First detection and evaluation of ivermectin resistance in *Haemonchus contortus* from sheep in Zhaosu, Xinjiang, China

Waresi Tuersong

Huazhong Agriculture University

Xingrun Yan

Huazhong Agriculture University

Caixian Zhou

Huazhong Agriculture University

Chunqun Wang

Huazhong Agriculture University

Wenda Di

Huazhong University of Science and Technology School of Foreign Languages

Jian Chen

Huazhong Agriculture University

Yanqin Zhou

Huazhong Agriculture University

Min Hu (✉ mhu@mail.hzau.edu.cn)

Huazhong Agriculture University <https://orcid.org/0000-0001-8381-6328>

Short report

Keywords: *Haemonchus contortus*, ivermectin resistance, larval developmental assay, faecal egg count reduction test

Posted Date: August 4th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-52502/v1>

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Abstract

Background: In 2017 in Zhaosu, Xinjiang, China, a series of sheep death suspected to have reduced the potency of ivermectin (IVM) have raised the vigilance of herdsman. At the same time, veterinary practitioners warned of the potential risk of the lack of efficacy of IVM in the treatment of *Haemonchus contortus*.

Methods: In this study, the efficacy of IVM against *H. contortus* was assessed under controlled experimental conditions by using larval developmental assay (LDA) and faecal egg count reduction test (FECRT) to determine whether the reduced efficacy in sheep in Zhaosu was caused by IVM resistance.

Results: In total, 1560 adult worms were isolated from the abomasum of post mortem examination of dead sheep which received recent IVM (0.2 mg/kg) treatment before death and eggs were collected from female worms for usage in LDA. The LDA result revealed that significant difference in resistance ratio (RR) was detected between the IVM-susceptible and -resistant isolates (6.09 for Zhaosu strain) of *H. contortus*. The Zhaosu strain was maintained in laboratory for FECRT, which showed that the efficacy of IVM was 0% at 1, 2 and 3 times dose, but at 4.8 times dose, it reached 87.5% at 14 days post-treatment.

Conclusion: The findings suggested that controlling *H. contortus* of small ruminants in Zhaosu requires an integrated parasite management strategy that should include monitoring of infection and anthelmintic resistance, and increasing the awareness of herdsman on the importance of using the appropriate drug usage to improve nematode control in sheep.

Background

Zhaosu is located in the southwest of Yili Kazak Autonomous prefecture, Xinjiang, China, which is the only county without desert in Xinjiang. In Zhaosu, sheep are the most important small domestic ruminants which usually are reared in smallholder farming systems with great numbers. The local sheep industry has a variety of production systems including indoor and outdoor units with a sheep population of approximately 740000 (According to the statistics of Zhaosu animal husbandry and Veterinary Bureau in spring, 2020). After mid-May each year, most of the sheep breeding herd is kept outdoors and domesticated sheep often share pasture with other livestock species such as cattle and horses so that such environment significantly increases the chance of endo-parasite and ecto-parasite infections, and there is a potential risk for substantial clinical and economic losses caused by parasitism unless effective control is implemented.

Infection by *Haemonchus contortus*, a highly pathogenic and economically important parasite, is a serious problem for extensive small ruminant farming system in warm climatic regions on a worldwide basis [1]. Control of this parasite is achieved through a combination of anthelmintic treatment and management strategy, particularly rational administration of anthelmintic, good hygiene and land management to interrupt transmission [2, 3]. Fortunately, three anthelmintic groups are available and have been used with great success for controlling this parasite in the past decades, including

Benzimidazoles, Imidazothiazoles and Macrocyclic lactones [4, 5, 6]. Among them, Ivermectin (IVM) belonging to macrocyclic lactones is a highly effective, broad-spectrum and gold standard anthelmintic drug that has been used widely since 1990's to control both endo- and ecto-parasites in a wide variety of hosts [7]. Unfortunately, IVM resistance is becoming a serious problem to animal health and its resistance in *H. contortus* is a threat to sustainable livestock production as well as a growing worldwide concern [8, 9]. Therefore, there is no guarantee that all chemicals will be uniformly effective in any region in the world.

