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Original Contribution

INVESTIGATION ON THE BENZIMIDAZOLE RESISTANCE IN GASTROINTESTINAL STRONGYLIDS OF SHEEP IN SOUTHEASTERN BULGARIA

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ABSTRACT
Benzimidazoles are the most commonly used anthelmintic drugs for the chemical control of sheep strongylidoses in Bulgaria. The intensive and not always proper application could be able to cause a reduction of their efficacy. To establish benzimidazole resistances among gastrointestinal strongylids, two in vitro assays were performed – Egg Hatch Assay (EHA) and Larval Development Assay (LDA). The effective concentrations required to inhibit 50 % of egg hatching (egg dead - ED_{50}) or larval development (larva dead - LD_{50}) were: for EHA from 0.0114 to 0.2023 μg/ml and for LDA from 0.0017 to 0.5817 μM thiabendazole. All the data were analysed. Based on the reference we assumed that ED_{50} values showing a presence of resistance are over 0.1 μg/ml and LD_{50} - over 0.12 μM thiabendazole. According to the results discovered here, in two of 13 sheep flocks examined, a benzimidazole resistance was detected with values of ED_{50} between 0.1132 and 0.2023 μg/ml and for LD_{50} between 0.2940 and 0.5817 μM thiabendazole.

Key words: benzimidazoles, larval development assay, egg hatch assay, sheep, anthelmintic resistance, strongylids

INTRODUCTION

Gastrointestinal strongylids (Phylum Nematoda, order Strongylida) are ones of the most prevalent and important parasites affecting small ruminants worldwide (1). They are responsible for significant losses of reducing sheep productivity and even death (2). The sheep breeding is still one of the leading livestock industry in Bulgaria. The geographical location, climate conditions of the country and pastural breeding of small ruminants almost year-round determine the high prevalence of gastrointestinal strongylids (GIS). Currently, in Bulgaria sheep and goats are kept in small private farms under extensive conditions and the management of gastrointestinal strongylidoses are largely based on the use of chemotherapy. To date, three main families of broad spectrum anthelmintics are used to control the helminth infections in grazing livestock – benzimidazoles, imidazothiazoles and macrocyclic lactones (3, 4). The benzimidazoles (BZs) are usually the first choice of sheep and goats deworming because of their low cost and broad spectrum of activity. The difficult financial situation of the owners of small ruminant farms in recent years has led to their uncontrolled and often wrong use. In consequence of these and other factors some GIS are able to select a resistance against BZs. Prichard et al. (5) defined this phenomenon as following: "Resistance is present when there is a greater frequency of individuals within a population able to tolerate doses of compound than in a normal population of the same species and is heritable" (see 6). Other factors contributing to the emergence of resistance are the treatment frequency, use of anthelmintics in sub-optimal doses, nematodes in "refugia" (7), keeping the goats and sheep together (8),

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treatment in a prepatent period (9), way of animals growing, species diversity of GIS in the population (10), geographical conditions of the region (11). The resistance is difficult to be overcame once developed. According to Alvares–Sanchez et al. (12) resistant strains seldom revert to susceptibility.

Benzimidazole (BZ) resistance has been demonstrated for first time in 1964 in *Haemonchus contortus* (13). Interesting is that thiabendazole (TBZ) and its mode of action were described for first time in 1961 (14) and only three years later Drudge et al. (13) found already a resistance selected. After commercialization of BZs in world markets, a resistance has been reported in number of GIS species of small ruminants from all parts of the world. It was established in *H. contortus*, *Trichostrongylus colubriformis*, *Trichostrongylus axei*, *Trichostrongylus vitrinus*, *Ostertagia circumcincta*, *Ostertagia trifurcata*, *Ostertagia davtiani*, *Cooperia curticei*, *Nematodirus spathiger*, *Nematodirus filicollis*, *Oesophagostomum venulosum* and other (15).

