COMPARATIVE EFFICACY OF ORAL FORMULATIONS OF IVERMECTIN AND LEVAMISOLE UNDER *IN VITRO* CONDITIONS AGAINST *HAEMONCHUS CONTORTUS*

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ABSTRACT: Anthelmintic resistance (AR) is the major constraint towards controlling parasitism of livestock. This study was executed to evaluate relative efficacy of oral formulation consisting of ivermectin (IVM) and levamisole (LEV) towards *Haemonchus contortus* under *in-vitro* condition. The worms were isolated from abomasa (n=384), collected from nearby slaughter house. The egg hatch test (EHT), adult motility test (AMT), and larval development test (LDT) were performed for estimation of lethal concentration (Lc) 90. A typical sigmoid dose response was converted to linear function by using Probit transformations. Different concentrations of LEV (0.172, 0.086, 0.043, 0.021, 0.010, 0.005 µl/mL), and IVM (0.002, 0.001, 0.0005, 0.00025, 0.000125, 0.0000625 µl/mL) were incubated with 100 eggs / concentration. The LC 90 values of LEV and IVM were found higher than values suggested by World Association for the Advancement of Veterinary Parasitology (WAAVP), which is (0.1µg/ml). The dose-dependency stipulates development of (AR) anthelmintic towards *H. contortus.*

Keywords: *Haemonchus contortus*, Levamisole, Ivermectin, *In-vitro*.

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INTRODUCTION

Helminths are the most important GI tract parasites which decrease feed conversion ratio due to reduced appetite, disturbance in food metabolic function and malabsorption of nutrients (Hamid *et al.*, 2016). This ultimately results in decreased growth rate, production, reproductive efficiency and increases the susceptibility of the immunocompromised animal to other harmful pathogens (Khan *et al*., 2017). These parasites can cause pathological changes to GI tract as well as abomasal mucosa, which results in serious morbidities, epidemiological changes, constant economic losses, biochemical disturbances and hematological changes (Maharana *et al*., 2016). These were the major factors which intently driven the scientists to conduct variety of research on different aspects of life cycle of *H. contortus* worldwide.

Haemonchus contortus prevalence reported from various parts of the world including Tunisia (79.66%), Turkey (10%), Cyprus (55%), Nigeria (87%), Germany (76.3%), Togo (81%), Eastern Ethiopia (60%), Egypt (33.06%), Iran (77.5%), Iraq (32.35%), Sudan (32%), India (74.92%) and Bangladesh (58%) (Umur and Yukari, 2005; Mesele *et al*., 2014). In Pakistan, the abattoir-based epidemiology of *H. contortus* has been reported in many areas including Lahore (24.92%), Gujranwala (38.33%), Shiekhupura (39.17%), Kasur (37.25%) and Multan (29.34%) (Gadahi *et al*., 2009).

Chemotherapy is the most extensively use approach for controlling GI tract helminths but, cost of treatment and AR are major constraints in failure of chemotherapeutics in the field (Tariq, 2015). The development of AR was first reported by George *et al*. (2012) against Phenothiazine. Among all anthelmintics used in small ruminants, benzimidazole was firstly reported to lose efficacy (Drudge *et al*., 1964). The anthelmintics currently available include nitrophenols, salicylanilides, benzimidazoles, imidazothiazoles and macrocyclic lactones (Coles *et al*., 2006). In 1980s, Macrocyclic lactones were marketed to use against parasites (Chabala *et al*., 1980) however, resistance has already been reported against this group (Echevarria *et al*., 1996). Anthelmintic resistance in small ruminants have been reported worldwide including Paraguay (Martin *et al*., 2012), Mexico (Whelan *et al*., 2010),

Canada (Falzon *et al*., 2014), Australia (Playford *et al*., 2014), New Zealand (Waghorn *et al*., 2006) and Italy (Zanzani *et al*., 2014). Reports on anthelmintic resistance against different classes of anthelmintics including: thiabendazole, levamisole, phenothiazine derivatives, refoxamide, ivermectin and organophosphates have been published globally (Sprenger *et al*., 2013; Leathwick and Besier, 2014; Cintra *et al*., 2016).

In Pakistan, oral formulation of IVM has recently been gone under in vitro trials to evaluate its efficacy against *H. contortus*. The oral formulation of IVM has been tested first time against different life cycle stages (egg, larvae, and adult) of *H. contortus* procured from slaughterhouse of Faisalabad. This study provides the first report on comparative susceptibility of *H. contortus* worms to oral formulations IVM in native sheep and goat population of district Faisalabad.