Our scant understanding of the resistance mechanism of IVM in the *H. contortus* populations limits our ability to develop evidence-based mitigation strategies [10, 11]. In spite of some reports on IVM resistance in *H. contortus* in other countries, there still lacks of information about IVM resistance in Xinjiang, China. However, concern of reduced IVM efficacy was raised to the Animal Husbandry and Veterinary Bureau in Zhaosu in recent years. Given the need for identifying the reasons causing several deaths of sheep in a herd in which IVM had been used regularly, the aim of our work was to confirm the presence of IVM resistance and to evaluate the resistance level in Zhaosu by using LDA in concert with FEC reduction tests (FECRT). The final objective of this study was to provide a reliable data basis for local sheep industry.

Methods

Haemonchus contortus strains

Two isolates of *H. contortus* were used in the study:

- i) Haecon-5 strain: Presented by Professor Robin B. Gasser of the University of Melbourne; susceptible to all commercial anthelmintics.
- ii) *Haemonchus contortus* Zhaosu strain: collected from the abomasum of dead sheep in Zhaosu County, Xinjiang Uygur Autonomous Region, China in 2017 (43°09'–43°15'N 80°08'–81°30'E). This region has an average annual rainfall of 511.8 mm, with long cool winter and short mild summer (Fig. 1).

Chemicals

The commercial IVM powder (Shyuanye Inc, Shanghai, China) was used as a source of IVM in LDA. The stock solution (1 mg/mL) was prepared in dimethyl sulfoxide (DMSO) and was diluted in DMSO to generate a series of working solutions. IVM Injection (Yuanzheng Pharmaceutical Co., Hebei, China) was used in the faecal egg count reduction test (FECRT).

Culture of larvae and morphological larval identification

The *H. contortus* female adults collected from the abomasum of dead sheep from Zhaosu were minced and then mixed with the sterilized sheep feces, which were then submitted to the College of Veterinary Medicine, Huazhong Agricultural University, Wuhan. The sample was incubated at 27 °C for 7 days,

subsequently the third-stage larvae (L3) of Zhaosu strain of *H. contortus* were collected and morphologically identified. In order to further perform FECRT, around 6-months old goats free of parasites were infected with 8000 L3s per goat of Zhaosu strain and Haecon-5 strain, respectively. All experimental goats used in this study were housed in a cage without access to grass and feces.

Recovery of *H. contortus* eggs

Fresh *H. contortus* eggs were recovered from the faeces of each donor goat using a similar method from a previous report [12]. In short, approximately 100 g of faeces were macerated in a mortar with 800 ml of sugar water. The suspension was filtered using the 450 µm and 125 µm fine sieves and filtrate was covered with a piece of plastic film by setting on the surface of suspension liquid for about 40 minutes. Then, the plastic film was removed and its surface was washed with normal saline. The collection solution was further filtered using the 50 µm and 38.5 µm fine sieves, the eggs on the surface of the sieves (38.5 µm) are collected and used immediately for larval development assays.

Larval development assay (LDA)

Larval development assay (LDA) was carried out as modified from the original method [13]. Briefly, the egg suspension (2 ml) recovered from faeces was added to T25 cell bottle containing 400 µl of growth medium (1 g yeast extract plus 90 ml dH₂O; autoclaved for 15 min; added with 10 ml of 10× concentrated Earle's solution). Each bottle contains about 5000 eggs which were incubated at 27 °C for 24 h. On the next day, after the eggs hatched, 99 µl of larval suspension (~100 larvae) and 1 µl IVM working solution (final DMSO concentration in experimental group and control wells was 1% v/v with triplicate assay wells) were added to the wells of 96-well plates and incubated at 27 °C for another 6 d (Fig. 2A). Final IVM concentrations ranged from 12.5 ng/µl to 0.2 ng/µl (12.5, 10.0, 5.0, 2.5, 1.25, 0.4, 0.2) for Zhaosu strain and 5 ng/µl to 0.1 ng/µl (5.0, 2.5, 1.25, 0.41, 0.205, 0.1 ng/µl) for Haecon-5 strain, control group received DMSO alone. At the end of the test, the number of L3s was counted in each well and the resistance ratio (RR) was calculated [14].

