Veterinary Parasitology, 35 (1990) 211–218
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

Effect of Ivermectin Treatment on Anti-Hypodermin C Titers of Asturiana Cattle Naturally Infected with *Hypoderma lineatum*

M. PRIETO[1], A. ESPI[1], J.A. GARCIA PALOMA[2] and F. PARRA[3]*

[1]*Laboratorio de Sanidad Animal, Gijón, Asturias (Spain)*
[2]*Centro de Experimentación Agraria, Villaviciosa (Spain)*
[3]*Departamento de Biología Funcional (Bioquímica), Universidad de Oviedo, Oviedo, Asturias (Spain)*

(Accepted for publication 5 October 1989)

ABSTRACT

Prieto, M., Espi, A., Garcia Paloma, J.A. and Parra, F. 1990. Effect of ivermectin treatment on anti-hypodermin C titers of Asturiana cattle naturally infected with *Hypoderma lineatum*. *Vet. Parasitol.*, 35: 211–218.

The collagenase (hypodermin C) from soluble crude extracts of *Hypoderma lineatum* 1st-instar larvae was purified by reverse-phase HPLC and used in a new indirect ELISA test. This pure protein had several advantages over the use of crude larval extracts allowing a much better discrimination between infested and non-infested cattle. The anti-hypodermin C titers of 19 Asturiana cattle were estimated over the course of a natural *H. lineatum* infestation cycle, in which the effect of ivermectin treatment was also investigated. The results showed differences in the onset and ending of the infestation with respect to those described for other European countries. The ivermectin treatment proved to be very effective and treated animals had relatively low anti-collagenase titers.

INTRODUCTION

A reliable serological method for diagnosis of animals which are warble fly infested could be of economic importance in areas of moderately extensive cattle breeding where eradication programs are not performed.

Asturias, the region where this work has been done, lies on the northern coast of Spain and can be divided into two main areas: a mountainous one of extensive cattle breeding and a coastal strip of land of less extensive farming. Whereas the parasite *H. lineatum* could be detected in almost 100% of the animals in the first area, and was subjected to eradication programs, the presence, to a lesser extent, of this parasite in the other area does not justify such a general campaign of eradication.

The success of limited antiparasite treatments in these regions relies on the precise detection of the presence and, possibly, the stage of the parasite devel-

*Author to whom all correspondence should be sent.

0304-4017/90/$03.50

opment in affected animals. This may be important to decide when the treatment should be done especially in regions, as the one under study, with very variable weather conditions, which can affect (Tarry, 1980) the number of parasitized animals and the moment in which the infestation takes place.

The level of infestation can be assessed by several immunological methods, such as passive hemagglutination (Boulard, 1985) or ELISA (Sinclair and Wassall, 1983). These techniques are not influenced by previous infestations owing to the absence of detectable circulating antibodies 14 weeks after the disappearance of the infection (Sinclair et al., 1984).

In this work we have investigated the antibody levels against hypodermin C in naturally infested Asturiana cattle, and the effect of ivermectin treatment on the evolution of the antibody titers, using a new indirect ELISA test.

MATERIALS AND METHODS

Antigens

H. lineatum crude extracts were made by homogenization of esophageal larvae in 0.05 M Tris-HCl buffer, pH 7.6. The homogenate was centrifuged for 90 min at $12.000 \times g$ (Lecroisey et al., 1979) and the supernatant used for HPLC purification of hypodermin C or as a crude antigen in ELISA tests.

High-performance liquid chromatography

Samples of crude extract from *H. lineatum* 1st-instar larvae (HL1) were fractionated by reverse-phase high performance liquid chromatography, on a C3-Beckman Ultrapore RPSC column, equilibrated with 25% acetonitrile in 0.1% trifluoroacetic acid (TFA). The runs were carried out in a Waters chromatograph equipped with a variable ultraviolet detector (Waters 481). Absorbance measurements were made at 280 nm in the sensitivity range of 1.0. Larval proteins were eluted with a linear gradient of acetonitrile from 25 to 50% in 0.1% TFA. All runs were carried out at room temperature at a constant flow rate of 0.7 ml min^{-1}.

Antisera

Cattle sera were prepared from blood taken at specific times, as indicated elsewhere.

A rabbit antiserum to the larval crude extract was made by intramuscular injection of larval homogenates (500 μg) in complete Freund's adjuvant. Four injections were given at one week intervals and the animals were bled a week after the last injection. The sera obtained were stored at $-20\,^{\circ}C$ until used.

