence of eggs of *Haemonchus contortus*. Microscope was used to examine the fecal samples and eggs were counted through McMaster egg counting technique as described by Zajac and Conboy (2012).

Direct microscopic examination: A glass slide was used to take small sample of faeces. After that for the purpose to make suspension 2 to 3 drops of water were added into faeces. In next step detritus was distant. At last, a cover slip was putted and sample was studied underneath microscope.

McMaster egg counting technique: With the help of this technique, eggs per gram (EPG) of feces of sheep were determined. A McMaster slide which have two chamber having 1.5mm depth and 0.15ml volume was used in this particular technique. In first step sample of 2 gram was made by using weighing balance after that in beaker with sample ZnSO4 saturated solution in quantity of 28ml was added. In next step the mixture were stirred to break pellets and to get homogenized mixture. Then mixture was strained eth the help of a 20 cm diameter strainer. For the purpose to separate eggs from solution, the solution is then shifted into a test tube after doing so the eggs started floating on surface of the solution. In last few steps, with utmost care eggs were picked through pipette and eggs were place on McMaster. At last, the eggs were counted by placing McMaster slide under the. The EPG was calculated as:

EPG = No. of eggs counted in both chambers x 50

Anthelmintic Resistance Trial: Coles *et al*. (1992) described two main tests to detect anthelmintic resistance. The assessment of anthelmintic resistance was done through FECRT (Faecal Egg Count Reduction Test) and EHA (Egg Hatch Assay) tests.

Grouping of Animals: *Haemonchus contortus* was detected in forty sheep which had more than 500 eggs per gram. Four groups of 10 animals of these sheep were then randomly made and name was given alphabetically. The drugs were given per oral (PO) according to bodyweight (BW) of the experimental animals. Four anthelmintics were used to check their efficacy as Albendazole (10 mg/kg BW), Levamisole (7.5 mg/kg BW), Ivermectin (0.2 mg/kg BW) through sub-cutaneous route and Oxfendazole (4.5 mg/kg BW).

Fecal sample collection and analysis: Pre-treatment was done at day 0 and fecal samples were collected from all sheep present in each group and post-treatment at day 7, 14 and 21. FECRT was performed on these samples.

Fecal egg count reduction Test (FECRT): FECRT is an *in vivo* technique which is very important. This technique is used commonly to test anthelmintic resistance throughout the world. The comparison between pre-treatment and post-treatment was done based on their egg per gram and then by using formula percent eff reduction was calculated.

$$\% \text{ Egg Reduction} = \frac{(\text{EPG pre} - \text{treatment mean}) - (\text{EPG post} - \text{treatment mean})}{(\text{EPG pre} - \text{treatment mean})} \text{X } 100$$

If the eggs of *H. contortus* are not reduced upto 95% by an anthelmintic then parasite's population which is being tested considered as resistant.

Egg Hatch Assay: Egg hatch assay is an in vitro approach based on the capacity of egg hatching which are incubated with different concentrations of drugs. Oxfendazole and albendazole resistance against *H. contortus* were determined by this assay. For this purpose, 3% dimethyl sulfoxide solution was used to prepare oxfendazole and albendazole with 0.005-2.6 µg/ml concentrations. Eggs of *H. contortus* that was freshly isolated were incubated for two days in 24 well cluster plates at 22 °C. Lugol's iodine was added to each

well after 2 days of incubation to stop the further hatching. Effective dose-50 (ED-50) was calculated by using Probit analysis and resistant was considered at ED-50 value more than 0.1 µg/ml.

Statistical Analysis: Significant difference was find out by using one way analysis of variance (ANOVA) to statistically analysed data on EPG for FECRT. Probit analysis were used to analysed results of Egg Hatch Test. SPSS Version 20.0 software were used to analysed data statistically. Level of probability ($P<0.05$) was shown results are statistically significant.

