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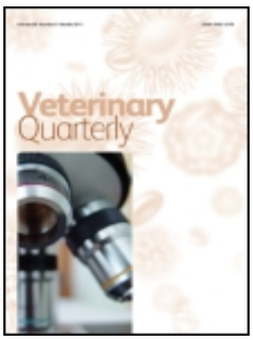

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Isoenzyme studies on *Theileria* (Protozoa, Sporozoa). Enzyme activity associated with the erythrocytic stage

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SUMMARY

Bovine blood containing piroplasms of Theileria parva, as well as non-infected blood, was lysed and subjected to iso-electric focussing.
Staining for 13 different enzymes revealed parasite-associated bands of glucose phosphate isomerase (GPI) activity, not of any of the other enzymes. There were no variations between individual donor animals in the host cell GPI bands and these bands did not interfere with the recognition of the parasite-associated bands, so that purification of the piroplasms was unnecessary. Blood from cattle infected with T. mutans also gave parasite-associated bands of GPI, but no such bands were seen in zymograms of blood from cattle infected with a Theileria sp. from Japan. Depending on the level of parasitaemia, up to four parasite-associated bands were found in one strain of T. parva and up to three in two other strains. Among the disadvantages of using piroplasm material for the study of isoenzymes of T. parva is the fact that animals often die before their parasitaemia is sufficiently high, and that some strains never give rise to a high parasitaemia.

INTRODUCTION

The genus *Theileria* includes some of the most pathogenic disease agents of domestic ruminants. Because of taxonomic problems and immunological differences, additional criteria are needed for their characterization. Isoenzyme patterns of parasitic protozoa are widely used as a tool for distinguishing between species and strains (13), but few studies have been made of the enzymes of theilerial parasites. At least two stages in the life cycle of *Theileria* can be obtained in sufficient quantities for such work; the erythrocytic piroplasm stage is collected by bleeding animals with a high parasitaemia and the schizont stage in lymphoblasts can be cultured *in vitro*. Allsopp and Wagner (1) used polyacrylamide gel electrophoresis and iso-electric focussing to compare the activity of a few enzymes in bovine lymphoblastoid cells infected wit *T. parva* and in uninfected cells. Musisi (11, 12) examined several enzymes in cultured lymphoblastoid cells of cattle and African buffalo, infected with *T. parva* and *T. lawrencei* (which we consider as biologically different strains of *T. parva* (14)), using thin-layer starch gel electrophoresis. Melrose and Brown (9) compared the activity of several enzymes in zymograms, obtained by thin-

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layer starch gel electrophoresis, of bovine erythrocytes infected with the piroplasm stage of *T. parva* and *T. annulata* and of uninfected red cells.

Melrose *et al.* (10), also using starch gel electrophoresis, examined glucose phosphate isomerase isoenzyme patterns of *T. parva* and *T. annulata* in infected lymphoblastoid cell cultures. Some of the results obtained by the above authors will be commented upon in the discussion.

In this paper we report on enzyme studies of the piroplasm stage of bovine *Theileriae* by means of isoelectric focussing.

MATERIALS AND METHODS

Friesian calves were infected with one of three strains of *T. parva,* designated as Boleni, Muguga, and Uganda. The Boleni strain, from Zimbabwe, has the characteristics of *T. parva bovis* (8), causing Rhodesian malignant theileriosis, the Muguga strain, from Kenya, and the Uganda strain cause classical East Coast fever. Transmission was performed with *Rhipicephalus appendiculatus* ticks or by cryopreserved tick-derived stabilates (4). The calves were bled late in the disease reaction in order to obtain samples from as high a parasitaemia as possible., Blood was also collected from an animal infected with a Nigerian strain of *T. mutans* (Katsina strain) and from an animal infected with a strain of *Theileria* sp. from Japan (Fukushima strain), assigned by some workers to *T. sergenti* (15). Blood was also obtained from animals free of blood parasites and used as control material.

The blood was collected in acid-citrate dextrose (ACD) and centrifuged at 2-4° C. The supernatant and the buffy coat were removed and the pellet was washed three times in an isotonic salt solution. The concentrated erythrocytes were then subjected to one of the following treatments:

a. Various intensities of ultrasonication followed by differential centrifugation in order to concentrate the liberated piroplasms.
b. Haemolysis, by means of various quantities of saponin or distilled water for various periods; after the removal of the saponin by washing with physiological saline or restoring of the isotonic state of the water treated samples, the material was concentrated by refrigerated centrifuging.
c. Removal of leucocytes by passing of the cells through a sulphoethyl cellulose/Sephadex G-25 column (7).
d. None.

