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Dose and stage dependency for the development of local skin reactions caused by *Trypanosoma congolense* in goats

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Summary

Intradermal inoculation of metacyclic forms of *Trypanosoma congolense* propagated in vitro caused skin reactions in goats similar to the local skin reaction (chancre) induced by the bite of an infected tsetse fly. The onset, size and duration of these local skin reactions were dose-dependent. Whereas one cultured metacyclic *T. congolense* was sufficient to cause a local skin reaction in a goat, over 10^7 bloodstream forms of *T. congolense* were necessary to elicit a detectable skin reaction and while *T. congolense* parasites present in lymph did not cause local skin reactions, trypanosomes collected from oedematous fluid of the chancre did. – Using non-dividing irradiated bloodstream forms it was estimated that 10^8 *T. congolense* were required to induce a detectable local skin reaction. – Intradermal needle inoculation of procyclic forms (uncoated trypomastigotes) of *T. congolense* propagated in vitro induced an intense inflammatory response which was similar to that found in the early phases of the reaction elicited by metacyclic trypanosomes. This suggests that the uncoated trypomastigotes which are known to be present in the saliva of infected tsetse may play a role in the initial development of the chancre. – The data obtained for the local skin reaction suggest the presence of an intracutaneous dividing stage of *T. congolense* which is intermediate between the metacyclic and bloodstream forms.

Key words: *Trypanosoma congolense;* goat; local skin reaction; chancre.

Introduction

When a trypanosome-infected tsetse fly bites an animal to obtain a bloodmeal, it deposits saliva containing anticoagulants (Lester and Lloyd, 1928) and

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a mixture of infective metacyclic trypanosomes and immature uncoated proventricular and epimastigote forms in the skin (Otieno and Darji, 1979). Several days later and preceding the appearance of parasitaemia, a localized skin reaction, commonly called a chancre, develops at the bite site. While chancres can be induced by all three species *(Trypanosoma brucei, T. congolense* and *T. vivax)* of tsetse-transmitted trypanosomes that infect domestic livestock, the size of the reaction is trypanosome species dependent in that the lesions caused by *T. brucei* are more severe than those produced by *T. congolense* or by *T. vivax* (Emery et al., 1980; Akol and Murray, 1983). Furthermore, there are certain stocks of *T. congolense* that do not induce any detectable skin reactions although they cause infection (Luckins and Gray, 1979).

Not only does the size of the chancre depend on the species of trypanosome but it is also affected by the species of host. The skin reactions to infective tsetse bites are significantly smaller in wild Bovidae than they are in domestic livestock (Murray et al., 1981). As these wild Bovidae were found to be more resistant to trypanosomiasis than domestic livestock (Murray et al., 1982), it is possible that the initial skin reaction plays a significant role in host susceptibility and that the small reactions observed are the first indication of a higher degree of resistance.

As an initial step in evaluating the role of the skin in host susceptibility, a series of experiments were carried out to identify the influences on size and severity of the chancre reaction caused by *T. congolense.* The variables investigated included the number of trypanosomes inoculated into the skin and the contribution that various developmental forms of the parasite might make. As it is now known that hypersensitivity reactions, possibly to tsetse saliva, can develop in goats (Nash, 1970) and cattle (Akol and Murray, 1983) after repeated tsetse bites, the current studies were carried out using parasites derived from cultures or mammalian blood and administered by intradermal syringe inoculation to exclude any possible effects caused by tsetse saliva.

Materials and Methods

Experimental animals

Adult castrated male East African/Galla cross-bred goats weighing 20 to 40 kg were obtained from an area of Kenya which was free of trypanosomiasis. All goats were drenched with anthelmintics and a coccidiostat (5 mg/kg body weight of Panacur 2.5%, Hoechst, Frankfurt, W. Germany; 7.5 mg/kg body weight of Ranide, Merck Sharp and Dohme [MSD], Rahway, USA; 50 mg powder of Amprol 20% [MSD] per kg body weight over 5 days) 3 weeks before being housed in flyproof isolation units, where they were given free access to hay and water with a daily ration of concentrates (Belfast Millers, Nairobi) of about 300 g per animal. All goats were negative for anti-trypanosomal antibodies by the indirect immunofluorescence test performed on acetone-fixed trypanosomes (Wilson, 1969).