A varying degree of BZ resistance in GIS has been widely reported in many European countries: Austria, Belgium, Czech Republic, Denmark, France, Germany, Greece, Holland, Ireland, Italy, Slovakia, Sweden, Turkey, United Kingdom (7, 16).

The information about the presence of anthelmintic resistance (AR) in Bulgaria is limited and incomplete. In contrast, many studies in neighboring countries have been done. Because of this reason we aimed to investigate the resistance against BZs in GIS of sheep in some areas of southeastern Bulgaria.

MATERIALS AND METHODS

Study area, farms and breeding management

The survey was conducted in 2012 on 13 sheep farms mainly located in southeastern Bulgaria. The local breeds and crosses in herds dominated. The age of animals varied from 12 to 36 months. Visiting each farm a questionnaire was filled allowing information on farming practices as growing and deworming of sheep including ectoparasites treatment. All herds were kept on pasture and were not dewormed against GIS during the last 10 weeks.

Experimental design

A total of 13 mixed faecal samples were obtained. Each of them represented a collection of individual samples taken rectally from 10 % of the sheep in herd. All samples were divided into two parts - one stored anaerobically with water in solid plastic containers at room temperature (18 - 22^0 C). The second part was used for the preparation of coproculture by mixing with vermiculite in a ratio of 2:1 (faeces/vermiculite) and then stored in a thermostat at 26^0 C during 10 days. An egg suspension was made by sugar flotation technique. Infective larvae were extracted from faeces by Baermann technique.

Anthelmintics and assays

Drug dilutions were prepared by dissolving a pure substance of TBZ in distilled water and a few drops of hydrochloric acid were added. Egg hatch assay (EHA) was performed according to Prelezov et al. (16). Five TBZ dilutions in µg/ml were used (0.02; 0.04; 0.05; 0.08; 0.1). Larval development assay (LDA) was carried out according to Vàrady et al. (17). Ten TBZ concentrations in µM were prepared (0.01; 0.02; 0.039; 0.078; 0.156; 0.313; 0.625; 1.25; 2.5; 5).

Data analysis

The data were analyzed by log-probit model (18) to determine the drug concentration which prevents 50 % of egg embrionation (ED_{50}) or larval development (LD_{50}). Logarithm of doses against percentage of hatched eggs or developed larvae as probit transformation were used. Dose-response line was plotted by Graph software to estimate the correct final concentration.

RESULTS AND DISCUSSION

Our results obtained were for ED_{50} from 0.0114 to 0.2023 µg/ml TBZ and for LD_{50} from 0.0017 to 0.5817 µM TBZ **(Table 1).** For interpretation of the values established in this study and the presence of anthelmintic resistance (AR), several important issues should be discussed before. Of paramount importance is the sensitivity of the tests used here. The reliability of EHA is high but there are some limitations. It is able to detect AR when resistant worms are at least 25 % of the population (19). This value in LDA is reduced to 10 % (20). Nevertheless, the two *in vitro* tests have shown well comparable results in field investigations (21). Therefore, EHA and LDA were chosen to be performed in this study.

Another important issue is the clarification of ED_{50} and LD_{50} values which are indicative for AR developed. In the international reference many contradictions on the problem exist. According to the guide of World Association for the Advancement of Veterinary Parasitology (W. A. A. V. P) the ED_{50} value at least 0.1 μg/ml TBZ is considered to be indicative for BZ resistance (22). It is not clear the accurate LD_{50} showing the presence of resistance. In the manual of LDA commercialized kit (DrenchRite®), GIS strains expressing values up to 0,078 μM TBZ seem to be low susceptible while those of them giving values from 0.156 to 0.313 μM – weakly resistant. When LD_{50} is 1.25 μM or more a moderately resistance is presented. This statement is supported by the results of Coles et al. (6) that the effective concentration of TBZ at LDA which blocks the development of non infective larvae (L_1 and L_2) to infectious stage (L3) in sheep nematodes was 0.02 μg/ml.