Immunoelectrophoresis

Crude larval extracts and HPLC purified hypodermin C were analyzed by electrophoresis on 1% (w/v) agarose gels in 0.05 M Tris-barbital buffer, pH 8.6. The antigens were then allowed to react overnight, at room temperature, with a rabbit antiserum raised against the larval extract placed in a central well. The dried gel was stained with Coomassie Blue R and destained afterwards in 7.5% acetic acid containing 45% ethanol.

ELISA

One hundred μl per well of crude extracts, containing about 20 μg of protein, or HPLC purified hypodermin C in 0.1 M carbonate buffer, pH 9.6, were added to ELISA plates (Fastbinder, Costar) and incubated overnight at 4°C. The plates were then blocked with 200 μl per well of phosphate-buffered saline containing 0.05% Tween 20 and 0.5% ovalbumin, for 45 min at 37°C. Cattle sera were added (1/60 in PBS-Tween) and incubated for 1 h at 37°C. After 3 washings with PBS-Tween buffer, 100 μl per well of rabbit antibovine IgG HRP-conjugated (Nordic) (1/3000 in PBS-Tween) were added and the mixture was incubated for 1 h at 37°C. After 3 washes, as described above, the plates were developed using OPD as the substrate. The reaction was stopped with 100 μl per well of 3 N sulfuric acid. The absorbance at 450 nm was measured in a Titertek Multiskan (Flow Laboratories) spectrophotometer.

Animals

In this study we used 19 Asturiana cattle estimated to be 4 months–8 years old, from a farm located at Villaviciosa on the Asturias (Northern Spain) coast.

Three of the animals (4 months old), which were naive to *H. lineatum*, were used as negative controls.

The remaining 16 animals, naturally infested by the fly were divided into two groups of 8. In one of them (Group I) the infestation was not interrupted by any kind of treatment, whereas the animals of the other group were treated, on 15 October 1987, with 200 μg kg^{-1} of ivermectin (Ivomec; Merck and Co. Inc.). Blood samples were taken from all animals before the treatment (2 July; 30 August; 15 October) and afterwards (15 March; 18 May). The sera samples were kept at −20°C until used.

RESULTS

Crude extracts of *H. lineatum* 1st-instar larvae (HL1) were processed by reverse-phase HPLC, using a linear gradient of acetonitrile (25 to 50%), to obtain pure individual antigens. The elution profile of the larval proteins is

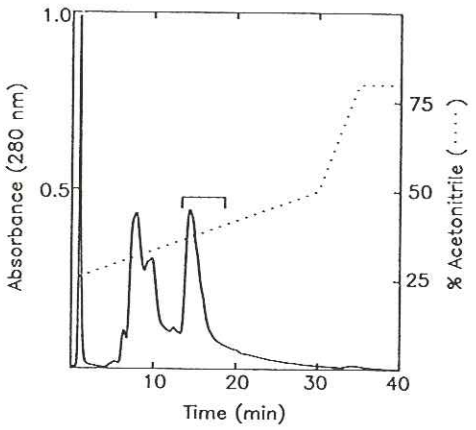

Fig. 1. Separation of HL1 proteins by reverse-phase HPLC. The fractions corresponding to hypodermin C (⊓) were pooled and used in ELISA tests (Table 1) or analysed by immunoelectrophoresis (Fig. 2).

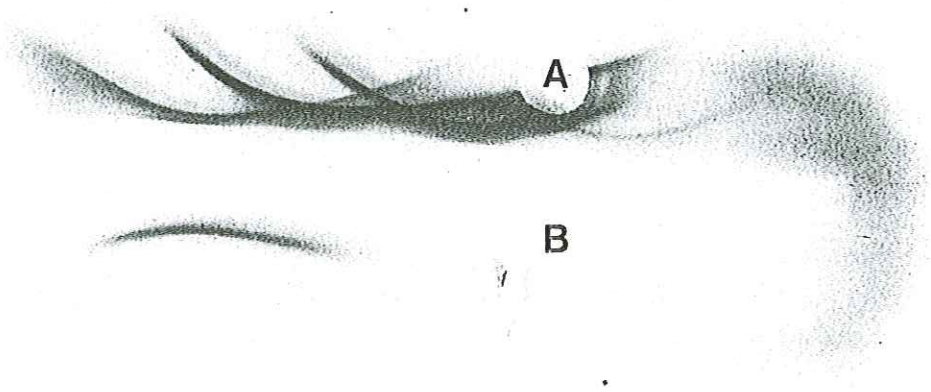

Fig. 2. Analysis of HL1 crude extracts and homogeneous hypodermin C by immunoelectrophoresis. (A) HL1 extract. (B) purified hypodermin C. The central slot contained a rabbit anti-HL1 serum.

shown in Fig. 1 in which the bracketed major peak, eluting at 45% acetonitrile, corresponds to hypodermin C. This single purification step allowed us to obtain homogeneous hypodermin C in high yields to be used as the antigen for the ELISA tests.

