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Evaluation of nitroxynil and closantel activity using ELISA and egg counts against *Fasciola hepatica* in experimentally and naturally infected cattle

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**Summary** — The responses of cattle infected with *Fasciola hepatica* to treatment with nitroxynil or closantel were monitored by faecal egg counts and by ELISA assay of anti-fluke antibodies. A first trial with experimentally infected heifers showed an increase in anti-fluke antibody titre as early as 2 weeks post-infection. Eggs were first detected in the faeces 10 weeks after infection. Egg output increased steadily over the next 8 weeks and then rapidly decreased. Treatment of a 20-week infection with nitroxynil was followed by a slow decrease in antibody titre 4 weeks later. This decrease continued over the next 40 weeks, but returned to pre-infection levels in only 2 out of 4 animals. The faecal egg count fell to zero 2 weeks after treatment and remained so for the following 30 weeks, although 1 animal produced a few eggs 32 and 34 weeks post-treatment. Within this period, neither diagnostic technique discriminated between this persistently infected animal and the others. In a second trial, 45 cattle from a naturally infected herd were treated with nitroxynil or closantel. The faecal egg counts of the treated cattle were zero within the following 2 months, whereas there were eggs in the faeces of the control (untreated) group. Nevertheless, the treated cattle showed a small, non-significant drop in anti-fluke antibody titre. These results demonstrate the need for new tools to monitor and evaluate accurately the efficacy of anthelmintic treatment.

**Résumé** — Évaluation de l’activité fascioli cide du nitroxynil et du closantel au cours d’infestations par *Fasciola hepatica*, chez les bovins, infectés expérimentalement ou naturellement. Des bovins infectés expérimentalement ou naturellement par Fasciola hepatica ont été traités par des fascioli cides, le nitroxynil ou le closantel. L’efficacité de ces traitements a été évaluée au cours des mois suivants, par des examens coprologiques et par des examens immunologiques par la technique ELISA. Au cours de l’infestation expérimentale, les anticorps anti-Fasciola hepatica sont détectés après 2 sem d’infestation et atteignent un plateau 6 sem après. Le diagnostic coprologique ne devient...
POSITIF QU'À LA 10e OU 12e SEMAINE APRÈS L'INFESTATION, PUIS S'ACCRÖIT JUSQU'À LA 18e SEMAINE ET Chute pour atteindre de très faibles valeurs à la 20e semaine. Un traitement par le nitroxinil à cette date se traduit 4 sem après par une chute lente des taux d'anticorps anti-Fasciola hepatica. Ils atteignent le seuil de négativité 10 mois après le traitement, pour 2 animaux sur 4. La recherche d'œufs de douves reste négative pendant les 30 sem suivant le traitement chez tous les animaux. À la 32e et 34e semaine, un des bovins, bien que maintenu dans des installations protégées, a présenté des œufs de douves à l'examen coprologique et conservait par ailleurs des taux d'anticorps relativement élevés. Des traitements avec le nitroxinil ou le closantel dans une exploitation naturellement infestée se traduisent au cours des 2 mois suivants, contrairement au lot témoin, par une négativation des coprologies suggérant une excellente activité sur les douves adultes. Cependant, une faible décroissance des taux d'anticorps anti-F hepatica évoquent la persistance d'éléments parasitaires. Les 2 méthodes de diagnostics utilisées classiquement pour apprécier l'efficacité des traitements fasciolicides sur tous les stades évolutifs de la population parasitaire se révèlent peu sensibles, notamment au cours des 2 mois suivant le traitement. De nouveaux outils sont nécessaires pour une évaluation rapide et précise de l'activité de ces traitements sur le terrain.

fasciolose / bovin / fasciolicides / coprologie / immunodiagnostic

INTRODUCTION

Several flukicides are now available to control fasciolosis in cattle. The activity of each of these drugs is variable against the different stages of the parasites' development. Some, like triclabendazole are very effective against immature flukes (Richards et al, 1990), others, such as nitroxynil or closantel, are highly efficient against adults (Rapic et al, 1988). The activity of each molecule on the different stages of the fluke can be accurately evaluated in experimental assays by necropsy of the infected animals. However, the methods available for assessing flukicide activity under field conditions are indirect. The most generally used methods are egg counting and immunological studies.

This study evaluates the efficacy of 2 flukicides that are most active against adult flukes. The treatments were assessed simultaneously by counting faecal worm eggs and by immunological diagnosis. The first study involved an experimental infection of the cattle, which was followed, 20 weeks later, by a nitroxynil treatment. This procedure was used to establish the methods for assessing fluke control. It was followed by a field trial on a farm that had a history of Fasciola hepatica infestation. At this farm, nitroxynil or closantel treatments were given in the traditional way, 1 month after winter herd housing.

