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PROTECTIVE IMMUNITY AGAINST *TRYPANOSOMA DANILEWSKYI* STRAIN FCC 1 IN JUVENILE CARP (*CYPRINUS CARPIO*)

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Abstract: Juvenile carps were used in the infection of *Trypanosoma danilewskyi* strain FCc 1 in the laboratory experiments to asses the protective immunity developed in fish after the clearance of trypanosomes from their blood. The fish recovered from infection were protected from re-infection as well as challenges of infection for at least until 195 days p.i. due to the presence of protective immunity. Fish can be passively immunized against *Trypanosoma danilewskyi* strain FCc 1 infection by injecting immune plasma from recovered fish. Moreover, the immune plasma was capable of lysing live trypanosomes at optimal temperatures. Fish can also be immunized by injecting lysed trypanosomes in immune plasma at 10°C and 20°C.

Key words: *Trypanosoma danilewskyi*, immunopathogenicity, trypanocidal antibodies, IgG, immune serum.

INTRODUCTION

Trypanosoma danilewskyi is a natural haemoflagellate parasite of common carp, *Cyprinus carpio* (Woo, 1981 a). Acquisition of protective immunity against this parasite has been documented by Barrow (1955) as the presence of trypanocidal antibodies in the blood of infected fish and the lytic ability of these antibodies was potent until 3-4 weeks after the disappearance of trypanosomes from the blood. Lom (1973) found 80 % mortality in experimentally infected goldfish, *Carassius auratus*, but he failed to re-infect the fish that recovered from initial infection although no trypanosomes were found in their blood, indicating the presence of protective immunity developed by the fish.

Trypanosomes and trypanoplasms induce a humoral immune response in variety of freshwater and marine fishes (Woo, 1981 a; Sypek & Burreson, 1983; Burreson & Frizzell, 1986; Jones & Woo, 1987 and Islam, & Woo, 1991) and cellular immune response in carp (Overath *et al.*, 1999 and Woo, 1999). During the infection of *Cryptobia salmositica* and *Cryptobia bullocki* in summer flounder, *Paralichthys dentatus* (L), peritoneal macrophages were found indicating the importance of cellular immunity (Sypek & Burreson, 1983 and Woo, 1999).

This study was carried out to investigate the development of protective immunity and resistance during the infection of *T. danilewskyi* strain FCc 1 in experimentally infected juvenile carp.

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MATERIALS AND METHODS

Four months old juvenile carps measuring 5-6 cm in length were obtained from Experimental Fish Facility, Zodiac Department, Agricultural University, Wageningen, The Netherlands and maintained in glass aquaria at 20°C for two weeks prior to infection. The *T. danilewskyi* strain FCc 1 was the same as used in previous studies (Ahmed, 1994; Ahmed *et al.*, 2001). All groups were fed an equivalent of 3 % of the body weight daily. The whole study was performed in 4 experiments.

In experiment 1, four groups A & B each containing 20 carps and C & D each containing 21 carps, were inoculated with 50,000 live trypanosomes/fish in groups A & C and 100,000 live trypanosomes/fish in group B, while 0.2 ml PSG/fish was inoculated in fish of group D (as a control). From groups C & D, 3 fish were killed every ten days starting from day 20—80 post infection. Blood plasma from fish in groups B, C & D were collected during different sampling times and stored at -80°C until use. All fish that died during the infection were diagnosed for the presence of trypanosomes. Parasitemia was estimated by rapid Matching Method.

In experiment 2, surviving fish from groups A (18 fish) and B (15 fish) from experiment 1 were considered as groups E and F, were challenged with 100,000 live trypanosomes/fish on day 195 post infection. A new group of 10 naive fish (group G, experiment 2 as a control) was also inoculated with a similar dose of trypanosomes. A wet preparation of blood from infected and control fish was examined weekly under phase contrast microscope and parasitemia was estimated.

In experiment 3, a volume of 0.2 ml of immune plasma B and C were injected in 10 naïve carps intraperitoneally in two new groups H and I respectively. The same volume of plasma D was injected in 10 naive carps in group J as control. All fish were challenged with 100,000 live trypanosomes/fish 24 hours after the injection of plasma. The blood of the challenged fish was examined weekly and parasitemia was estimated.

In experiment 4, 1000 live trypanosomes were incubated in 50 µl of immune plasma B, C and plasma D with an equal volume of PSG buffer (pH 8) at 20°C, 10°C and 5°C in microtiter plates (ten wells for each plasma) for 1 and 2 hours. The contents of each well were observed under phase contrast microscope to estimate trypanolysis (Table 1). After incubation at 20°C and 10°C, the contents of each well for plasma B were inoculated into 10 naïve carps in two group K and K' respectively, the contents of each well for plasma C were inoculated in 10 naïve carps in two groups L and L' and the contents of each well for plasma D were inoculated in 10 naïve carps in two group M and M'. The blood of the fish from these groups was examined weekly for the presence of trypanosome.

