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Isoenzyme characterization of *Trypanosoma evansi* isolated from capybaras and dogs in Brazil

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Trypanosoma evansi was seen in blood samples taken randomly from both wild and semi-captive capybaras caught in Mato Grosso do Sul State, Brazil and in sick dogs brought into local veterinary clinics Trypanosome stocks from capybaras and dogs were significantly different in their patterns of growth in mice, while the trypanosomes from dogs were mostly dyskinetoplastic By isoenzyme electrophoresis all the trypanosomes were of the most common type of *T evansi* found elsewhere

Key words *Trypanosoma evansi*, Capybara, Isoenzyme, Dyskinetoplasty

Introduction

Semi-captive capybaras (*Hydrochaeris hydrochaeris*) are currently being bred in a pilot study for their skin and meat production, in the Pantanal region of Mato Grosso do Sul State, Brazil A large number are infected with *Trypanosoma evansi* and many become cachectic and die, some local dogs suffer in a similar manner

Behavioural and morphological studies were undertaken Since we found no description of the enzymic characterization of Brazilian *T evansi*, isoenzyme electrophoresis was also carried out in order to ascertain whether or not these trypanosomes were genetically different from *T evansi* seen in Colombia and other parts of the world (Gibson et al , 1980, 1983, Boid, 1981, 1988)

Materials and methods

Capybaras

The capybara is the largest living rodent, the adults weighing between 30 and 50 kg It is semi-aquatic and is found in Central America and extensively throughout the tropical areas of South America east of the Andes The Pantanal region of Brazil is at the heart of its range

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In the wild it lives in groups of between 2 and 25 animals (Alho et al., 1987) always near water, where it grazes on grass and aquatic vegetation In the pilot study semi-captive capybaras are kept in open enclosures of 15 ha^2, in groups of up to 50 animals

Isolates came from 2 animals caught in the wild and 4 animals kept in semi-captivity (Table 1)

Dogs

Isolates were obtained from dogs brought into veterinary clinics in Campo Grande, Mato Grosso do Sul State All three infected dogs had lived in the state for at least a year, two in the Pantanal region (Table 1)

Trypanosomes

Infections were detected by microhaematocrit centrifugation (Woo, 1969) and the trypanosomes identified in thin blood films stained with 10% Giemsa

Samples of blood from the original hosts were passaged once by intraperitoneal inoculation into mice or splenectomized rats in Brazil Samples of infected blood from these laboratory rodents, with 7 5% glycerol and 5 $\mu g\,ml^{-1}$ heparin, were cryopreserved in liquid nitrogen and sent to the U K

TABLE 1

Origin of stocks

E 18	Female juvenile capybara, in good condition, wild Isolated 11 March 1986
E 22	Male adult capybara, in good condition, wild Isolated 10 December 1986
E 60	Male juvenile capybara, very thin, semi captive Isolated 10 June 1986
E 110	Female juvenile capybara, in good condition, semi captive Isolated 12 September 1985
E 139	Female adult capybara some wasting, semi captive Isolated 9 June 1986
E 152	Female juvenile capybara, in good condition, semi captive Isolated 9 June 1986
H	Female dog, 18 months old From a farm on the edge of the Panatal region Mato Grosso do Sul State
K	Male dog, six years old When diagnosed, had just returned from a farm in the Pantanal, Mato Grosso do Sul State
R	Male dog, born and bred in Mato Grosso do Sul State

All capybara samples were isolated from animals on or near Nhumirim farm Corumba county, Mato Grosso do Sul State Brazil 18 59′ Lat 56 39′ Long

Dog samples came from animals brought into veterinary clinics in Campo Grande, Mato Grosso do Sul State during 1986

The stabilates were thawed and passaged through female mice Initially Balb/C mice were used, as in our experience they are more susceptible to infection with trypanosomes than other common laboratory strains of mice Once established, the larger Bristol Swiss mice were used to increase the yield of blood Differences in the patterns of infection between the two strains of mice were not apparent Initially all mice were immunosuppressed by irradiation at 600 rad, however this quickly proved unnecessary for the stocks from dogs

Parasitaemias were determined by examination of tail blood using the 'matching' method (Herbert and Lumsden, 1976) Initially, examination began three days after inoculation, but from experience the virulent stocks from dogs were later examined from the first day onwards