$$\text{Resistance ratio (RR)} = \frac{\text{LD}_{50} \text{ for Zhaosu strain}}{\text{LD}_{50} \text{ for Haecon-5 strain}}$$

Faecal egg reduction test

A faecal egg count reduction test (FECRT) was performed according to the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluation of anthelmintic efficacy in ruminants [15]. In an attempt to further confirm the results of the LDA and the cause of death of sheep, four consecutive efficacy trials involving *H. contortus* experimental infections were carried out in goat. Thirty days after infection, goats in the treatment group were treated with IVM (IVM sub-cutaneous injection) at different doses including 0.2 mg/kg (standard dose), 0.4 mg/kg (2 times dose), 0.6 mg/kg (3 times dose) and 0.96 mg/kg (4.8 times dose), respectively. Goats in the control group were left untreated.

One day before the treatment and 14 d after the treatment, goats were checked for faecal egg counts (EPG) and the efficacy of the treatment was evaluated by the faecal egg count reduction test (FECRT), according to the formula, based on the guidelines recommended by WAAVP [15]. In addition, after 20 days of treatment at 0.4 mg/kg, the goats infected with Zhaosu strain were slaughtered to collect adults and calculate the worm burden. Percentage faecal egg count reduction (FECR) was determined according to the formula $FECR (\%) = [1 - (T2/T1)] \times 100$ where T1 (before treatment) and T2 (post treatment) are the arithmetic mean egg counts in the group treated with IVM, respectively [16].

Results

Case history

This investigation was carried out in July 12, 2017. According to the sheep owners, infected sheep became weak, lethargic and recumbent, despite treatment orally with IVM (0.2 mg/kg) before death. According to local veterinarians and farmers' statement, sheep in most farms in Zhaosu suffered from sporadic deaths in the different herd over the preceding two months (May to July 2017). Sheep were drenched orally with IVM (mainly) two to four times, each spring (April to May) and each autumn (September to November) in this region.

Post mortem examination

In July 2017, a 1-year-old male sheep which had been sick for several weeks and had received oral ivermectin (0.2 mg/kg) before its death was submitted to post mortem examination. Post mortem examination revealed that the faeces are firm and scant, the dead sheep was emaciated and malnourished, and had severe anemia. A total worm count got 1560 *H. contortus* worms in its gastric compartment (Fig. 2B). Sectional examination of the viscera didn't reveal other significant pathological changes and the cause of death was concluded to be severe anaemia and referable to the gastric *H. contortus* infestation.

Larval developmental assay

The results of dose response experiments with IVM against *H. contortus* larvae in vitro LDA revealed a good separation between susceptible Haecon-5 strain (control) and Zhaosu-resistant strain. In the control group, about 98% of the hatched larvae developed through to L3. In contrast, Zhaosu strain showed dose responses ranging from full development (0.2 ng/μl) to 100% inhibition on the development (12.5 ng/μl) with increasing IVM concentrations, indicating that the larval development assay was able to define the response of *H. contortus* larvae to the drug. The IC₅₀ values were 1.25 ng/μl and 0.205 ng/μl for Zhaosu strain and Haecon-5 strain, respectively. A comparison on the IC₅₀ value between Zhaosu strain and Haecon-5 strain showed that resistance ratio (RR) is 6.09, suggesting that Zhaosu strain was highly resistant to IVM. Then, we used this resistance ratio as a reference and conducted FECRT using different IVM doses to calculate IVM efficacy against Zhaosu strain.