RESULTS

Fecal egg count reduction test: Table 1, 2 and 3 were shown faecal egg count reduction test result performed in sheep at 7th, 14th and 21st day of test respectively. 84. 15% efficacy of albendazole were found at day 7 and oxfendazole 87.07%, while levamisole 96 % and ivermectin were showed efficacy 98%. At day 14th again calculated the efficacy of these anthelmintic and results were showed an efficacy of oxfendazole 87.69 and albendazole 89.62 while ivermectin and levamisole were shown 99% effective. An efficacy of these anthelmintic was again calculated at day 21 that was 93.2% for oxfendazole, 88.32% for albendazole, 98% for levamisole and 100% for ivermectin. However, the mean efficacy i.e. 89.2%, 87.43%, 97.66% and 99% were respectively came out of these anthelmintic. The results of fecal egg count reduction test were revealed that *H. contortus* in sheep population were resistant to oxfendazole and albendazole because their mean efficacy is less than 95% at day 7, 14 and 21 while levamisole and ivermctin was susceptible for *H. contortus*.

Egg Hatch Test: Figure 1 and 2 showed the results of egg hatch test for albendazole and oxfendazole respectively. The value of LC50 is 0.89 µg/ml for albendazole and 1.09 were found for oxfendazole. According to WAAVP the LC50 value for oxfendazole was higher than 0.1 µg/ml that is discriminating dose for benzimidazole group. So that resistant developed against oxfendazole and albendazole for *H. contortus*.

Table 1. Effect of different anthelmintics on fecal egg count/egg per gram (EPG) (Mean ± SE) and % efficacy against *Haemonchus contortus* in Mirpur Azad Kashmir at day 7.

Anthelmintic	Pre-treatment EPG Day 0	Post-treatment EPG Day 7	Efficacy % Day 7	Remarks
Albendazole	915±10^{a}	145±32.33^{a}	84.15%	resistance
Oxfendazole	890±81^{a}	80±23.00^{b}	87.07%	resistance
Levamisole	820±70^{a}	30±13.00^{c}	96.00%	susceptible
Ivermectin	950±14^{a}	20±11.00^{d}	98.00%	susceptible
Control	810±69^{a}	885±65.00^{e}	---	---
P value	.375	.000	---	---

Values bearing different superscript letter in each column are statistically significantly different ($P<0.05$)

Table 2. Effect of different anthelmintics on fecal egg count/egg per gram (EPG) (Mean ± SE) and % efficacy on *Haemonchus contortus* in Mirpur Azad Kashmir at day 14.

Anthelmintic	Pre-Treatment EPG Day 0	Post-Treatment EPG Day 14	Efficacy % Day 14	Remarks
Albendazole	915±10^{a}	95±24.09^{b}	89.62%	resistance
Oxfendazole	890±81^{a}	80±30.00^{c}	87.60%	resistance
Levamisole	820±70^{a}	5±5.00^{d}	99.00%	susceptible
Ivermectin	950±14^{a}	5±5.00^{d}	99.00%	susceptible
Control	810±69^{a}	945±70^{e}	---	---
P value	.375	.000	---	---

Values bearing different superscript letter in each column are statistically significantly different ($P<0.05$)

Table 3. Effect of different anthelmintics on fecal egg count/egg per gram (EPG) (Mean ± SE) and % efficacy on *Haemonchus contortus* in Mirpur Azad Kashmir at 21.

Anthelmintic	Pre-treatment EPG Day 0	Post-treatment EPG Day 21	Efficacy % Day 21	Remarks
Albendazole	915±10[a]	105±28.33[b]	88.52%	resistance
Oxfendazole	890±81[a]	60±20.00[c]	93.2%	resistance
Levamisole	820±70[a]	15±7.00[d]	98.00%	susceptible
Ivermectin	950±14[a]	0±0.00[e]	100%	susceptible
Control	810±69[a]	1075±91.0[a]	---	---
P value	.375	.000	---	---

Values bearing different superscript letter in each column are statistically significantly different (P<0.05).