Inbred AJ and Balb/C mice as well as Wistar rats were maintained in the small-animal unit at ILRAD on a commercial pelleted ration.

Trypanosomes

Stabilates. T. congolense IL 1180 is a cloned derivative of STIB 212, which is a stabilate prepared after a single passage in rats of an isolate made from a lion *(Panthera leo)* in the Serengeti region of Tanzania in 1971 (Geigy and Kauffmann, 1973). STIB 212 was passaged twice in mice, preserved as stabilate IL 20E-8 and then cloned twice. After the first cloning it was preserved as stabilate IL 933 which was cloned again and preserved as stabilate IL 968. This last stabilate was passaged once in mice and preserved as stabilate IL 1180. The *T. congolense* procyclic form population was prepared from the bloodstream forms of IL 687, an uncloned stabilate derived as described by Moloo (1981) from STIB 212.

Isolation of trypanosomes. Stabilated populations of *T. congolense* were expanded in irradiated (500 to 600 rads) mice or rats. During the first peak of parasitaemia, the rodents were exsanguinated and the trypanosomes were isolated on diethylaminoethyl (DE 52) cellulose anion-exchange columns (Whatman Chemical Separation Ltd., Great Britain) according to the method described by Lanham and Godfrey (1970). The concentration of the trypanosomes in the column eluate was calculated using a Neubauer haemocytometer.

Irradiation of trypanosomes. Trypanosomes suspended in phosphate saline glucose (PSG), pH 8.0, containing 20% (w/v) foetal bovine serum (FBS), were exposed to 60,000 rads of gamma-irradiation delivered by a caesium source (^{137}Cs). Following irradiation the concentration of live trypanosomes was calculated.

In vitro cultivation. Metacyclic trypanosomes of the *T. congolense* clone (IL 1180) were propagated in vitro at 28°C in the presence of purified bovine dermal collagen (Vitrogen™, Collagen Co., Palo Alto, California) following the procedure described by Gray et al. (1981) with some modifications (Hirumi et al., 1982). The cultures were initiated by placing the proboscides of infected tsetse flies *(Glossina morsitans centralis)* in HEPES-(25 mM-)buffered Eagle's minimum essential medium with Earle's balanced salt solution (Gibco, Paisley, Scotland) and 20% (v/v) heat-inactivated foetal bovine serum (Flow Laboratories, Irvine, Scotland). The cultures contained long slender promastigotes, epimastigotes and metacyclic trypanosomes which became infective to mammals (mice, goats and cattle) from day 40 onwards in culture. Metacyclic forms were separated from the other forms by passing through DE 52 cellulose columns (Lanham and Godfrey, 1970) and collected by low speed centrifugation at 1000 *g* for 20 min.

Non-infective procyclic form (uncoated form) cultures were initiated by placing bloodstream forms obtained from infected mouse blood in the absence of the bovine dermal collagen but in the presence of bovine fibroblast feeder layers in the same medium. The cultures were maintained at 28°C over 30 days by changing the medium (½–¾ volume) every other day and collected by slow speed centrifugation.

Tsetse flies

Glossina m. centralis were bred and reared at ILRAD at 25°C and 70% relative humidity. Teneral tsetse were fed on the flanks of goats infected with *T. congolense* IL 1180 during the first wave of parasitaemia. The tsetse were maintained by feeding them daily except sundays, on the ears of uninfected rabbits. On day 30 post-emergence the tsetse were allowed to probe on warm slides at 37°C and the saliva was examined for metacyclic trypanosomes at 320× magnification. Once proven infected, five single tsetse in tubes of 2.5 by 7.5 cm with netting at one end and a cork on the other were allowed to feed on the cleaned and shaved flank of each experimental goat.

Experimental design

A series of experiments employing ten fold dilutions in PSG, pH 8.0, was carried out in goats using different developmental stages of *T. congolense.* Metacyclic forms propagated in vitro were tested in 4 goats (Table 1), while bloodstream forms of the same clone (Table 2) were investigated in 3 goats. Similarly, irradiated bloodstream forms were tested in 5 goats while procyclic forms of *T. congolense* IL 687 propagated in vitro were inoculated into 3 goats. In most experiments, each dilution of trypanosomes was tested in duplicate in each animal, but when biopsy material was

required up to 6 inoculation sites per dilution were employed in a single goat. Trypanosome suspensions in 0.1 ml PSG, pH 8.0, were inoculated into the skin of the flank of goats using a disposable tuberculin syringe and a 25 gauge (0.50×16 mm) needle. Each inoculation site was marked with a felt-tipped pen.