Faecal egg count reduction test (FECRT)

In FECRT experiment, faecal eggs were detected in all five groups including four experimental groups and one control group prior to administration of IVM, group mean egg counts still remain at similar level to that before treatment on different days after treatment at 600 ~ 1800 eggs per gram for the experimental and control groups (Table 1, Fig. 2C). Following IVM administration, in groups 1, 2 and 3, the FECs averaged EPG almost unchanged on day 14 post treatment (Table 1). However, for the 4th IVM administration group, average EPG ranged from 800 to 100 with the time going from Day 0 to Day 21 after treatment and FECR was 87.5% (Table 1). In this high dose group, although FEC significantly reduced, interestingly, it gradually increased with time and EPG reached 200 on day 28 post-treatment (Table 1). Based on faecal egg count reduction, in four experimental groups, efficacies were 0%, 0%, 0% and 87.5% on day 14 post treatment, respectively (Table 1). In addition, on day 21 post-treatment in goat received 2 times dose IVM (0.4 mg/kg), 980 (46% vs 54%) adult worms were isolated from the abomasum of post mortem examination (Fig. 2D).

Discussion

As *H. contortus* has shown a strong ability to develop resistance, IVM resistance in this parasite is extensively studied and well documented [17]. Today, most of the field isolates of *H. contortus* from different continents were resistant to IVM, including North America [18], South America [19], Europe [20], Australasia [21], Africa [22] and Asia [23]. However, no registered cases of IVM resistance in *H. contortus* and IVM efficacy situation have been reported in Xinjiang. The present work constitutes the first document of IVM resistance and follows reported treatment failures of IVM in sheep in Zhaosu, Xinjiang.

After mid April 2017, the practitioner noticed that there was a reduction in the average weight of sheep in different farms. They also observed that sheep suffered from exercise tolerance and subcutaneous oedema and sporadic death across different farms, and mortality of sheep over two months was about 10%. The clinical symptoms, morbidity and mortality of sheep over two months were suspected to be caused by haemonchosis, despite long-term regular oral drenching with IVM. The history of IVM usage and the post mortem findings strongly suggested IVM resistance had led to a failure of *H. contortus* control in this region. Post mortem findings and experimental results from LDA and FECRT in the present study confirmed that this suspicion was true, and indicated that IVM was completely ineffective. Therefore, the high level of resistance to IVM in *H. contortus* detected in this study is deeply concerning.

In the present study, it is important to highlight that surviving females showed no significant suppression in fecundity at 1, 2 and 3 times dose, but reduction in egg generation has been observed from the first day to 20 days after treatment at 4.8 times dose. Similar findings (reduced fecundity by IVM treatment) were also obtained in sheep (*Cooperia curticei*) [24], cattle (*Cooperia curticei*) [25] and pig (*Oesophagostomum dentatum*) [16] nematodes. A recent study in the free-living nematode *Caenorhabditis elegans* showed that calcium signaling plays a crucial role in fertilization [26]. Interestingly, another study in filarial human parasitic nematode *Brugia malayi* reported that some genes in the Ca^{2+} signaling pathway and a number

of other genes involved in fertilization were down-regulated following exposure to IVM, which caused adverse effects on ovulation [27]. In view of the above report, the reduction in ovulation of the female worm of *H. contortus* may also be related with dysregulation of genes involved in the fertilization after IVM exposure.

Development of IVM resistance in this case might have been affected by a number of factors, such as excessive and frequent treatment, under-dosing with IVM, and its management strategies [28, 29]. Firstly, high frequency of treatment is long recognized as a major causal factor for anthelmintic resistance [30], especially when using the same drug for a long time. IVM had been used for at least the last seven years without the use of other classes of anthelmintics in Zhaosu. There is no doubt that many livestock owners routinely use IVM which may remove sufficient nematodes to achieve a good clinical result, but also exacerbate the resistance situation by allowing resistant worms to survive. We suggested that it could be the main reason for the prevalence of IVM-resistant worms in this region which would steadily increase over time. Secondly, lack of farmers' awareness to the importance of ensuring appropriate doses and an underestimation of animal weights that appears to have been common in Zhaosu, therefore, inadequate dose was a potential factor for resistance to develop [31, 32, 33]. Thirdly, the absence of detection and managing IVM resistance in the long term caused the selection pressure for the resistance in *H. contortus* to increase with time. In the majority of cases, FECRT is the only currently available method of simultaneously assessing the efficacy of anthelmintics and resistance level of species, especially in remote pastoral areas like Zhaosu. However, lack of technical support and epidemiological survey appears to be a barrier to its wide adoption and livestock owners are not aware of the anthelmintic resistance situation in their properties. Based on the results of this study, more comprehensive investigation are needed in the future to further confirm the clinical and economic impact of *H. contortus* IVM resistance on sheep in the local production system.