MATERIALS AND METHODS

Experimental procedures

Experimentally infected cattle (Experiment I)

Five 6-month-old Friesien heifers, that were free of liver fluke infection on the basis of faecal examination and immunological diagnosis, were infected with metacercaria of F hepatica produced under standard conditions on Lymnaea truncatula (Baeza et al, 1994). One animal (No 4) was given 800 metacercariae and the 3 others (No 1–3), 400 metacercariae per os. One animal (No 5) was kept as an uninfected control. They were bled weekly for 5 months then every 2 weeks for 7 more months. The sera were stored until assay for antibodies. The progress of the infection was monitored by bimonthly faecal examinations, starting 10 weeks post infection (PI).

On week 20 PI, heifers 1–4 were each given 10 mg•kg⁻¹ nitroxynil (Dovenix ND, Rhône-Mérieux, France) subcutaneously. The animals
were kept indoors for 60 weeks and silage and water were supplied ad libitum.

Naturally infected cattle (Experiment II)

A total of 45 adult Charolais cattle on a farm known to be naturally infected with *F. hepatica* were examined by faecal egg counts and immunological diagnosis in mid-December 1992 immediately after they entered their winter housing. They were treated at this time for lice with lindane (Veticide ND, Vetoquinol, France). Their anti- *Fasciola* antibody activity was assayed and they were allocated at random to 1 of the 3 groups of 15 animals. They were given subcutaneous injections on January 12 1993, as follows:

Group 1: nitroxynil (10 mg·kg⁻¹) (Dovenix ND, Rhône-Mérieux, France)
Group 2: closantel (10 mg·kg⁻¹) (Flukiver ND, Janssen, France)
Group 3: untreated

All the animals were kept indoors, fed with hay and given water ad libitum. They were bled monthly and their faeces were sampled for fluke egg counts.

**Immunological tests**

*F. hepatica* antigens were obtained from liver flukes that were collected from cattle bile ducts at the slaughterhouse. The flukes were washed in normal saline and kept in this medium for 3 h. The excretory/secretory (ES) products that were collected were used as antigens.

The antibody titres of the sera were determined by ELISA (Boulard et al, 1985). Briefly, wells of micro-ELISA plates (Linbro, Titertek, Flow Laboratory) were coated with 100 µl ES (5 µg protein·ml⁻¹) in 0.1 M carbonate buffer pH 9.6. Bovine sera were diluted (1:300) in PBS, and studied in triplicate. The second antibody was horseradish peroxidase conjugated rabbit antibovine IgG (H+L) (Nordic Immunology, Tebu, France) (diluted 1:3 000 in PBS). The substrate was 2,2-azino-di-(3-ethylbenzthiazolinsulphonate) (ABTS, Boehringer Mannheim, Meylan, France).

Positive and negative sera were used as standards on each plate to provide interassay control. Results were expressed as a percentage of this positive serum using the following calculation: 

\[
\frac{((\text{sample serum mean OD}) - (\text{standard negative serum mean OD}))}{(\text{standard positive serum mean OD} - \text{Standard negative serum mean OD}))} \times 100.
\]

**Faecal egg examinations**

The number of faecal eggs per gram (epg) were counted after sedimentation (Dorsman and Bijl, 1982). *F. hepatica* eggs were distinguished from the eggs of paramphistomes by staining with a 0.1% methylene blue solution (Boray and Pearson, 1960).

**RESULTS**

In experimentally infected heifers (Experiment I), the course of infection showed the appearance of a few eggs in the faeces by week 10 (fig 1). The maximum epg occurred after 18 weeks of infection. Nitroxynil treatment was applied in week 20. All the subsequent epg remained negative for the following 40 weeks, except for 1 animal initially infected with 800 metacercariae, which excreted 0.1 then 0.2 epg on weeks 52 and 54 PI, despite the fact that it was housed in an environment that had no risk of reinfection with *F. hepatica*. No fluke eggs were detected in the faeces of the uninfected heifer.

The antibody response (fig 2) showed the humoral response during the primary experimental infection of the 4 heifers. ES-specific antibodies rose above the control level by 2 weeks PI and reached a plateau by week 6. The antibody level remained constant until the 25th week PI, despite the fact that the cattle were treated on week 20. The antibody titres dropped gradually over the following 35 weeks and ultimately returned to the control level in only 2 of the 4 heifers. The antibody titres in the other 2 heifers decreased slowly and the animal which excreted a few eggs on weeks 52 and 54 PI had the highest level of circulating antibodies. The antibody titres of the unin-
infectected animal remained constant (20%) during the 60 weeks of the experiment.

In naturally infected cattle (Experiment II), each group initially contained 20–54% negative animals for fluke eggs (table I), but only one animal in each group was immunologically negative.

No egg was found in the faeces of the 2 treated groups throughout the post-treatment period (February–March) (table I),

Fig 1. The faecal egg counts of 4 calves following experimental infection on week 0, with 400 metacercariae of *F. hepatica* (animal 1 — ■ —, animal 2 □, animal 3 * — ) or 800 metacercariae (animal 4 — △ —). The animals were treated with 10 mg kg⁻¹ nitroxynil on week 20 (†).