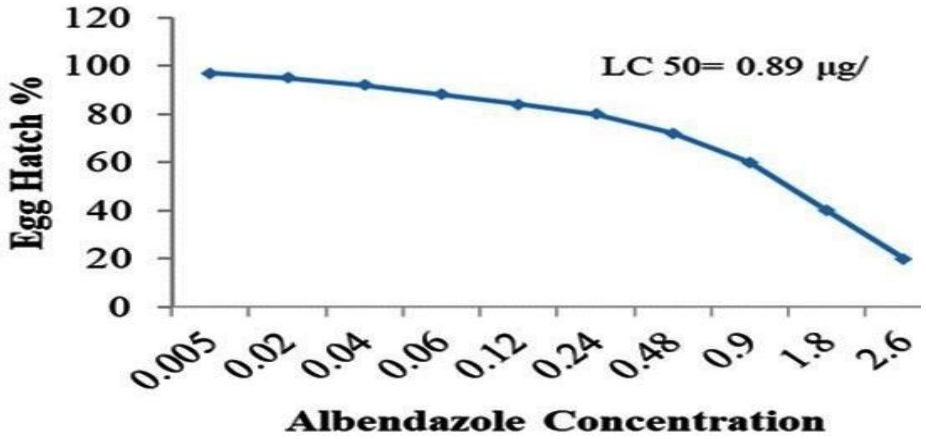

Figure 1. Effect of different concentrations of Albendazole on egg hatching % *of Haemonchus contortus.*

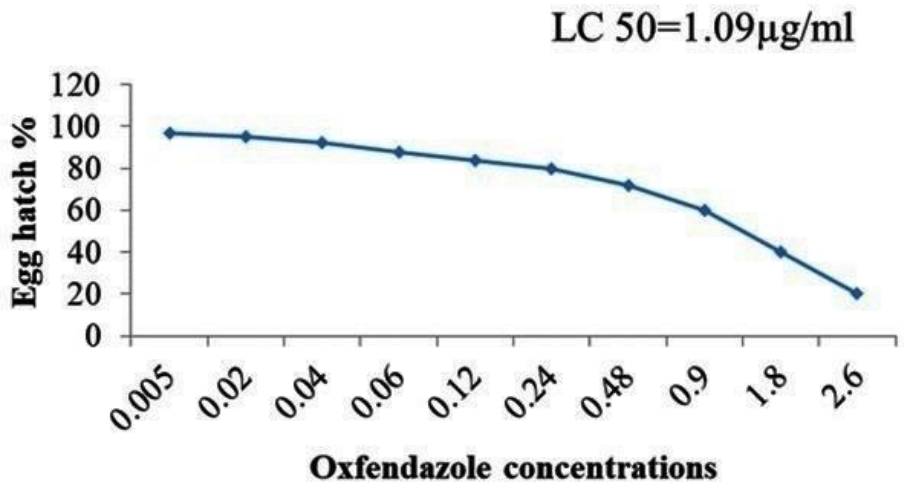

Figure 2. Effect of different concentrations of Oxfendazole on egg hatching % of Haemonchus contortus.

DISCUSSION

Nematodes infection mainly gastrointestinal nematodes (GIN's) play primary role in production losses, increase in managemental cost of animals and cause of animal death. (Teref *et al*., 2012) Helminthes have greatly reduced small ruminant production due to disease in the tropics and elsewhere. (Puspatisari *et al* 2009). In Indian occupied Kashmir Tariq et., al (2010) stated 54.3% GINs prevelance. Similarly a study was conducted in Rawalpindi, Islamabad region to explore the prevelance of gastrointestinal nematodes. It was found that the overall prevelance of GINs was 66.45% by Chaudhry *et al*. (2016). Saddiqi *et al*. (2011) presented similar results of prevelance of nematodes about 63.7 % in Rawalpindi region of Pakistan.

In various parts of the world, pharmaceutically derived anthelmintics have been used against infection of helminth in domestic animals (Shen *et al*. 2019). Data is available in reports that there is existence of AR (Anthelmintic Resistance) counter to nematodes and resistance against the drugs have developed (Chaparro *et al*. 2017).