The samples, treated as indicated under a, b, c, or d were then processed in one of the following ways:

1. They were subjected to 4 successive pressing cycles in the X-press (5).
2. They were subjected to three cycles of rapid freezing and thawing in liquid nitrogen and water of 37° C.

1,4 Dithioerythritol was added as an antioxidant to all final samples to a final concentration of 2 mM, and all samples were stored at —70° C.

Initially, only control samples and samples parasitized with *T. parva* which had been treated in the X-press were rapidly thawed and subjected to iso-electric focussing.

Iso-electric focussing was carried out on an LKB (Bromma, Sweden) 2117 Multiphor connected to an LKB 2103 Power Pack, on LKB ampholine polyacrylamide gels, at a temperature of 10° C. Plates with a pH range of 3.5-9.5, 5.5.-8.5, and 4.0-6.5 were used, with different times, electrode solutions, and settings of the power supply according to the pH range, as indicated by the manufacturer. After focussing for 90 to 150 minutes (according to the pH range of the plate), the different enzymes were visualized by specific staining, as described by Brewer (3) and Groen (6).

The zymograms were examined for the presence of the following thirteen enzymes:

lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), malate dehydrogenase (oxaloacetate-decarboxylating) (NADP) (EC 1.1.1.40), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44), glyceraldehyde-phosphate dehydrogenase (EC 1.2.1.12), glutamate dehydrogenase

(NADP) (EC 1.4.1.3), glucokinase (EC 2.7.1.2), pyruvate kinase (EC 2.7.1.40), 6-phosphofructokinase (EC 2.7.1.11), glucose phosphate isomerase (GPI) (EC 5.3.1.9), phosphoglucomutase (glucose cofactor) (EC 2.7.5.5) and acetylesterase (EC 3.1.1.6).

After the initial work with the material treated in the X-press had shown that *T. parva*-associated isoenzyme activity could be detected only for glucose phosphate isomerase (GPI) (see below), staining of the various samples, after isoelectric focussing as described above, was limited to this enzyme.

Enzymes, coenzymes, and substrates were obtained from Boehringer, Mannheim, histochemical dyes from Sigma, St. Louis.

RESULTS

In samples infected with *T. parva*, additional bands were detected only of GPI activity, not of any of the other 12 enzymes, as compared to uninfected blood.

All samples showed a series of GPI bands of host cell origin which were identical in number and location in samples from all individual cattle, whether or not infected with *Theileria*. These host GPI bands were usually fainter in ultrasonicated, haemolysed, and column-passed samples. These preliminary treatments of the samples did not offer any advantage over samples treated only in the X-press or by freeze-thawing, as neither the host bands nor the haemoglobin apparently interfered with the recognition of the parasite-associated bands, and the latter were less intense after our attempts at purification and haemolysis. Host GPI bands were not detected in plates with a pH range of 4.0-6.5, whereas parasite-associated GPI bandse were not seen in plates with a pH range of 5.5-8.5. No overlap occured between host and parasite GPI bands.

Plates with the full pH range of 3.5-9.5 were at least as suitable for recognition of the parasite bands as those with the narrow pH range of 4.0-6.5. The latter showed only the parasite bands, but fainter and more diffuse.

The simple method of destruction of the cells by freezing and thawing gave results which were at least as good as those obtained with the X-press.

Samples containing the Muguga strain of *T. parva* gave four parasite-associated GPI bands when parasitaemia was high (over 10 per cent), but samples with a low parasitaemia only gave three such bands. The Uganda strain only gave three bands, even when parasitaemia was 15 per cent, while the Boleni strain also gave no more than three fairly faint bands at its maximum parasitaemia of 5 per cent.

In a single experiment iso-electric focussing of a sample prepared from blood infected with *T. mutans* also gave parasite-associated GPI bands; these were three in number, of which at least two were in different sites from *T. parva* bands. No parasite GPI bands could, however, be demonstrated to occur in samples of blood with a parasitaemia of approximately 5 per cent of the Japanese *Theileria* sp.

Figure 1 is an example of a plate with pH range 3.5-9.5, bearing samples of three strains of *T. parva* as well as a sample of uninfected control blood.

DISCUSSION AND CONCLUSIONS

GPI was the only enzyme giving parasite-associate isoenzyme bands. We were unable to show additional bands of glyceraldehyde-phosphate dehydrogenase in infected material, contrary to Melrose and Brown (9) and Musisi (12). However, the former authors used a different technique and reported only a low parasite-associated activity of this enzyme, while Musisi (12) worked with the schizont stage in lymphoid cells.

Unlike Allsop and Wagner (1), we were unable to show additional lactate dehydrogenase bands in infected cells, but their experiments were carried out on the schizont stage.