For comparison, three standard stocks were also grown in the same way, using irradiation as required These were MCAM/KE/80/KETRI 2479 (KETRI 2479) and MCAM/KE/80/KETRI 2480 (KETRI 2480) from camels in northern Kenya (Gibson et al , 1983) and MHYD/CO/73/E 8 (E 8) from a Colombian capybara (Gibson et al , 1980)

Trypanosomes were separated from the mouse blood by ion-exchange chromatography on a DEAE-cellulose column (Lanham and Godfrey, 1970) The soluble extract for enzyme electrophoresis was prepared using the method of Gibson et al (1983)

Enzyme electrophoresis

Electrophoresis was carried out on cellulose acetate plates (CAE) or on thin-layer starch gel (TSGE), depending on which gave the best resolution

The following 11 enzymes were examined by TSGE as described previously (Gibson et al , 1978, 1980) EC 2 6 1 2, alanine aminotransferase (ALAT), EC 2 6 1 1, aspartate aminotransferase (ASAT), EC 5 3 1 9, glucose phosphate isomerase (GPI), EC 2 7 5 1, phosphoglucomutase (PGM), EC 1 1 1 40, 'malic' enzyme (ME), EC 1 1 1 37, malate dehydrogenase (MDH), EC 1 1 1 42, isocitrate dehydrogenase (ICD), EC 1 1 1 103, threonine dehydrogenase (TDH), EC 3 2 2 1, nucleoside hydrolase (NH), EC 3 4 11, two peptidases substrate L-leucylglycylglycine (PEP-1), L-leucyl-L-alanine (PEP-2)

The above enzymes were also examined by CAE (Lanham et al , 1981), except for ALAT and ASAT which gave poor resolution CAE plates (76 × 94 mm) were developed using reduced volumes of TSGE recipes Developers were prepared just before electrophoresis as agar underlays 2–3 mm thick, preset in flat dishes (e g 25 ml of developer in a dish 105 mm × 105 mm square)

After electrophoresis the CAE plate was lightly blotted and applied to the preset underlay Using forceps, the plate was gently lowered onto the developer, excluding all air bubbles

This combination was incubated at 37°C until optimal resolution was attained, after which the plate was removed from the underlay and fixed by soaking for 5 min in two changes of 5% acetic acid, followed by two changes of distilled water The plate was then left to dry in air

A further five enzymes not previously used by Gibson et al (1983) were examined by CAE There were EC 1 1 1 44, phosphogluconate dehydrogenase (6PGD), EC 5 3 1 8, mannose phosphate isomerase (MPI) EC 3 4 13 9, proline dipeptidase (PEP-D),

EC 1 15 1 1, superoxide dismutase (SOD), EC 2 7 1 40, pyruvate kinase (PK) The electrophoretic conditions for these enzymes on CAE are given in Tables 2 and 3

Developer underlays for 6PGD, MPI, and PEP-D (and for SOD after modification) were similarly prepared from TSGE recipes of Le Blancq et al (1986) For

TABLE 2

Stock buffers for cellulose acetate electrophoresis (CAE)

A	0 1 M Tris/0 1 M maleic acid/0 01 M EDTA(Na_2)/0 01 M Mg $(CH_3COO)_2$ pH 7 4 (adjusted to pH 7 4 with 5 M NaOH)
B	0 008 M sodium dihydrogen orthophosphate/0 192 M disodium hydrogen orthophosphate pH 8 0
C	Tris barbital/sodium barbital pH 8 6–9 0 (Helena Labs, Beaumont, Texas, U S A , Cat No 5805, dissolve 1 sachet in 500 ml $I=0$ 1)
D	0 2 M KH_2PO_4/KOH pH 7 0 (adjusted to pH 7 0 with KOH)
E	0 25 M Tris/0 055 M boric acid/0 005 M EDTA (Na_2)/0 0075 M $Mg(CH_3COO)_2$ pH 7 6
F	0 1 M Tris/0 3 M boric acid pH 7 6
G	0 2 M Tris/0 08 M citric acid pH 6 0

