Variations of biological indicators as highly presumptive markers for fasciolosis in experimentally-infected sheep

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SUMMARY

The aim of this study was to select parameters showing the greatest variations during Fasciola hepatica infections in sheep for establishing high-presumption diagnoses for fasciolosis in Algeria. Eight biological parameters (leukocyte, erythrocyte, eosinophil counts, serum bilirubin, IgG and IgM concentrations and serum enzyme (ALT, AST) activities) were studied in 4-month old female sheep mono-infected (n = 5) with 150 F. hepatica metacercariae and bi-infected (n = 5) (the reinfection with 150 metacercariae was made 6 weeks later). Erythrocyte counts and serum enzyme (ALT and AST) activities showed insignificant variations in infected sheep compared to uninfected controls (n = 5), whereas significant increases of leukocyte and eosinophil counts were noted in infected sheep. They peaked on the 5th week in both infected groups and also on the 13th week in bi-infected sheep. Bilirubin concentrations significantly increased in the bi-infected group from week 13 to peak at week 15, while insignificant variations in the mono-infected group were found. Significant increases of IgG and IgM levels were also observed in both infected groups. After peaking at weeks 3/5, IgG concentrations remained relatively constant and highly elevated in both groups, while IgM concentrations steadily decreased before another peak at week 11. Because of low costs for measurements of eosinophil counts and bilirubin, such tests may be routinely used to establish high-presumption diagnoses for fasciolosis in Algeria.

Keywords: Fasciola hepatica, Algeria, bilirubin, eosinophils, immunoglobulins, leukocytes, sheep.

RÉSUMÉ

Variations d’indicateurs biologiques considérés comme de possibles marqueurs de la fasciolose chez des moutons infestés expérimentalement

Le but de cette étude est de sélectionner les paramètres qui présentent les plus grandes variations au cours d’une infestation par Fasciola hepatica chez le mouton et qui peuvent permettre un diagnostic de forte présomption de la fasciolose en Algérie. Huit paramètres biologiques (numérations érythrocytaire, leucocytaire et en éosinophiles, concentrations sériques de la bilirubine, des Ig G et des Ig M, activités sériques enzymatiques (ALT, AST)), ont été étudiés chez des brebis femelles âgées de 4 mois, mono-infestées par 150 métacercaires de F. hepatica (n = 5) ou bi-parasitées (la seconde infestation par 150 métacercaires a eu lieu 6 semaines après la première) (n = 5). La numération érythrocytaire et les activités sériques enzymatiques (ALT, AST) n’ont pas varié de façon significative chez les brebis infestées par rapport aux témoins non parasités (n = 5), alors que des augmentations significatives de la numération leucocytaire et du comptage des éosinophiles ont été observées chez les animaux infestés. Des valeurs maximales ont été obtenues pendant la 5ème semaine dans les deux groupes parasités et également pendant la 13ème semaine seulement chez les animaux bi-infestés. Les concentrations en bilirubine ont augmenté significativement dans le groupe bi-parasité dès la 13ème semaine pour atteindre des valeurs maximales à la 15ème semaine, tandis que les animaux mono-parasités n’ont présenté que des variations non significatives. Les concentrations en Ig G et Ig M ont également augmenté de façon significative chez les deux groupes parasités. Après l’obtention d’un pic à la 3ème/5ème semaine, les concentrations en Ig G sont restées relativement constantes et fortement élevées dans les deux groupes alors que celles en Ig M ont progressivement diminué avant d’atteindre un nouveau pic à la 11ème semaine. En raison des faibles coûts du comptage des éosinophiles et du dosage de la bilirubine, ces tests pourraient être utilisés en routine afin d’établir un diagnostic de forte présomption de la fasciolose en Algérie.

Mots-clés: Fasciola hepatica, Algérie, bilirubine, éosinophiles, immunoglobulins, leucocytes, moutons.

Introduction

Distematoses due to Fasciola hepatica is nowadays considered as one of the main five parasitoses in Algeria. In the northeastern part of this country, high prevalences of natural infections are recorded in cattle, especially in the littoral zone (nearly 45% at El Tarf, 42% at Skikda, and 27% at Jijel, unpublished results). Because of its insidious nature, the disease is only detected at slaughterhouses during liver inspection, as no serological or coprological screenings for fasciolosis are performed in farms.

A large-scale screening for fasciolosis in the Algerian livestock (mainly cattle and sheep) is difficult to carry out, as analysis laboratories specialized in veterinary serology and/or coprology are few in number and the cost of such analyses is still too high to make investigations in numerous ruminants. In view of this situation, it is interesting to establish high-presumption diagnoses by using haematological parameters, as such tests are little priced and can be routinely made by unspecified analysis laboratories throughout the Algerian territory.