In nematodes which are of veterinary importance AR (Anthelmintic Resistance) has been developed intensely against groups which belongs to antinematodal drug and this is reported not only in Pakistan but also in whole of the world (Papadopoulos *et al*. 2012). Anthelmintics disaster has been reported in Pakistan by many authors Saeed *et al*. (2010), and Muhammad *et al*. (2015). Furthermore, presence of resistance against nematodes is identified in goats precisely with respect to Pakistan (Saeed *et al*. 2010). The movement of host have a great positive impact on the anthelmintic resistance and resistant strains as it effects the frequency and spread of these two (Muchiut *et al*. 2018). Ali *et al*. (2018), studied that helminthiasis mostly present in goat, sheep, buffalo and cattle in Pakistan's irrigated areas. The results from study by Raza and co. identify that the percentage of presence is 52%, 62%, 47% and 51% in goat, sheep, buffalo and cattle respectively. In the particular study, hey have stated that fecal egg counts percentages of ivermectin and levamisole were 99% and 97.6 % respectively. The results of Muhammad *et al*. 2015 for ivermectin in sheep which is salt range breed as well as belongs to karakul breeds are nearly alike to the results of Ali *et al*. and results of Chaparro *et al*. (2017) about levamisole against Haemonchus contortus are comparable to this study but on the contrary results considered by Muhammad *et al*. (2015) and Akhter *et al*. (2014) for levamisole are conflicting as they have reported resistance against levamisole. I can be assumed that this type of ambiguity in results regarding levamisole's resistance is because of its low use in Azad Kashmir.

Efficiency of different drugs to counter Haemonchus contortus can be detected by different tests like egg hatch assay (EHA) and fecal egg count reduction test (FECRT) and these were exercised to check drugs like Valbazen, Levamisole and Dectomax. Four groups were formed with 8 goats for each one which were infected naturally. One of two tests to check efficiency of Dectomax have indicated that it has 91.8% efficacy followed by 88.6% of Valbazen and 83.4% of Levamisole after 10th and 14th day after treatment to counter Haemonchus contortus. The results of another test which is egg hatch recommended that all the three drugs (anthelmintics) have been failed to couter H. contortus particularly goats (Akhter *et al*. 2014).

A farm which belong to sheep and doubted to have resistance of ivermectin was examined by testing through faecal egg count reduction test. The animals were separated in 5 groups and every group possess 10 animals for the sake of experiment. First group from those 5 were identified as control and other four groups were individually treated according to dose recommended by manufacturer with doramectin, levamisole, albendazole and moxidectin. Results from test showed that two drugs doramectin and albendazole had low efficiency which was 15 % and 87% respectively otherwise other two performed well as moxidectin showed 99% and Levamisole showed 100% effectiveness (den Brom *et al*. 2007).

Hamad *et al*. (2013), did an experimental test to check the efficacy of Nicotiana tabacum leaves against H. contortus in sheep. A catastrophic level of resistance was showed against H. contortus which belong to sheep and oxfendazole as it belongs to benzimidazole group and results was obtained through EHA and FECRT. Adult motility test (AMT), FECRT and EHA were used to check the efficacy of leaves of tobacco to counter nematodes. Efficacy of oxfendazole has been lost and same is the case with Albendazole. Kowal *et al*. (2016) have described that the albendazole's efficacy have been drooped down to 87% which is very low to counter Haemonchus contortus. A study showed that the albendazole has its effect in percentage is 87.43 which is very low (Akhtar *et al*. 2014). In this study it is stated oxfendazole has lost its effectiveness as high level of resistance has been developed against it and its efficiency is up to 89.2% and this result was supported by the similar findings of dos Santos *et al*. (2017). Saeed *et al*. (2010) also supports our results as a low-level efficiency of oxfendazole was stated in their study.

Conclusion: Different doses of oxfendazole as well as albendazole was used to complete egg hatch test. Liquid concentration (LC50) of both drugs were calculated and for albendazole it was 0.82ug/ml and for oxfendazole it was 1.09ug/ml anf this dose of both drugs were more than the dose of benzimidazole which is 0.1µg/ml and this express the existence of anthelmintic resistance and supports the findings of faecal egg count reduction test. These results are supported by the study of Muhammad *et al*. (2015). Results of Egg Hatch Test are similar to the results of faecal egg count reduction test.

Conclusion: In Azad Jammu and Kashmir resistance against oxfendazole as well as albendazole supposed to be the result of non-technical staff and may be due to excessive and overdose of anthelmintics.

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