To serve as a control in each experiment, one goat was inoculated intradermally with similar numbers of horse red blood cells collected in heparinized containers and diluted in PSG, pH 8.0. In addition, 1 goat was inoculated intradermally with 0.1 ml aliquots of PSG, pH 8.0, at several sites.

Studies were also carried out to evaluate the capacity of *T. congolense* parasites within the chancre to induce skin reactions. During the height of the local skin reaction, 9 days after a goat had been infected with tsetse-transmitted *T. congolense* IL 1180, oedematous fluid was collected from 2 chancres by squeezing the lesions by hand after they had been pricked with a lancet. From each chancre the fluid was drawn through a 23 gauge (0.60×25 mm) needle attached to a 1 ml syringe containing 0.2 ml PSG, pH 8.0. Each syringe was inverted several times before approximately 0.1 ml of PSG plus chancre fluid was inoculated intradermally into 4 sites (2 for each chancre) on the shaven flank of an uninfected goat. Each site was marked with a felt-tipped pen. In a second experiment using 3 goats, oedematous fluid of the chancre was collected in a similar way from a goat bitten at several spots by tsetse flies infected with *T. congolense* IL 1180. The fluid collected from 4 chancres was mixed with 0.4 ml of PSG and half of it was inoculated intradermally at 2 sites in one goat, while the other half was passed through a sterile filter with 0.22 μm pore size (Millex, Millipore Corporation, Bedford, Massachusetts) and the filtrate inoculated intradermally at 2 sites in a second goat. The third goat was inoculated intradermally with 0.1 ml of PSG, pH 8.0, at 4 sites and served as a control.

To investigate the ability of *T. congolense* forms present in lymph to induce skin reactions, the efferent lymphatic vessel draining the skin site bitten by tsetse infected with *T. congolense* IL 1180 was cannulated as described for goats by Dwinger (1985). Fresh lymph was collected daily following tsetse-transmitted infection and inoculated intradermally in aliquots of 0.1 ml at 4 marked skin sites on the shaven flank of a goat. Each day a different goat was inoculated over a period of 9 days.

Sampling techniques

Skin thickness at the site of injection was measured using vernier callipers prior to inoculation and, thereafter, every day for 15 days. The normal skin thickness in the East African/Galla goat is 0.3–0.4 cm.

Two ml blood samples were collected from the jugular vein into EDTA-coated vacutainer tubes twice weekly for 3 weeks. Packed red cell volume percent (PCV) was measured and the buffy coat was examined for trypanosomes using phase-contrast microscopy (Murray et al., 1977).

Skin biopsies were taken after sedating the animal with 0.3 to 0.5 ml of 2% xylazine hydrochloride (Rompun, Bayer, Leverkusen, W. Germany) given by intramuscular inoculation. The skin was shaved and disinfected and a sample of skin containing the inoculation site measuring approximately 2 cm × 0.5 cm and including epidermis, dermis and panniculus carnosus muscle (cutaneous muscle), was excised. The sample was immediately placed in formol-sublimate fixative: a mixture of 900 ml of 50% saturated aqueous mercuric chloride and 100 ml of formalin (Drury and Wallington, 1980). The wound was closed with silk mattress sutures. For histological examination, skin sections 5 μm thick were cut from paraffin-embedded blocks and stained with Mayer's haematoxylin and eosin and, in some cases, with Giemsa or carbol chromatrope (Cook, 1974).

Statistical analysis

The results of the measurements of several skin reactions in 3 to 4 goats are presented as the arithmetic mean ($\bar{x}$) ± the standard deviation (S.D.).