The purified protein was assumed to be the larval collagenase (hypodermin C) on the basis of its electrophoretic mobility on non-denaturing PAGE (Pruett

TABLE 1

Comparison of HL1 extracts and homogeneous hypodermin C as antigens in ELISA tests, for the detection of *H. lineatum* parasitized cattle

Number of cattle	Infection	Absorbance (450 nm) ± SE	
		HL1 extract	Hypodermin C
6	*H. lineatum*	1.03 ± 0.06	0.99 ± 0.08
3	–	0.67 ± 0.08	0.16 ± 0.04

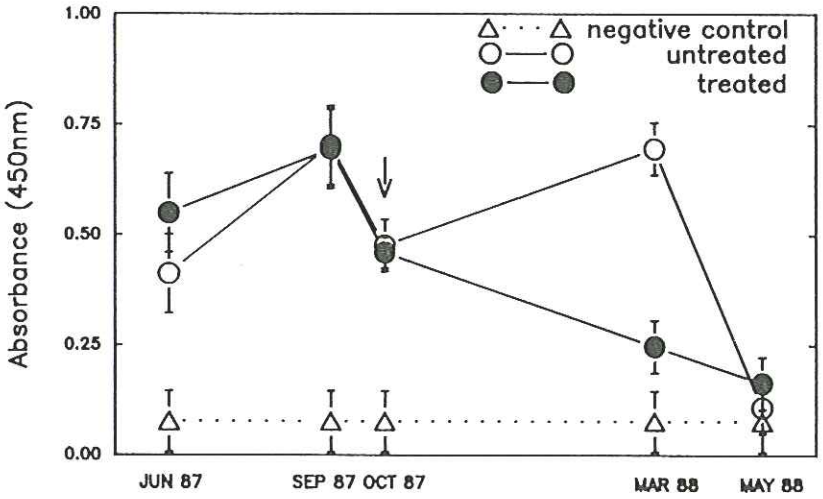

Fig. 3. Anti-hypodermin C titers (mean ± SE) measured by ELISA on serum samples from naive Asturiana cattle (△) or naturally infected (○,●) with *H. lineatum*. The arrow indicates the ivermectin treatment performed in animals from Group II (●).

and Barrett, 1984). In this type of electrophoresis the purified protein had the same mobility as the fastest band of the larval crude extract (data not shown). In a further test, we compared the material from the HPLC peak eluting at 45% acetonitrile with the crude HL1 extract by immunoelectrophoresis. As can be seen in Fig. 2, a single precipitin band was obtained corresponding to homogeneous hypodermin C, co-migrating with the fastest band in the crude extract.

The effects of using a pure antigen instead of crude extracts in ELISA tests can be seen from the results shown in Table 1. The data clearly reveal a much better signal/noise ratio when pure hypodermin C was used, with relatively very low absorbance values obtained from control uninfested calves.

The anti-hypodermin C titers over the course of a natural *H. lineatum* infestation cycle, measured by ELISA, are plotted in Fig. 3, where the effect of ivermectin is also shown. The first blood samples (June 1987) showed high antibody titers in all animals, increasing to a maximum in September 1987.

The samples taken in October 1987 showed similar antibody levels in all cattle; on this date half of the animals (Group II) were treated with 200 μg kg^{-1} of ivermectin, a dose which seems to be effective in the control of all stages of development of the parasite (Boulard et al., 1988b). The blood samples taken following the ivermectin treatment (March 1988) showed a clear difference in anti-hypodermin C antibodies between treated (Group II) and untreated (Group I) cattle. The titers obtained from animals in Group II were very close to the negative controls, whereas the titers from Group I had the highest antibody levels. The last serum sample assayed (May 1988) showed, in both groups, titers in the range of the negative controls.

All grubs that survived to form warbles in the backs of the animals were manually counted during the February–March period preceding (1987) and following (1988) the ivermectin treatment. Whereas all cattle showed warbles (1–30) at the beginning of the study (February 1987) only untreated animals (Group I) had viable grubs (1–15) in February 1988. In all cases the grubs were expelled from the animals by April 1987 or 1988.

DISCUSSION

Recently, cattle antibodies to HL1 soluble antigens over the course of an infestation cycle have been investigated by Western blotting (Pruett et al., 1988). The results showed differences in their detection and persistence. From those data hypodermin C is one of the most immunogenic proteins, the first to be detected, having no common antigenic determinants with the other two major antigens (hypodermins A and B).