In ruminants infected with F. hepatica, different changes in haematological characteristics, hepatic functions, and immunoglobulin levels have already been described by several authors (see review by BEHM and SANGSTER [3]). Among changes occurring in blood components, hypereosinophilia was the most known, as it developed in all host species [12].
Significant variations of several hepatic enzymes, i.e. gamma-glutamyl transpeptidase, sorbitol dehydrogenase, or glutamate dehydrogenase, in infected ruminants were also reported by Anderson et al. [2], and Bulgin and Anderson [5]. As changes of these biological parameters might serve as indicators to diagnose fasciolosis, it was interesting to routinely use several of them for establishing high-presumption diagnoses. In view of this necessity, the aim of this work was to select parameters showing the greatest variations compared to unexposed controls i) during a primo-infection of sheep with *F. hepatica*, and ii) during a re-infection of the same ruminants.

**Materials and Methods**

**ANIMALS AND EXPERIMENTAL PROTOCOL**

The study was carried out in 2003, in the region of Jijel (north-eastern Algeria). The choice of sheep for this experiment was made on the basis of a major physiological criterion as for many authors sheep are the preferential definitive hosts of *F. hepatica* and the damage induced by fasciolosis in this species are known to be important [3]. A total of 15 female lambs, 4-month-old, crossbreed (local Ouled-Djellal race x imported New Zealand race) and weaned, were chosen for this study. All lambs were intensively reared.

For this experiment, these lambs were removed from the flock before being permanently reared in the sheepfold during the whole time of the study. A previous screening using the ELISA method [7] demonstrated the absence of *Fasciola* infection in these lambs at the onset of the experiment. Five lambs were used as controls (group 1). The second group was composed of five animals, each infected with a single dose of 150 metacercariae, while the five lambs of the third group were each infected with 150 larvae at the onset of the experiment and again with other 150 metacercariae 6 weeks after the first infection [8]. Metacercariae of *F. hepatica* originated from experimental infections of *Galba truncatula* (University of Limoges, France) and were administered to each lamb via the introduction of a bolus in the oesophagus. Infected lambs were slaughtered 12 weeks after the first metacercaria infection, i.e. at the end of the 12th week for the group 3 (bi-infected group) and at the end of the 18th week for the group 2 (mono-infected group). The six parameters studied were the numbers of erythrocytes, leukocytes and eosinophils, the serum bilirubin concentrations, and the serum ALT and AST activities. Their choice was based on the two following criteria: the measure of each parameter must be easy, with a low cost (haematological parameters) and/or the parameters must be specific enough of hepatic damage (ALT, AST, and bilirubin). Two immunoglobulins (IgG and IgM) were also measured to detect more specific immune reactions.

**STATISTICAL ANALYSIS**

Mean values recorded in the three groups of sheep were compared using a two-way analysis of variance. The Student t-test [18] was used to compare the mean values recorded from week 4 to week 10 and from week 12 to week 18 in bi-infected sheep. Differences were considered as significant when P values were less than 0.05.

**Results**

**HAEMATOLOGICAL ANALYSES**

No significant variation of erythrocyte counts was evidenced between controls and infected (mono- or bi-) sheep (data not shown).

Compared to controls, the number of leukocytes (figure 1) was significantly increased in infected sheep. It peaked at week 5 in the mono-infected group and clearly decreased after week 9. A first peak at week 5 and another higher peak at week 13 were found in the bi-infected group. Significant effects for sheep group (F = 30.97, P < 0.001) and the date of blood collection (F = 15.66, P < 0.001) were noted. In the bi-infected group, the variations of leukocyte numbers did not significantly differ from week 3 to week 18.

Similar findings were noted for eosinophils (figure 2). In infected lambs, their numbers peaked at week 5 (in both groups) and at week 13 (in bi-infected sheep only). The group of lambs (F = 38.62, P < 0.001) and also the date of blood collection (F = 35.82, P < 0.001) exhibited significant influences. However, there was no significant difference between the values of the mono-infected group and those recorded in bi-infected sheep. In the bi-infected group, the numbers of eosinophils noted at the 12th week and after were significantly different (t = 2.06, P < 0.001) from those recorded from week 4 to week 10 and from those observed in controls at the 12th and 13th weeks (p < 0.001).
BIOCHEMICAL ANALYSES

Serum enzyme activities (ALT, AST) were similar in the 3 groups during the whole period of the experiment (data not shown).

Besides, during the first 12 weeks, the bilirubin concentrations (figure 3) found in infected sheep, were closely related to those of controls. However, this parameter markedly increased at the 13th week, peaked at week 15 and thereafter slowly decreased until the 18th week in the bi-infected group. Significant effects for the group of lambs (F = 8.56, P < 0.001) and the date of blood collection (F = 9.57, P < 0.001) were noted but, in fact, only the bi-infected group was different from the two others. The bilirubin concentrations noted after week 12 in this group were also significantly different (t = 1.82, P < 0.001) from those recorded from week 4 to week 10.

IMMUNOLOGICAL ANALYSES

Compared to controls, an increase of IgG concentrations (figure 4) was noted in infected sheep from week 2 to weeks 5 and 6. Afterwards, relatively constant values (always significantly increased when compared to controls) were recorded until the end of the experiment. The group of sheep significantly influenced (F = 313.60, P < 0.001) the values of this parameter, while insignificant variations were noted for the date of blood collection. In the bi-infected group, the concentrations recorded after the 12th week were significantly different (t = 4.03, P < 0.001) from values found between week 4 and week 10.