Results

Intradermal inoculation of metacyclic forms of T. congolense propagated in vitro

Intradermal inoculation of metacyclic forms of *T. congolense* propagated in vitro induced local skin reactions that were macroscopically as well as microscopically similar to those elicited by metacyclic *T. congolense* induced by a tsetse fly bite. Macroscopically the reaction showed at its peak all the characteristics of a local inflammatory response: swelling, redness, heat and painful to the animal, with a two fold increase in skin thickness. Histologically, the reaction was characterized by infiltration of neutrophils and lymphocytes in papillary dermis and around vascular trunks in reticular dermis and hypodermis. During the peak of the reaction the majority of the cells were lymphocytes (8–12 μm diameter), lymphoblasts (>12 μm diameter) and macrophages with some plasma cells present, but neutrophils could still be found. Trypanosomes were readily seen, especially between collagen bundles in the reticular dermis and inside lymph vessels in the hypodermis at 5 and 6 days after inoculation. The onset, size and duration of the local skin reaction were to a large degree dose-dependent (Table 1). A single metacyclic trypanosome inoculated into the skin caused a skin reaction 7 days later, in 3 of the 8 inoculated sites; this reaction lasted 32 h as assessed by a skin thickness greater than 0.5 cm. On the other hand, inoculation of 10^5 metacyclic trypanosomes gave a detectable reaction 5 days later and by 6 days after inoculation reached an average maximal size of 0.77 cm. In this case, all 24 inoculated sites resulted in local skin reactions which were larger than 0.50 cm over a period of 72 h (Table 1). All 4 goats became parasitaemic at an average of 10 days after inoculation.

Intradermal inoculation of T. congolense transferred with oedematous fluid of the chancre

Two of the 4 injection sites developed skin reactions that were first detected on days 8 and 10 after inoculation. By day 10 both sites were macroscopically indistinguishable from a chancre reaction caused by the bite of an infected tsetse. The skin thickness increased up to an average thickness of 1.02 cm on day 13 and thereafter decreased. Trypanosomes were first seen in the blood of the goat on day 12 after the intradermal inoculation. When oedematous fluid of the chancre, collected from a second goat, was passed through a filter of pore size 0.22 μm and inoculated intradermally into a goat, no detectable skin reaction developed nor did the goat become infected. On the other hand, unfiltered chancre fluid, induced a skin reaction at one of the 2 sites inoculated. The skin reaction was first detected 7 days after inoculation, reaching a maximal size of 0.82 cm 3 days later. The goat became parasitaemic 12 days after the intradermal injection.

Table 1. Kinetics of development of skin reactions in goats* induced by intradermal inoculation of metacyclic forms of *Trypanosoma congolense* propagated in vitro

Number of trypanosomes inoculated	Days to detection of reaction	Maximal size of reaction (cm)		Days to maximal size of reaction	Duration of reaction >0.5 cm (hours)	Number of inoculation sites	Number of reactions
		$\bar{x}$	S.D.				
1	7.0	0.53	0.07	7.0	32	8	3
10	6.6	0.56	0.05	7.0	36	8	6
10^2	6.0	0.55	0.08	6.8	27	8	7
10^3	5.7	0.58	0.07	6.8	48	8	7
10^4	5.1	0.62	0.05	6.5	51	8	8
10^5	4.9	0.77	0.13	6.2	72	24	24

* A total of 4 goats were used. Each animal received all dilutions in duplicate, except the highest concentration of parasites which was inoculated at 6 sites in each goat.

$\bar{x}$ = mean S.D. = standard deviation

Table 2. Kinetics of development of skin reactions in goats* induced by intradermal inoculation of bloodstream forms of *Trypanosoma congolense*

Number of trypanosomes inoculated	Hours to detection of reaction	Maximal size of reaction (cm)		Days to maximal size of reaction	Duration of reaction >0.5 cm (days)	Number of inoculation sites	Number of reactions
		$\bar{x}$	S.D.				
10^2	–					4	–
10^3	–					4	–
10^4	–					4	–
10^5	–					4	–
10^6	48	0.55	–	4	3	4	1
10^7	31	0.66	0.04	4	3	16	3

* A total of 3 goats were used. Two of the animals each received all dilutions in duplicate, while the third goat was inoculated at 12 sites with the highest concentration of parasites only.

$\bar{x}$ = mean S.D. = standard deviation – = no reaction

Intradermal inoculation of T. congolense transferred with lymph

No detectable skin reactions were seen following intradermal inoculation of lymph collected during the first 9 days after tsetse challenge of a goat, although lymph collected from 4 days onwards caused infection with an average incubation period of 8 days after inoculation of the goats. Lymph at 4 days after infection contained very few parasites, but by 8 days about 5×10^5 trypanosomes per ml were present.