Conclusion

In conclusion, this study is the first report on IVM resistance in *H. contortus* in Xinjiang, which is the initial step and important opportunity to understand and discuss the wider issue of anthelmintic usage and efficacy on sheep farms in Zhaosu. Local veterinarians and farmers should be aware of the relationship between the infection rate and the severity of haemonchosis and the possibility of resistance development with long term application of IVM in sheep production systems in order to implement more cost-effective control strategy. Therefore, wider investigation based on worm egg counts for worm prevalence, and faecal egg count reduction testing for drug resistance in the field in sheep is timely in order to detect the emergency of drug resistance as early as possible and mitigate possible risk factors for further anthelmintic resistance development.

Abbreviations

FECR: faecal egg count reduction; IVM ivermectin; LDA: Larval development assay; FECRT: faecal egg count reduction test; RR: resistance ratio.

Declarations

Ethics approval and consent to participate

All of the experimental animals used in this study were treated in strict accordance with the recommendations in the Guide for the Regulation for the Administration of Affairs Concerning Experimental Animals of the People's Republic of China. The protocol was approved by the Ethics Committee of Huazhong Agriculture University (permit HZAUGO-2016-007).

Consent for publication

Not applicable.

Availability of data and materials

Data sharing is applicable to this article.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by the National Natural Science Foundation of China (Grant no. 31872462), the National Key Basic Research Program (973 program) of China (Grant no. 2015CB150300) and the Natural Science Foundation of Hubei Province (Grant No. 2017CFA020) to MH.

Authors' contributions

MH conceived and designed the project. WT performed most experiments with the help from XY in LDA and prepared the first draft of the manuscript. CW, WD described the case and collected the samples from Zhaosu. XY, CZ, JC were responsible for feeding goats, and treatment. YZ commented on project design. MH revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Tables

Table 1: Result of Fecal Egg Count Reduction Test (FECR%) and ivermectin (IVM) efficacy against *Haemonchus contortus* of Zhaosu strain in goat

Experimental group		Day of treatment						
		Day0	Day7	Day14	Day21	Day28	Day35	Day42
Haecon-5 (control)	FEC	1200	1200	1300	1200			
Zhaosu strain FEC6 600 600 700 700	FEC	600	600	700	700			
	FECR(%)		0	-16.6	-16.6			
	Efficacy		NE	NE	NE			
1 times dose IVM								
Haecon-5 (control)	FEC	1800	2000	1900	2100			
Zhaosu strain FEC6 600 600 700 700	FEC	1200	1300	1500	1500			
	FECR(%)		-8.3	-25	-25			
	Efficacy		NE	NE	NE			
2 times dose IVM								
Haecon-5 (control)	FEC	900	1200	1200	1200	1300		
Zhaosu strain FEC6 600 600 700 700	FEC	900	900	900	900	900		
	FECR(%)		0	0	0	0		
	Efficacy		NE	NE	NE	NE		
3 times dose IVM								
Haecon-5 (control)	FEC	1200	1400	1400	1300	1400	1300	1300
Zhaosu strain FEC6 600 600 700 700	FEC	800	0	100	100	200	200	200
	FECR(%)		100	87.5	87.5	75	75	75
	Efficacy		100%	ES	ES	ES	ES	ES
4.8 times dose IVM								

NE=no Efficacy

ES= Efficacy Significantly

Figures

Figure 1

Map of Xinjiang, China: Red star represents exact location of the Zhaosu County which *H. contortus* specimens were collected. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

(A) Larval development assay; (B) Adult *H. contortus* collected from abomasum surface of the dead sheep in Zhaosu (Dehydration by alcohol); (C) Images of *H. contortus* eggs; (D) Adult *H. contortus* collected from abomasum surface of the goat treated with 2 times dose IVM.