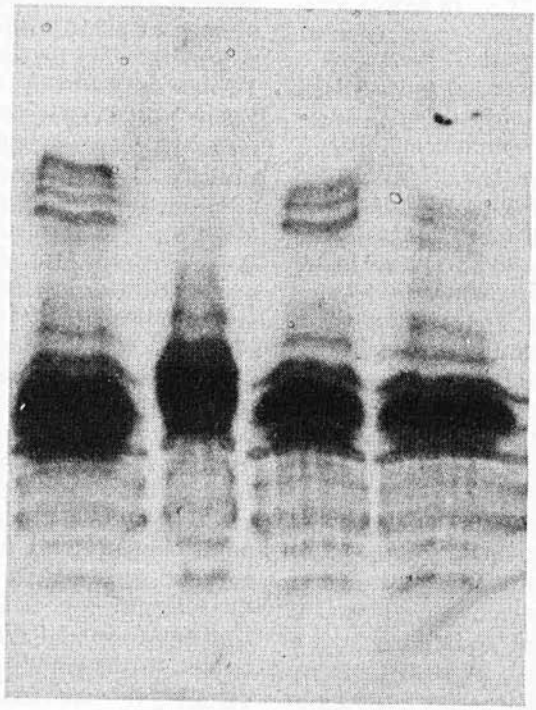
Our findings indicate that there is no individual variation between different cattle in host GPI bands, thus confirming those of Ansay (2).

Musisi (12) found differences between *T. annulata* and *T. parva* in parasite-associated bands of GPI, glyceraldehyde-phosphate dehydrogenase, and aldolase;

Melrose and Brown (9) and Melrose *et al.* (10) also found differences in isoenzyme patterns of GPI between these species. Our findings confirm species differences, as with *Theileria* sp. (Japan) no parasite-associated bands of GPI could be seen in the pH range where those of *T. parva* and *T. mutans* were present. However, it was not possible to be sure whether any parasite bands were masked by haemoglobin. The single experiment with *T. mutans,* in which parasite-associated bands of GPI occupied positions different from those of *T. parva,* should be repeated for confirmation.

We have possibly also shown strain differences, in that the Muguga strain gave one more band than either the Uganda or the Boleni strains; however, the extra band could not be detected when parasitaemia was low and no conclusion is possible with respect to the Boleni strain, where a high parasitaemia could not be obtained. It has been shown in cross-immunity tests in cattle that the three strains are immunologically different (15), so that we were unable to detect the difference between the Uganda and Boleni strains, although this difference is so great that a calf immunized with the Boleni strain by infection and treatment, followed by homologous challenge, died on subsequent challenge with the Uganda strain. Musisi (12) had no success in differentiating between strains of *T. parva* (*T. parva* and '*T. lawrencei*') by isoen-

Fig. 1. Zymogram of bovine blood.



1 = infected with *T. parva* (Muguga),
2 = uninfected.
3 = infected with *T. parva* (Uganda).
4 = infected with *T. parva* (Boleni).

Isoelectric focussing was carried out for 90 minutes on a plate with a pH range of 3.5-9.5 at a gel temperature of 10° C. 1 M H_3PO_4 was used as the anode solution, 1 M NaOH as the cathode solution. Fixed power was 30W, with a maximum voltage of 1500 V.

GPI bands were stained by incubation of the gel in 100 ml 0.1 M Tris-HCl (pH 8.0), containing 2 mM $MgCl_2$, 50 mg disodium D-fructose-6-phosphate, 10 mg NADP, 6 mg MTT, 2 mg PMS, 50 µl glucose-6-phophate dehydrogenase, 50 µl 6-phosphogluconate dehydrogenase, and 1 g agar.

zyme patterns of aldolase and glyceraldehyde-phosphate dehydrogenase but obtained some evidence of strain differences from the GPI isoenzyme patterns. Melrose *et al.* (10) established differences in the GPI isoenzyme patterns of different strains of *T. annulata*.

The use of red cell material does not appear to be very suitable for the study of intraspecific strain differences in *T. parva*: 1. The number of bands is small, at least for GPI, and their position does not appear to vary greatly between different strains. 2. Animals often die of acute theileriosis before parasitaemia is sufficiently high for the preparation of satisfactory samples. 3. Strains of the *bovis*- and *lawrencei*-types do not give rise to a sufficiently high parasitaemia. 4. To obtain each sample it is necessary to infect a calf, which almost invariably dies, making the method expensive both in animal lives and in financial resources. For these reasons we are now investigating the use of schizont material, obtained in cultures of infected lymphoblastoid cells (15). The use of red cell material is, however, so far the only means of comparing species of which the schizont cannot yet be cultured *in vitro*.

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