Intradermal inoculation of bloodstream forms of T. congolense

Skin reactions were induced by bloodstream forms of *T. congolense* IL 1180 only when large numbers of parasites were used. Three of the 16 sites inoculated with 10^7 trypanosomes, showed 31 h later distinct reactions similar in appearance to a chancre, reaching a maximum thickness 4 days after inoculation (Table 2). One of the 4 inoculations of 10^6 trypanosomes gave a smaller nodule, while none of the lower dilutions gave any detectable skin reaction (Table 2).

The 3 goats developed patent infections an average of 4 days after inoculation of bloodstream forms.

Intradermal inoculation of irradiated bloodstream forms of T. congolense

In order to assess the finite number of trypanosomes required to cause a detectable skin reaction, bloodstream forms of *T. congolense* were irradiated to prevent further multiplication without killing the parasites. At least 10^8 irradiated parasites were needed to elicit a small nodule of 0.44 cm thickness (a 10–50 percent increase over normal skin thickness) 24 h after inoculation, which lasted only 1 to 3 days. None of the goats became infected.

Histologically, the reaction was similar to the early stage of the chancre showing oedema, disruption of collagen fibres, proliferation of vasoformative cells and infiltration of mostly neutrophils and some mononuclear cells. Trypanosomes were readily observed in the hypodermis at 4 h after inoculation.

Intradermal inoculation of irradiated metacyclic forms of T. congolense propagated in vitro

Metacyclic trypanosomes of *T. congolense* IL 1180 cultured in vitro were also irradiated and inoculated intradermally into 3 goats (with a maximum of 10^5 metacyclic forms per inoculation site) but no reaction could be elicited nor did the goats become infected.

Intradermal inoculation of procyclic forms of T. congolense propagated in vitro

To assess the possible role that procyclic forms of *T. congolense* might play in initiating chancres, a range of doses (10^3–10^8) of uncoated parasites propagated in vitro were inoculated intradermally in goats. The inoculation of 10^8

parasites lacking a surface coat caused, by the following day, a small skin nodule of 0.58 cm in thickness which persisted for 1 to 2 days.

The goats inoculated with procyclic forms did not become infected, nor did they show any evidence of immunity. When challenged intradermally with bloodstream forms of a closely related *T. congolense* stock, the animals became parasitaemic.

Intradermal inoculation of horse red blood cells (HRBC)

Control goats inoculated with ten fold dilutions of HRBC showed no detectable skin reactions. Histologically there was a slight cellular infiltration in all skin layers consisting of neutrophils, lymphocytes and plasma cells during the first 3 days after injection of 10^8 HRBC per inoculation site. In the same way, intradermal inoculation of PSG induced no skin reaction.

Discussion

The development of the local skin reaction in goats has been shown to be influenced by the number of parasites inoculated, the developmental stage of the parasites employed, but not to require the presence of tsetse saliva. Major differences in biological activity were found to exist between procyclic, metacyclic and intracutaneous forms as well as trypanosomes recovered from lymph and blood in terms of their capacity to induce skin reactions.

Intradermal inoculation of metacyclic forms of *T. congolense* propagated in vitro elicited local skin reactions in goats similar to those induced by tsetse flies. This observation has also been reported in cattle (Akol, 1985) and rabbits (Luckins et al., 1981). The present study showed that even a single metacyclic trypanosome could cause a local skin reaction. Although a chancre is not a prerequisite for infection in the case of *T. congolense,* the appearance of a chancre is indicative of ensuing infection (Morrison et al., 1985). Therefore one can assume that in our experiments a single metacyclic form of *T. congolense* can cause infection in goats.

On the other hand, Fairbairn and Burtt (1946) found that at least 300–450 metacyclic *T. rhodesiense* were needed to initiate infection in man with or without the occurrence of a local skin reaction. However, Harley and Wilson (1968) assumed that a single metacyclic trypanosome of *T. congolense* could infect a mouse.