MATERIALS AND METHODS

Study area and sample collection: The district Faisalabad is located at North East of Punjab, which lies on longitude 73°74 east, and latitude 30°31.5 north, at on the elevation (604) feet above sea level. The total livestock population of the district is 2.93 million out of which small ruminant population contributes highest share consisting of 0.53 million heads. Adults *H. contortus* were procured from the slaughter house of Ghulam Muhammadabad, Faisalabad. The efficacy trials were conducted at MPL University of Agriculture, Faisalabad.

Study animals and collection: The planned study was performed in 2018 during March to August (spring and summer seasons). A total number of 384 abomasa from sheep (n=232) and goats (n=152) were collected immediately after slaughtering. The abomasa were then transported to the MPL, Department of Parasitology (DOP), Faculty of Veterinary Science (FVS), University of Agriculture, Faisalabad (UAF).

***In-vitro* anthelmintic activity:** Tests including; EHT, LDT and AMA were applied for testing the anthelmintic activity in accordance with the guidelines of WAAVP Coles *et al*. (1992). Oral formulations of LEV (1.5% w/v) and IVM (10mg/ml) were used for performing the assays.

a) Egg hatch test: The female *H. contortus* were triturated with the help of pestle and mortar. The resulting material was centrifuged at 300 *xg* for 2 minutes to make a suspension in Eppendorf tubes. After centrifugation, the tubes were agitated with vortexer and added up saturated salt solutions. Then the suspension obtained was one more time centrifuged at 300 *xg* for 2 minutes for floating the eggs. The supernatant containing eggs was collected in separate Eppendorf tubes and eggs were adjusted to a level of 100 eggs/mL in McMaster chamber (Soulsby, 1982).

The tests were executing as per guidelines published Coles *et al*. (1992). Briefly, 0.2 mL suspension contain 100 eggs approximately was inoculated in 24 wells micro-titration plates. Equal volume two-fold diluted concentration consisting of IVM (0.0020, 0.001, 0.0005, 0.00025, 0.000125 and 0.0000625 µl/mL), & LEV (0.172, 0.086, 0.043, 0.0215, 0.01075 and 0.00538 µl /mL) were added in the wells. Phosphate Buffer Saline (PBS) was added up as negative control. The incubation was done at 27°C for a period of 48 hours. Following incubation, the lugol's iodine solution (LIS) was added up to the solution to inhibit/prevent hatching of eggs. Then the eggs were counted on each plate. Three replications of the test were performed.

b) Larval development test: The test LDT was performed following the guidelines devised (Hubert & Kerboeuf, (1992). In brief, 500 µL suspension contains100 eggs approximately was poured into the test tubes of 5 mL volume containing 150 µL of glucose as nutritive medium. 24 hours of incubation was done at 27°C. The test tubes were then added with different concentrations of LEV and IVM as mentioned above. The negative control was added only with diluent and eggs suspension. Another incubation was done for 7 days at 27°C. The same procedure was repeated thrice to describe effect of above mentioned anthelmintics on growth of larvae to L1, L2 and L3 stages under inverted microscope.

c) Adult motility test: Abomasa was incised longitudinally, examined and scraped carefully to remove any adhering worms. Adult worms (*H.contortus*) were isolated from abomasa of slaughtered animals and preserved in the PBS at 4°C and pH 7.2.

The test was performed as per guidelines devised by Coles *et al*. (1992). In brief, a total of 10 adult *H. contortus* were exposed to different prepared concentrations of LEV and IVM at room temperature as mentioned earlier. No anthelmintic was added to negative control wells. The activity of anthelmintics was dependent upon the mortality and motility of tested worms which was observed subsequently every two hours till 12 hours. The immotile worms were suspended in lukewarm PBS to monitor recovery of motility, if any. Three replications of the test were performed.

Statistical analyses: The Probit transformation has been applied to transform a typical sigmoid dose-response curve to linear function. Lethal concentration (LC) 90 was calculated using linear regression (for $y = 0$ on the probit scale). The data was analyzed statistically using SAS (Schork and Remington, 2010).

RESULTS

Non-significant difference in the hatching (%) of *H. contortus* eggs was found at different concentrations of LEV and IVM. The LC90 values of LEV and IVM were found 4.82 µg/ mL and 2.77 µg/mL, respectively that are elevated than the suggested value of an anthelmintic (0.1µg/ml). Association between effect of varied concentrations of LEV and IVM on hatching inhibition (%) as determined through EHA is presented in Figure 3.1a and 3.1b, respectively.