Table 1. *ED_{50} and LD_{50} values of TBZ in EHA and LDA.*

Flock №	ED_{50} in μg/ml	LD_{50} in μM
1	0.0217	0.0121
2	0.0177	0.0028
3	0.0183	0.0204
4	0.0148	0.0231
5	0.0114	0.0066
6	0.0138	0.0017
7	0.2023	0.2940
8	0.1132	0.5817
9	0.0189	0.0068
10	0.0137	0.0102
11	0.0267	0.0132
12	0.0135	0.0133
13	0.0221	0.0074

According to the data discussed above we assumed that the resistance to BZs is expressed only when the ED_{50} is more than 0.1 μg/ml and LD_{50} over 0.12 μM TBZ. Based on our results we confirmed that a BZ resistance is presented in 2 farms, out of all 13 farms tested. This is the firs official report about Bz resistance in GIS of sheep in Bulgaria.

It is theoretically possible to have many not investigated sheep herds resistant to BZs in the country. As a main argument we may note a few bad practices of sheep and goats breeding in small farms under extensive conditions. Firstly, the very frequent use of the same drug for a prolonged period of time. Increased drenching frequency has shown to correlate with the cases of BZ resistance (23). Moreover, it is not followed the rule that the dose of drug should be adjusted to the animal with the highest body weight in the herd. Underdosing is considered to be an important factor in developing resistance (24) because larger animals do not receive an optimal dose. Keeping the sheep and goats together is another very important factor for contributing a resistance. It is well known the differences in the pharmacokinetics of anthelmintics in sheep and goats. The bioactivity of BZs is much lower in goats than sheep (7) therefore, the effective doses used in sheep deworming are too low to achieve the same effect in goats. Thus, the goats receive drugs at sub-optimal doses for prolonged periods of time and resistance may occur. Accordingly, such resistant strains are able to be transferred between sheep and goat (15) So, sheep and goat herds should not be kept together, and common pastures should be avoided.

CONCLUSION

Based on the mistakes in small ruminants farming, it is likely that AR to exist in many parts of Bulgaria, but further and more scale studies should be conducted and more sensitive tests should be used to confirm this hypothesis. Therefore, to protect the small ruminant farms it is not enough to conduct just a routine diagnostic of strongylidoses. It should be introduced a parallel diagnosis of AR especially when the treatment regimes failure.

REFERENCES

1. Varady, M., Papadopoulos, E., Dolinska, M., Konigova, A. Anthelmintic resistance in parasites of small ruminants: sheep versus goats. *Helminthologia*, 48:137-144, 2011
2. Sykes, A. Parasitism and production in farm animals. *Journal Animal Production*, 59:155 – 172, 1994.
3. Entrocasso, C., Alvarez, L., Manazza, J., Lifschitz, A., Borda, B., Virkel, G., Mottier, L., Lanusse, C. Clinical efficacy assessment of the albendazole–ivermectin combination in lambs parasitized with resistant nematodes. *Veterinary Parasitology*, 155:249-256, 2008.
4. Traversa, D., Paoletti, B., Otranto, D., Miller, J. First report of multiple drug

resistance in trichostrongyles affecting sheep under field conditions in Italy. *Parasitology Research*, 101:1713-1716, 2007.