Similar findings were also noted for IgM (figure 5). However, in bi-infected sheep, two peaks, the first at week 3 and the other at week 11, were evidenced. The group of
Discussion

Among parameters measured, erythrocyte counts as well as serum enzyme AST and ALT activities only showed insignificant variations in infected sheep. These findings disagreed with reports made by other authors, as anaemia and increases of several hepatic enzyme activities (glutamate dehydrogenase, glutamate-oxaloacetate aminotransferase, gamma-glutamyl transferase) were reported in a variety of infected hosts under experimental conditions [3]. To comment these differences, the most valid explanation would be the level of experimental infections with F. hepatica larvae in sheep used in the different studies. In our opinion, the number of metacercariae ingested by each lamb in the present study (150 per animal in the mono-infected group and 300 in the bi-infected group) compared to unexposed controls (n = 5).

In contrast, other four parameters showed significant variations during the F. hepatica infection in sheep and they warrant special comments:

- Eosinophil peaks noted at week 5 (in both groups of lambs) and at week 13 (in the bi-infected group only) agree with the previous reports [10, 13, 15] in different definitive hosts infected with F. hepatica. According to these authors, the first peak would be correlated with the onset of young fluke burrowing through the liver, while the second peak would coincide with the arrival of adult flukes into the bile ducts. In contrast, CHAUVIN [6] attributed these peaks to the two periods of young fluke burrowing which succeeded in the livers of bi-infected sheep and explained eosinophil decreases, which occurred after each peak, by the development of a visceral response and, consequently, by a high need of circulating eosinophils for the damaged hepatic parenchyma. Even if the opinions of above authors on the explanation of the second peak are different, a significant increase of eosinophils was observed in the two groups of infected lambs and this parameter is worth taking into account as a good biological indicator to establish a high-presumption diagnosis for fasciolosis.

- A hyperbilirubinaemia was only found at week 15 in the bi-infected group. This finding disagreed with the report of JEMIL et al. [12], as these authors did not detect such variations in infected sheep. By contrast, PRACHE and GALTIER [16], and FERRE et al. [9] noted a significant increase of bilirubin concentrations from week 6 to week 14 in infected sheep. As this bilirubinaemia was not found in the mono-infected group in the present study, it can be assumed that fluke burdens present in the livers of mono-infected lambs would not allow the development of an obstructive phase. Conversely, a cholestasis would develop in the bi-infected group (these lambs have received a double dose of metacercariae) and would induce an increase of serum direct bilirubin concentration. If this hypothesis is considered valid, the greatest variations of this parameter would only be noted during heavy infections with F. hepatica. However, this parameter was specific enough of cholestasis, it must be taken into account.

- In both groups of infected lambs, IgM and IgG concentrations increased from week 2. The first immunoglobulin type showed two peaks at week 3 and at week 13 but it is difficult to determine if the second peak was due to the re-infection of sheep, as there were scarce reports on this fact. On the other hand, the high values of IgG recorded after week 5 or 6, agreed with the reports of CHAUVIN [6] and of PIACENZA et al. [14]. To explain the persistence of these IgG values, two hypotheses were proposed by several authors. According to HILLYER et al. [11] and CHAUVIN et al. [8], the increase of IgG would due to the excretory/secreted products released by adult flukes when they stayed in the bile ducts, thus supporting the synthesis of IgG. However, for CHAUVIN [6], the immunological and enzymatic processes acting in the increase of IgG concentrations would be more complex. As the IgG level early increased during a F. hepatica infection, it is a good indicator to determine the presence of flukes within the mammalian host. However, the detection of such immunoglobulins can only be performed using immunoenzymatic methods (e.g. ELISA) in specialized analysis laboratories.

The results reported in the present study demonstrated the significant influence of the date of blood collection on leukocyte and eosinophil counts, bilirubin concentrations, and IgM levels. As these parameters showed important fluctuations over time, even within uninfected controls (see figures 1 and 3),

![Figure 5: Variations of serum IgM concentrations (means ± standard deviations) according to time in mono-infected sheep (with 150 Fasciola hepatica metacercariae) (n = 5) and in bi-infected sheep (2 infections with 150 larvae at interval of 6 weeks) (n = 5) compared to unexposed controls (n = 5).](Image 1)
it seems necessary to propose highly selective thresholds for these markers to avoid false positive results. Nevertheless, it is important to note that these markers are indicators only for a diagnosis of high presumption for fasciolosis and not for a certainty diagnosis.

In conclusion, the above results demonstrated the interest of eosinophil counts and bilirubin to be routinely used as biological indicators for establishing a diagnosis of high presumption for fasciolosis. The low cost of such tests, coupled with the fact that they could be easily made by unspecialized analysis laboratories throughout Algeria, pleaded for the use of these parameters as biological indicators for establishing high-presumption diagnoses of fasciolosis in local cattle and sheep.

References