Significant association (P<0.05) was observed in the development (%) of *H. contortus* larvae at different concentrations of LEV and IVM. Association between effect of various concentrations of IVM and hatching inhibition (%) as determined through LDT presented in Figure 3.1c and 3.1d, respectively.

Significant association (P<0.05) in the motility (%) of *H. contortus* adults was found at different concentrations of LEV and IVM at regular time intervals of 2 hours respectively. Association between effect of various concentrations of LEV and IVM on adult motility (%) as determined through AMT is presented in figure-3.1e and 3.1f, respectively.

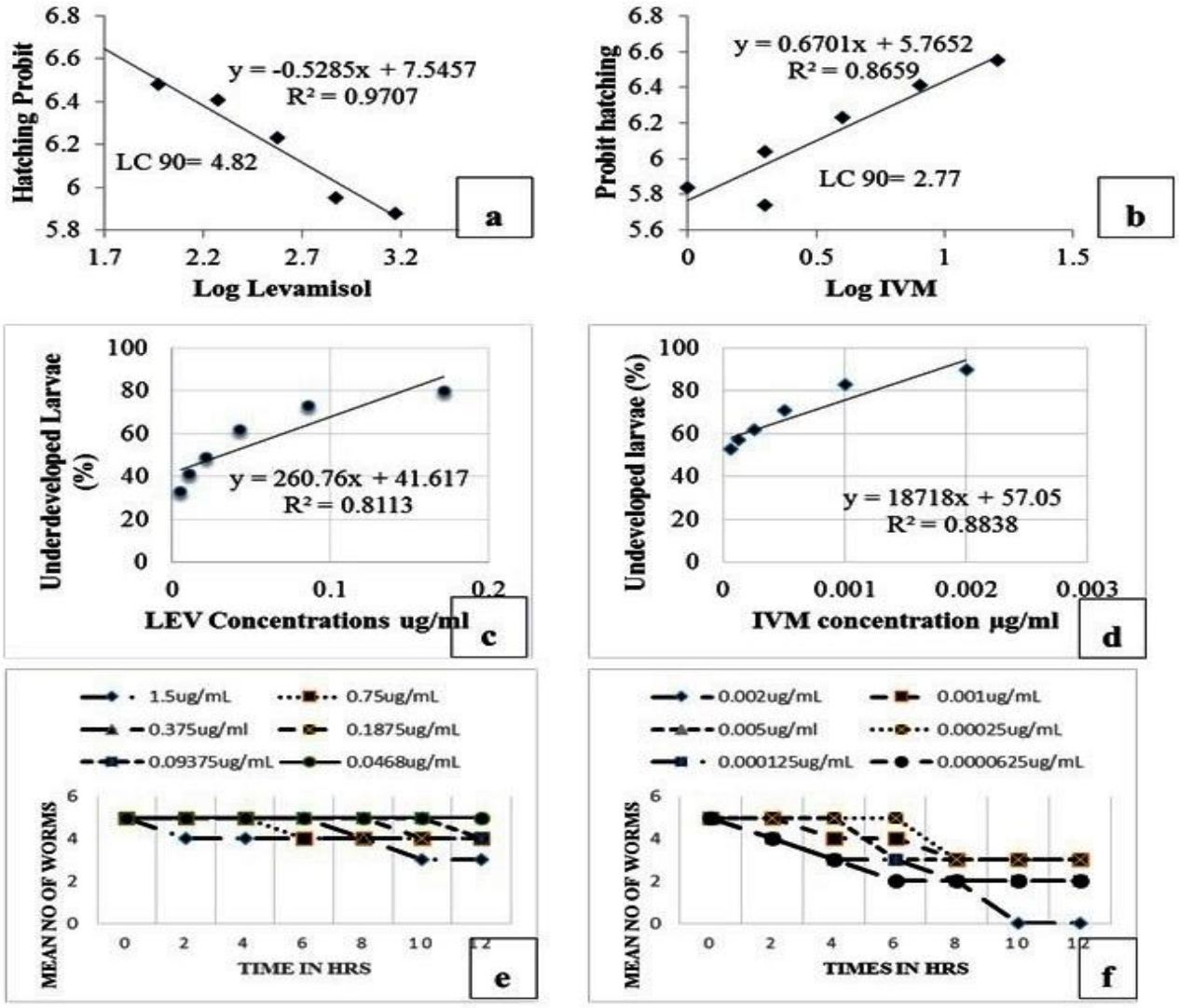

Figure-1. Dose dependent response of LEV and IVM by using EHT, LDT and AMA (a and b) Association between effect of different concentrations of LEV and IVM on Hatching inhibition (%) through EHA **(c and d)** Association between effect of different concentrations of LEV and IVM on development of Larvae (%) using LDT **(e and f)** Effect of LEV and IVM on motility of *H. contortus* adults *in vitro* at 0, 2, 4, 8, 10 and 12 hr. by using AMA