Fig 2. Anti-*F. hepatica* antibodies in the sera of 4 calves experimentally infected on week 0, with 400 (animals 1 — ■ —, 2 □, 3 — ) or 800 (animal 4 — △ —) metacercariae of *F. hepatica*. On week 20 PI, the animals were treated (†) with nitroxynil. Animal 5 — △ — was uninfected and untreated.
whereas the control group showed zero epg in February and 20% of the animals had positive epg at the end of March. Eggs of paramphistomes were present in the faeces of all the animals in all groups throughout the study. The treatment had no effect on paramphistomes. The mean antibody titre in the 3 treated groups fell slightly during the 3 months of the study (table II). At each period of sampling, the groups presented similar antibody titres, as shown by the confidence intervals established at $P < 0.05$.

**DISCUSSION**

The first experiment examined the effects of nitroxynil treatment on flukes 20 weeks after experimental infection. The flukicide activity, assessed by faecal eggs counts, was detected immediately after treatment in all the animals and no fluke eggs were found during the following 32 weeks. Eggs were first detected 10 weeks PI and the epg steadily increased over the following 3 months to week 18 PI and then rapidly declined. There was no correlation between the infective dose and the epg. These data agree with those of Doyle (1972) and Kendall and Parfitt (1975), who found that egg output was negligible 21 weeks after experimental infections. Since the faecal egg counts studies cannot detect fluke infections before 10 weeks PI and appear to be inaccurate for infections over 21 weeks old, this technique is unsuitable for monitoring the effectiveness of chemotherapy when
drugs are given during these periods, even following an experimental infection.

Whereas the faeces of all the heifers treated with nitroxynil had ceased to contain fluke eggs 2 weeks after treatment, 32 weeks later, 1 of them produced a few eggs over a period of 2 weeks. This suggests that a few flukes, probably immature at the time of treatment, may be affected and their prepatent period extended for 8 months. Flukicide drugs have previously been shown to have a high activity against the adult flukes and to retard the growth of immature flukes in infected cattle treated for up to 10 weeks (Buscher et al., 1987). Nevertheless, nitroxynil treatment also has a deleterious effect on the ability of the eggs of these retarded flukes to hatch and could have a negligible epidemiological impact (Stammers, 1976). To date, however, too little attention has been paid to the effects of flukicides on the prolonged growth of the immature flukes. This problem is epidemiologically important and tools to explore it, which are more accurate than egg counts, need to be developed.

These drawbacks make it difficult to accurately assess the efficacy of flukicides used in the field to treat a mixed-age fluke population using only faecal egg examinations.

The second experiment indicated that chemotherapy with nitroxynil or closantel was, according to the faecal studies, a clear success. However, because of the limitations of this assay method and the fact that few eggs were recovered in the control group, this interpretation of the egg count may not provide an accurate evaluation of the drugs' efficacy. The information provided by the immunological study could therefore be used to provide complementary data.

Considerable progress has been made in the development of immunological diagnostic tools for fasciolosis (Oldham, 1983; Boulard et al., 1985; Chauvin and Boulard, 1992). Circulating antibodies have been detected by ELISA as early as the second week after fasciolosis infection in cattle and, depending on the specificity, they appear to be good indicators of fasciolosis.

In agreement with previous studies (Oldham, 1983; Wyckoff, 1983; Malone, 1986; Boulard and Regnaud, 1989), the heifers infected with _F. hepatica_ in Experiment I developed high serum levels of antibodies against fluke secretory and excretory products. They were detectable within 2 weeks of infection. The antibody profiles of animals infected with 400 and 800 metacercariae were similar. The response reached a plateau at 6 weeks PI and remained stable for the next 3 months. Whereas the antibody value of the uninfected animal remained at the same level (20%) during the 60 weeks of the experiment. An early study (Boulard and Regnaud, 1989) showed that the immune response of 8 calves experimentally infected with 1 000 metacercariae and given no drug therapy remained at this level up to 8 months PI. Chemotherapy with nitroxynil, 20 weeks after infection, caused the antibody titre to decline 5 weeks later. However, the anti-fluke SE antibody titre 20 weeks after treatment could not readily distinguish an animal that was still infected and producing a few eggs 32 weeks after treatment from the fluke-free animals. This gradual decrease in the anti-fluke antibody titre, which lasted over 10 months after treatment, makes it very difficult to accurately assess the efficacy of the drugs.

This difficulty is increased under field conditions when a mixed age fluke population is treated, as in Experiment II. In this case, chemotherapy resulted in a decrease in anti-fluke antibody titre in both treated groups. However, during the same period, there was also a moderate decline in the mean antibody titre in the control group. These drops were not statistically different between December and the end of March within each group, or, for the same month between groups. Thus, immunological diag-
nosis is not an accurate means of evaluating the effects of flukicides.

While the results of the 2 methods used in Experiment II may suggest that the drugs are effective against adult flukes, the assays are not sensitive enough, even when used together, to compare the relative success of the 2 flukicides against immature parasites. There is an urgent need for new methods, which reflect more accurately the exact parasitological status of the host after treatment. The detection of circulating antigens in the serum, bile or faeces will probably be of great value in the future (El-Bahi et al. 1992).

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