5. Prichard, R.K., Hall, C.A., Kelly, J.D., Martin, I.C.A., Donald, A.D. The problem of anthelmintic resistance in nematodes. *Australian Veterinary Journal*, 56:239–250, 1980.
6. Coles, G.C., Jackson, F., Pomroy, W.E., Prichard, R.K., Samson-Himmelstjerna, G., Silvestre, A., Taylor, M.A. and Vercruysse, J. The detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, 136:167-185, 2006.
7. Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M.N., Afaq, M. Anthelmintic resistance: The state of play revisited. *Life Sciences*, 79:2413-2431, 2006.
8. Jackson, F. Anthelmintic resistance - the state of play. *British Veterinary Journal*, 149:123–138, 1993.
9. Van Wyk, J.A. Refugia – overlooked as perhaps the most potent factor concerning the development of anthelmintic resistance. *Onderstepoort Journal of Veterinary Research*, 68:55-67, 2001.
10. Waller, P.J. Control strategies to prevent resistance. *Veterinary Parasitology*, 46:133-142, 1993.
11. Papadopoulos, E., Himonas, C., Coles, G. Frough and flock isolation may enhance the development of anthelmintic resistance in nematodes. *Veterinary Parasitology*, 97:253-259, 2001.
12. Alvarez-Sanchez, M.A., Perez-Garcia, J., Cruz-Rojo, M.A., Rojo-Vazquez, F.A. Anthelmintic resistance in trichostrongylid nematodes of sheep farms in Northwest Spain. *Parasitology Research*, 99:78-83, 2006.
13. Drudge, J.H., Szanto, J., Wyant, Z.N., Elam, J. Field studies on parasite control in sheep: comparition of thiabendazole, ruelene and phenothiazine. *American Journal of Veterinary Research*, 25:1512-1518, 1964.
14. Brown, H.D., Matzuk, A.R., Ilves, I.R., Peterson, L.H., Harris, S.A., Sarett, L.H., Egerton, J.R., Yakstis, J.J., Capmbell, W.C., Cucker, A.C. Antiparasitic drugs. IV. 2-(4’-Thiazolyl)-benzimidazole, a new anthelmintic. *Journal of the American Chemical Society*, 83:1764-1765, 1961.
15. Borgsteede, F.H.M. Anthelmintic resistance in nematodes of sheep and goats. In: Coles, G.C., Borgsteede, F.H.M., Geerts, S. (eds), *Anthelmintic Resistance in Nematodes of Farm Animals*. European Community. Directorate General for Agriculture, Brussels, 1-16, 1994.
16. Prelezov, P., Kirkova, Z., Bauer, C., Georgieva, D., Koinarski, V., Ivanov, I. Survey on the anthelmintic efficacy of benzimidazoles against gastrointestinal strongylids of sheep in southeastern Bulgaria. *Trakia Journal of Science*, 6:82-86, 2008.
17. Varady, M., Corba, J., Letkova, V., Kovac, G. Comparison of two version of larval development test to detect anthelmintic resistance in *Haemonchus contortus*. *Veterinary Parasitology*, 160:267-271, 2009.
18. Healy, M.J.R. Estimating LD50s without a computer. *Parasitology Today*, 4: 25–27, 1988.Martin, P.J., Anderson, N., Jarrett, R.G. Detecting benzimidazole resistance with faecal egg count reduction test and in vitro assays. *Australian Veterinary Journal*, 66:236-240, 1989.
19. Dobson, R.J., Le Jambre, L.F., Gill, J.H. Menagement of anthelmintic resistance: inheritance of resistance and selection with persistent drugs. *International Journal of Parasotology*, 26:993-1000, 1996.
20. Varady, M., Cernanska, D., Corba, J. Use of two in vitro methods for the detection of anthelmintic resistant nematode parasites on Slovak Republic. *Veterinary Parasitology*, 135:325-331, 2006.
21. Coles, G.C., Bauer, C., Borgsteede, F.H.M., Geerts, S., Klei, T.R., Taylor, M.A. and Waller, P.J. World Association for the Advancement of Veterinary Parasitology (W. A. A. V. P) methods for the detection of anthelmintic resistance in nematodes of veterinary importance. *Veterinary Parasitology*, 44:35-44, 1992.
22. Chartier, C., Pors, I., Hubert, J., Rocheteau, D., Benoit, S., Bernard, N. Prevalence of anthelmintic resistant nematodes in sheep and goats in western France. *Small Ruminant Research*, 29:33-41, 1998.
23. Edwards, J.R., Wroth, R., De chaneet, G.C., Besier, R.B., Karlsson, J., Morcombe, P.W., Dalton-morgan, G., Roberts, D. Survey of anthelmintic resistance in Western Australian sheep flocks. 2. Relationship with sheep management and parasite control practices. *Australian Veterinary Journal* 63:139–144, 1986.