DISCUSSION

The degree of infection caused by parasites depends upon two types of factors viz; extrinsic and intrinsic; which affect the dynamics of host-parasite relationship (Francisco *et al*., 2009). Extrinsic factors include weather condition, amount of forage present, quality of forage, host density and development of (AR), which is one of the major causes of failure of worm control program (Body *et al*., 2011). Intrinsic factors include, gender, age, physiological condition, nutritional status, genetic differences as well as immune status of the animal (Waghorn *et al*., 2006; Marquez *et al*., 2008; Gasbarre *et al*., 2009). Extensive use of same dewormer in field or at farm level results in failure of anthelmintics and development of resilient/resistant parasites as the frequency of these dewormers is 6-9 times/year practiced in the field (Webb and Ottaway, 1986; Love *et al*., 1992; Bygarski *et al*., 2014). It has also been reported that, once parasites developed resistance against one group of anthelmentic, they also possess resistance against some other groups too (Calvete *et al*., 2012). The anthelmintic resistance against different classes of anthelmintics including thiabendazole, levamisole, phenothiazine derivatives, refoxamide, ivermectin and organophosphates has been reported worldwide (Chagas *et al*., 2013).

The result of the present study demonstrated the non-significance ($P>0.05$) in hatching percentage (%) of eggs showing that both drugs (LEV and IVM) have no ovicidal activity which is in agreement with (Webb and Ottaway, 1986 and Love *et al*., 1992; Sargison and Scott, 2011) who reported multiple factors which are responsible for success of parasites in developing resistance including excessive use of same anthelmintic, underdosing, poor management procedures and frequency of deworming (8-10 times a year) etc.

The results of LDT clearly showed that AR was present to some degree in both goat and sheep. The findings of LDT performed in this study were similar to (Neveu *et al*., 2007; Taylor *et al*., 2007 and Campos *et al*., 2016) Which could be due to the use of suboptimal doses.

The level of resistance among adult *H. contortus* populations in decreasing order was to LEV and IVM in the present study, which was probably due to less frequent use (3 times a year) of IVM compared with LEV (4 times a year) for the last many years. Frequent use of anthelmintics has been alleged to be a contributor towards development of AR (Shalaby, 2013). Saeed *et al*., (2007) have reported many causes of development of resistance which include: compromised quality of anthelmintics, haphazard and repeated use of antinematicidals, sub-optimum dose administration, absence of any tactical or strategic worm control program and poor managemental conditions on farms and in the field (Veterinarian file). *H. contortus* populations have been reported for their tendency for earlier development of resistance compared with other species of nematodes (Saddiqui, 2005; Saeed *et al*., 2007).

On the contrary side, there are chances of any in vitro assay to predict false positive or false negative readings under prevailing circumstances. The false-positive results may be attributed to the fact that in *in-vitro* experiments the worms exposed to much higher concentrations and longer period of time as compared to in the gut at predilection site (Dolinská *et al*., 2016). For this inference, there are multiple reasons reported including the binding of drug to gut contents, movement of drug down towards intestine, degraded by abomasal pH and gut microflora (Molento and Canever, 2018). False-negative results may be occurred due to assessment criteria and requirement of higher doses as compared to *in vivo* to observe the prompt reduction in the movement of worms and to consider a drug toxic against parasites. While, *in-vivo*, very minute disturbance in the intestinal movement may be sufficient to destabilize the position of worm in the intestine and ultimately causing the expulsion. Moreover, an *in vitro* assay may also misidentify the potential anthemintic if the activation of that drug requires the presence of host enzymes or assay is addressing a life cycle stage of parasite which is not concerned to be controlled in the field (Légaré and Ouellette, 2017).

Conclusion: The result of abovementioned study described that the haemonchosis is an endemic disease in the area and its prevalence is mostly related to epidemiological factors. Both the drugs have no ovicidal activity. The larvicidal and adulticidal activity of IVM is much better as compared to LEV. However, the lesser magnitude of the development of AR against IVM is there. The adult motility assay performed in the present study may be useful in determining the efficacy of potential anthelmintics against adult *H. contortus*. However, further *in vivo* and *in vitro* evaluation of the AMT is required to confirm the usefulness of the assay as it may misidentify the motility of worms.

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