TABLE 3

Electrophoretic conditions for cellulose acetate

Enzyme	Electrode and soaking buffer[a]	Volts	Time (min)
GPI	A, 1/8 dilution	250	35
ICD	B, 1/10 dilution	250	45
MDH	C 1/2 dilution	180	56
ME	C, 1/5 dilution	250	38
MPI	D, 1/12 dilution	250	40
NH	B, 3/40 dilution	250	25
PEP-D	C, 1/5 dilution	250	35
PEP-1	G, (no dilution)	200	30
PEP-2	G, (no dilution)	200	30
PGM	E, 1/3 dilution	250	35
PK	A, 1/4 dilution	250	45
SOD	F, (no dilution)	400	25
TDH	C, 1/5 dilution + 15 mM KCl	250	35
6PGD	A, 1/8 dilution	250	60

[a]10% sucrose (domestic sugar) added to buffer

SOD, *p*-iodonitrotetrazolium (INT) was substituted for NBT, care being taken to avoid undue exposure of the developer to light The underlay and CAE plate were developed by incubation in the dark at room temperature for 8–10 min, followed by exposure to uniform white light until optimum contrast of colourless bands against the red-pink background was obtained The plate was then fixed as above

PK was developed using a tetrazolium staining system* This method also stained for adenylate kinase, but weakly in comparison with the strong PK bands which were more clearly distinguishable

Results were photographed using a Polaroid MP4 Land camera and Type 667 film A green filter was used for the SOD CAE plates

Results

Observations on capybaras

The infection rate in wild capybaras was 27% ($n=22$), while in captive animals the level was 58% ($n=31$)

Observations on dogs

In the blood samples taken from dogs, all trypanosomes showed extreme dyskinetoplasty

Behaviour in mice

The *T evansi* isolated from dogs appeared initially more virulent than the stocks from capybaras On the second passage through mice after cryopreservation, the parasitaemias in 'dog' stocks rose quickly, in two or three days to a lethal peak of $\geqslant 10^{8\cdot7}$ trypanosomes per ml Trypanosomes were seen the day after inoculation from cryopreservation in stock K With stocks H and R blood films were not made until day 3, by which time the infections had reached $10^{7\cdot5}$ and $10^{8\cdot7}$ trypanosomes per ml respectively

Five of the 'capybara' stocks multiplied more slowly From cryopreservation E 139 took two passages over 37 days to show any trypanosomes, then a further passage and 6 days to reach a level suitable for harvesting sufficient numbers of organisms E 110 took eight days to show any trypanosomes and a further three passages over 24 days to reach the same level. initial parasitaemias relapsed over 4 to 10 days E 152 took 5 days to show any trypanosomes and a further two passages over 7 days to reach harvestable levels

Stocks E 22 and E 60 from capybaras proved difficult to grow in Brazil and failed to grow at all in mice after cryopreservation, although motile trypanosomes were seen in the stabilates after thawing

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*PK developer underlay Final concentrations, Tris-HCl, pH 8 0, 100 mM, $MgCl_2$ 10 mM, KCl 60 mM ADP 2 mM, NADP 1 mM, phosphoenol pyruvate 1 5 mM, glucose 10 mM, MTT 0 6 mM (or 0 25 mg ml^{-1}), PMS 0 16 mM (or 0 05 mg ml^{-1}), hexokinase 1 0 IU ml^{-1}, glucose-6-phosphate dehydrogenase 1 0 IU ml^{-1}, agar 0 6%

However, stock E 18 produced a lethal peak in less than 10 days on the first passage after cryopreservation, trypanosomes were seen in the blood after 3 days The behaviour of this stock falls between the others from capybaras and those from dogs

The number of days required for the stocks from capybaras and from dogs to reach a high level ($10^{8\cdot7}$ trypanosomes per ml mouse blood), was significantly different ($P=0\ 0138$, DF = 34, $n=9$, Mann-Whitney 'U' test)

The standard stocks varied E 8 and KETRI 2480 produced lethal peaks in two days after only one passage through mice after inoculation from cryopreservation, KETRI 2479 produced a relapsing infection, lethal on the second peak at around day 10 However, before cryopreservation all had been passaged many times through mice