Larval collagenase was also shown to be one of the major antigens of early detection in experimental infestations performed in rabbits (Boulard and Weintraub, 1973). Thus, hypodermin C appears to be the antigen of choice for the immunodiagnosis of hypodermosis. Accordingly, our results also show that ELISA tests, based on the use of pure hypodermin C as the antigen, more clearly differentiate between infested and control animals, in contrast with the poor discrimination obtained using crude HL1 extracts.

When the new indirect ELISA was used to investigate the antibody levels over a natural infestation cycle, the test proved the existence of high titers of anti-hypodermin C in the blood samples taken on 2 June 1987 in all assayed animals, clearly indicating that a new infestation had already begun. These results differ from those reported by others (Boulard et al., 1988a) in which, by this time, the previous year's infestation had still not come to an end. Our data also showed that there were viable mature flies in the area under study (Asturias, Spain) as early as the end of April or the beginning of May 1987. The high anti-hypodermin C titers observed in June could be the result of an intensified antigenic response, as a consequence of a previous infestation (Robertson, 1980).

In contrast with previous observations, on 20 May 1988 all antibody titers were close to the negative controls, indicating a delay in the infestation cycle compared with that in 1987 in which we could measure relatively high titers of anti-hypodermin C in the samples taken at about the same time of the year (2 June). Taking into account that weather conditions play an important role in the emergence of flies from pupae, as well as in mating efficiency (Tarry, 1980), excessive rainfall and cool temperatures could be responsible for the observed differences. In fact, in the area under study, the mean rainfall measured in April 1988 was 197 l m^{-2}, about four times the amount registered in April 1987 (54.5 l m^{-2}). The mean temperatures also showed a significant difference (cooler in 1988) which also can explain the observed apparent delay in infestation.

A conclusion drawn from our results is the benefit of a precise diagnosis of the anti-hypoderma antibody levels prior to the use of any antiparasite compound. This could avoid undesirable responses which can lead to animal losses, especially where weather conditions can significantly affect the development of the parasite.

The ivermectin treatment used proved to be very effective leaving the treated animals free of warbles at the time in which the untreated animals had 1–15 viable grubs.

ACKNOWLEDGMENTS

The authors wish to thank Dr. R.S. Cármenes for his help in processing HPLC data. This research was supported in part by the C.I.C.Y.T and by Consejería de Agricultura y Pesca del Principado de Asturias. A. Espi was a fellow of Caja de Ahorros de Asturias.

REFERENCES

Boulard, C., 1985. Advantages de l'immunodiagnostic de l'hypodermose bovine établi par l'hemaglutination passive par ELISA, a partir du sérum et du lacto-sérum, sur la numération des varons. Ann. Rech. Vet., 16 (4): 335–343.

Boulard, C. and Weintraub, J., 1973. Immunological responses of rabbits artificially infested with the cattle grubs *Hypoderma bovis* and *H. lineatum*. Int. J. Parasitol., 3: 379–386.

Boulard, C., Argente, G. and Hillion, E., 1988a. Hypodermose bovine 1re partie: description et incidence économique. Point Vet., 20 (111): 17–30.

Boulard, C., Argente, G. and Hillion, E., 1988b. Hypodermose bovine 2e partie: diagnostic et traitement. Point Vet., 20 (112): 17–27.

Lecroisey, A., Boulard, C. and Keil, B., 1979. Chemical and enzymatic characterization of the collagenase from the insect *Hypoderma lineatum*. Eur. J. Biochem., 101: 385–393.

Pruett, J.H. and Barrett, C.C., 1984. Induction of intradermal skin reactions in the bovine by fractionated proteins of *Hypoderma lineatum*. Vet. Parasitol., 16: 137–146.

Pruett, J.H., Temeyer, K.B. and Burkett, B.K., 1988. Antigenicity and immunogenicity of *Hypoderma lineatum* soluble proteins in the bovine host. Vet. Parasitol., 29: 53–63.

Robertson, R.H., 1980. Antibody production in cattle infected with *Hypoderma* spp., Can. J. Zool., 58: 245–251.

Sinclair, I.J. and Wassall, D.A., 1983. Enzyme-linked immunoabsorbent assay for the detection of antibodies to *Hypoderma bovis* in cattle. Res. Vet. Sci., 34: 251–252.

Sinclair, I.J., Tarry, D.W. and Wassall, D.A., 1984. Persistence of antibody in calves after an infection with *Hypoderma bovis*. Res. Vet. Sci., 37: 383–384.

Tarry, D.W., 1980. Warble fly infestation and climate. Vet. Rec., 106: 559–560.