While one metacyclic of *T. congolense* can cause a local skin reaction, over one million bloodstream forms were required to induce even a small nodule in the skin. The poor chancre development by bloodstream forms might be due to the fact that the parasites do not stay at the site of inoculation long enough to release inflammatory molecules, since trypanosomes do have the capacity to activate complement (Nielsen and Sheppard, 1977) and are known to contain proteolytic enzymes (Tizard et al., 1978). No skin reactions have been reported

to occur after intradermal inoculation of *T. congolense* bloodstream forms in goats (Emery et al., 1980), cattle (Bolton, 1965) or rabbits (Awad et al., 1969), although this may have been due to the lower number of parasites inoculated. However, it might also have been due to the different strain of *T. congolense* used, since it is known that some strains of *T. congolense* do not produce chancres in rabbits (Luckins and Gray, 1979) following tsetse transmission.

Inoculation of different numbers of metacyclic and bloodstream forms of *T. congolense* has established that the chancre reaction is dose-dependent in terms of onset, size and duration. The dose-dependency of the local skin reaction has been previously recognized in goats (Emery et al., 1980), rabbits (Willett and Gordon, 1957) and humans (Willett, 1956) following inoculation of bloodstream forms of the *Trypanozoon* subgenus. Development of chancres in rabbits following intradermal inoculation of metacyclic forms of *T. congolense* propagated in vitro was also found to be dose-dependent (Luckins et al., 1981).

Bloodstream forms did not elicit local skin reactions unless inoculated in very high numbers, while metacyclic forms did cause chancres, but were deposited by the tsetse fly in small numbers in the skin (Otieno and Darji, 1979; Thévenaz and Hecker, 1980) and were reported to be non-dividing forms (Shapiro et al., 1984). Our results of the transfer from one goat to another of a few drops of oedematous fluid of the chancre causing a local skin reaction 7 days later, suggest the existence of an intracutaneous dividing stage that causes a local skin reaction and is intermediate between the metacyclic and the bloodstream form. Once in the lymph vessels (or in the bloodstream) the parasites lose their capability to induce chancres, since the inoculation into goats of lymph draining the skin area bitten by tsetse flies caused infection without the formation of a chancre. Thus, the lymph probably contained trypanosomes which had already differentiated to bloodstream forms. Camera lucida drawings and measurements of the trypanosomes found in thin films of chancre fluid have shown that these parasites are longer than metacyclic or bloodstream forms (Roberts et al., 1969). Moreover, parasites observed in the local skin reaction by electron microscope have exhibited morphological features different from metacyclic and bloodstream forms (Dwinger et al., in press). The ability to elicit a chancre reaction by the transfer of unfiltered oedematous fluid of the chancre from one animal to another has also been achieved in rabbits (Luckins, personal communication).

The results with irradiated bloodstream forms indicated that the presence of up to 10^8 *T. congolense* was needed to cause a skin reaction in goats. However, no skin reactions could be elicited by inoculating irradiated metacyclic forms of *T. congolense* propagated in vitro. It is however quite possible that if higher numbers of irradiated metacyclic forms could have been inoculated, similar results would have been obtained to those following the inoculation of irradiated *T. congolense* bloodstream forms.

The intense inflammatory reaction caused by the intradermal inoculation

of uncoated trypanosomes propagated in vitro suggests that the uncoated forms deposited in the skin by the infected tsetse fly (Otieno and Darji, 1979) may also contribute to the formation of the chancre. Recently it has been reported that uncoated *T. congolense* forms activate complement by the alternative pathway (Ferrante and Allison, 1983). Complement activation may generate mediators of inflammation and thus contribute to the inflammatory response, which is an important part of the chancre reaction. The inflammatory basis of the chancre is indicated by the fact that it is possible to abrogate the local skin reaction in rabbits by systemic treatment with hydrocortisone (Seed et al., 1972).

In conclusion, while non-multiplying metacyclic trypanosomes are thought to differentiate to multiplying bloodstream forms, the above studies suggest that at least in the case of *T. congolense* a stage exists in the skin which is intermediate between the metacyclic and bloodstream forms. Furthermore, the fact that the size and intensity of the local skin reaction induced by *T. congolense* is dependent on the number of trypanosomes inoculated into the skin might be an important observation with respect to host resistance to trypanosomiasis. The smaller skin reactions to the bite of infected tsetse exhibited by trypanotolerant domestic and wild Bovidae (Murray et al., 1981; Akol, 1985), might possibly reflect that fewer numbers of parasites become established in the skin of these animals.

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