Morphology

Stained thin blood films confirmed that all the trypanosomes were members of the subgenus *Trypanozoon* according to the description by Hoare (1972) Trypanosomes from capybaras showed dyskinetoplasty in 30% of individuals, the remainder having easily visible kinetoplasts By contrast the trypanosomes from dogs showed up to 100% dyskinetoplastic individuals

Enzyme electrophoresis

CAE was used for most of the enzymes as preparation and running times are quicker However, the definition of the patterns for some enzymes was not always as good on CAE as on TSGE, for example some double bands on TSGE would appear on CAE plates as a large, diffuse single band TSGE was carried out on the poorer staining enzymes to allow the cross checking of our results with those previously published ALAT and ASAT were found to give poor results with CAE and so were examined only by TSGE The newly introduced enzymes were examined only by CAE as the bands obtained were of good resolution and showed no differences between stocks

With 15 of the enzymes examined in the 7 stocks, no variations in band patterns were found (Table 4), the bands obtained for MPI were too weak to produce a conclusive result

The profiles of 14 enzymes matched exactly those for the two standards, E 8 from a Colombian capybara and KETRI 2480 from a Kenya camel For PEP-1, a highly polymorphic enzyme, the patterns of the sample stocks matched the E 8 standard but not KETRI 2480

The standards E 8 and KETRI 2480 were previously analysed with a smaller range of enzymes by Gibson et al (1980, 1983) and were classed as Group 1 (zymodeme 58 in the WHO reference collection, D G Godfrey, personal communication) The additional enzymes examined gave exactly the same patterns in both the newly examined isolates and the standards Thus *T evansi* from the Pantanal is of the type commonly found throughout Africa and elsewhere

Some differences from KETRI 2479 were found The result from SOD confirms that KETRI 2479 differs from other typical stocks of *T evansi* (Gibson et al , 1983)

TABLE 4

Enzyme patterns

Stock	ALAT	ASAT	GPI	ICD	MDH	ME	NH	PEP-1	PEP-2
KETRI 2480	2	1	1	2	1	2	1	2	4
KETRI 2479	2	3	1	2	1	24	1	1	4
E8	2	1	1	2	1	2	1	6	4
All new stocks	2	1	1	2	1	2	1	6	4

Stock	PGM	TDH	6PGD	PEP-D	PK	SOD
KETRI 2480	2	1	1	1	1	1
KETRI 2479	3	1	1	1	1	2
E8	2	1	1	1	1	1
All new stocks	2	1	1	1	1	1

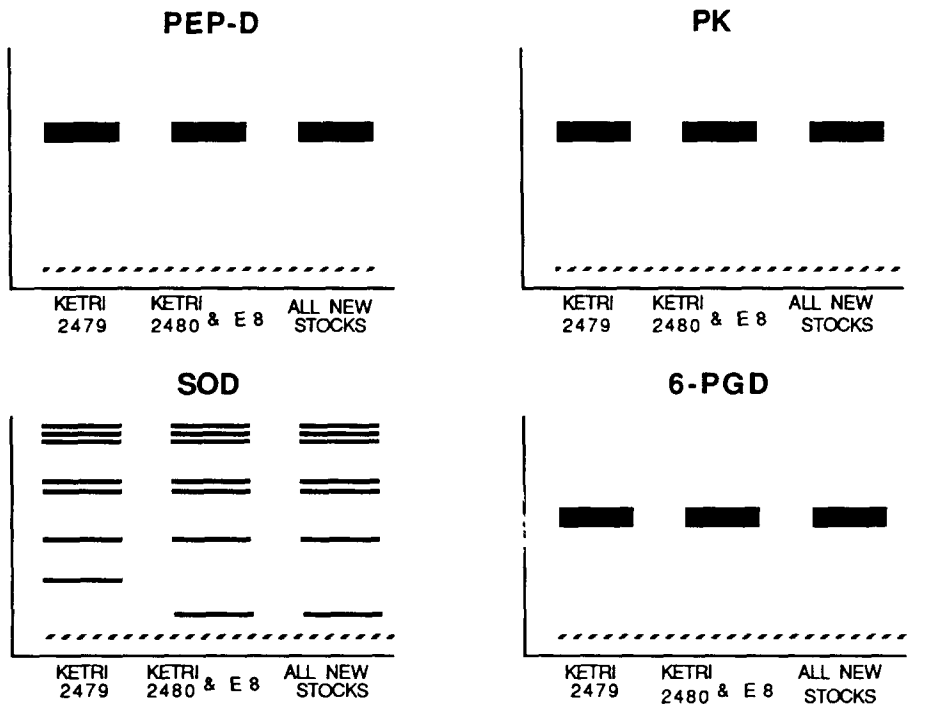

Fig 1 The banding patterns obtained with the enzymes PEP-D, PK, SOD and 6PGD All stocks show pattern 1 for each enzyme, except for KETRI 2479, which shows pattern 2 for SOD The dotted line shows the origin, where the lysates were applied

Discussion

T evansi has become widespread in South and Central America since its introduction to the continent, probably in the 16th century from Spain (Hoare, 1965) The disease

it causes (Mal de Cadeiras) first appeared in the Mato Grosso in 1850 (Miranda, 1904) Today *T evansi* is found in many species of wild and domestic animals throughout this region and the rest of Brazil (see Shaw, 1977)

The apparent behavioural differences among the Brazilian stocks of *T evansi* were not supported by genetic differences All were similar to other typical *T evansi* described from around the world (Gibson et al , 1980, 1983, Boid, 1981) This observation held true even after using an extended range of enzymes, which also demonstrated further the distinctiveness of the trypanosomes exemplified by KETRI 2479, found in Kenyan camels (Gibson et al , 1983)

Gibson et al (1980) reported no enzymic differences in *T evansi* isolates from capybaras in Colombia Boid (1981) found some enzymic differences in Sudanese camel isolates which were not found in our investigation However, he found (Boid, 1985) considerable variation between *T evansi* from Java and Indonesia, with the Indonesian stocks forming a more homogeneous group

Boid (1988) found, in 15 stocks from Sudan, intraspecific variation in ME, PEP-1, ALAT, G6PDH, PEP-4 and HK, these last three enzymes were not used by us Boid pointed out that the different variants recorded in Sudan and Kenya indicate that different sets of selection pressures are operating In addition more variants may be found in these areas as they are close to the tsetse belt and some limited gene flow from *T b brucei* populations may still be possible, Gibson et al (1983) record a tsetse transmissible stock in a Kenyan camel, in a herd also harbouring typical *T evansi* Populations in other continents may tend to become more homogeneous

Despite genetic similarity, fresh isolates from capybaras were difficult to grow in rodents while isolates from dogs grew rapidly Monzon et al (1986) also reported large differences in virulence to mice between stocks of *T equinum*, which is probably a dyskinetoplastic form of *T evansi* (Hoare, 1972)

There was a high degree of dyskinetoplasty in the dog samples In the past dyskinetoplastic strains of *T evansi* have been found in nature (Hoare, 1949), others have evolved spontaneously in laboratory passaged stocks (Hoare and Bennett, 1939), while totally dyskinetoplastic stocks have been produced by treating infected animals with organic dyestuffs (Hoare and Bennett, 1937) and with Berenil (Killick-Kendrick, 1964) Howsoever it occured in our isolates from dogs, its persistence in these hosts suggests a transmission cycle separate from that involving both the wild and semi-captive capybaras

Infections with *T evansi* appear confined to the capybaras and dogs in this part of the Pantanal In the same area, 203 cattle and 52 horses were examined over the same period using microhaematocrit centrifugation (Woo, 1969) and inoculation into laboratory mice and rats, but all were negative Thus at present the capybara appears not to be a reservoir of the disease for other domestic stock, although this position could change in the future In other areas of South America *T evansi* is found in both domestic livestock and wild animals living nearby, including capybara (Migone, 1910, Morales et al , 1976)

Since many captive capybaras die, the method of transmission must be discovered if effective control for commercial development is to be achieved The vectors may be flies of the genera *Tabanus* and *Stomoxys* (Hoare, 1965) or perhaps reduviid bugs (Manz, 1985) Another possible vector, also acting as a reservoir, is the vampire bat (Hoare, 1965) It is also unknown why the infection rate is higher in the semi-captive

capybaras than in the wild ones The greater density of semi-captive animals may offer better opportunities for transmission

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