SAS computer software package was used for statistical analysis of the data. Bonferroni (Dunn) t test was applied to different variables to compare their means in different groups.

RESULTS

Mortality in juvenile carps:

All fish inoculated with trypanosome in experiment 1 dev parasitemia and the peak of infection was obtained on 35 days p.i. (Fig. 1). In A, two fish (10 %) died on day 38 and 40 p.i. The blood of the dead fish re $1.6x10^6$ trypanosomes/ml and the peritoneal fluid has $1x10^3$ trypanosomes/m group B, five fish (25 %) died on day 35, 3 on 40 and 1 on 46 p.i. The dead fi $1.4x10^8$ trypanosomes/ml in the blood and $2x10^4$ trypanosomes/ml in the body In group C, three fish died (15 % mortality), one died on day 35 and 2 on day having parasitemia of $1.53x10^6$ trypanosomes/ml in the blood an $1.13x10^3$ trypanosomes/ml in the body fluid. No trypanosomes were found i fish of group D.

Resistance to re-infection in survived carps:

The fish that survived in experiment 1 were diagnosed for trypanoson their blood on day 84 p.i. Only seven fish (4 in group A and 3 in group B) had small number of parasites (243.8±12.6 trypanosomes/ml blood in group A 214.3±4.6 trypanosomes/ml blood in group B) and no trypanosomes were thereafter before they were challenged on day 195 p.i.

Out of 18 fish in group E, only 5 fish revealed 1.4±1.1 trypanosomes/ the blood on day 7, 2.1±1.2 trypanosomes/ml on day 14, 1.6±1.2 trypanosom on day 21 and 1.2±1.1 trypanosomes/ml on day 28 post challenge. No trypanos were found in any of the fish on day 35 or later. In group F, 4 fish had trypanos (1.4±1.1 trypanosomes/ml) on day 7, on day 14, 2.05±1.3 trypanosomes/ml, o 21, 1.3±1.1 trypanosomes/ml, and on day 28, 1.03±1 trypanosomes/ml in the b No trypanosomes were found thereafter. All control fish (group G) were infecte at the peak of infection, $4.4\pm0.01x10^6$ trypanosomes/ml in the blood was obtain day 35 p.i. (Fig. 2). Two fish died, one on day 35 and second on day 38 challenges. Both, blood ($1.6x10^6$ trypanosomes/ml) and the body ($1.1x10^3$ trypanosomes/ml) were positive with live trypanosomes. For the nu (log 10 mean ±SEM) of trypanosomes/ml of blood in fish see Table 1.

Passive immunity:

The immune plasma B from experiment 1, when inoculated in 10 r carps in group H, remained protected from challenges of infection. In the bloc only 4 fish very small numbers of trypanosomes (7.8±1.1 trypanosomes/ml) found on day 21. In group I fish were also protected against the challenge infection but a very small number of trypanosomes (1.5 ±1.1 trypanosomes/ml) found in 5 fish on day 14 post challenge. In group J, fish developed an infection the peak of infection ($2.8x10^7$ trypanosomes/ml) was obtained on day 35 challenge (Fig.3). Two fish died on day 39 with a parasitemia

$1.23x10^6$ trypanosomes/ml in the blood and of $1.15x10^3$ trypanosomes/ml in the body fluid.

Trypanolysis:

The immune plasma B and C from experiment 1 were effective in neutralizing the infectivity of the trypanosomes when incubated at 20°C and 10°C, both for 1 and 2 hours incubation time. The lysis of live trypanosomes was 96±11.3 % in plasma C at 20°C and 92.4±15.2 % at 10°C. Only a small number, 3 % and 5 % of swollen trypanosomes were present at 20°C and 10°C respectively after incubation. The lysis in immune plasma B was 93.2 ±9.5 % at 20°C and 89.4±10.7 % at 10°C. The effectiveness of immune plasma B and C was slightly lower when incubated at 10°C for 1 hour and almost ineffective when incubated at 5°C for 2 hours incubation time. Plasma D had no effect in neutralizing the infectivity of trypanosomes at any temperature or incubation time. There was some lysis in plasma D (4.95±1.1 %) that was not different from lysis in PSG (4.75±1.2 %) at 20°C. Moreover, the fish receiving plasma D incubated trypanosomes at 20°C and 10°C and then challenged, developed an infection (Table 2). The peak of infection ($1.1x10^6$ trypanosomes/ml from 20°C incubation, Fig. 4) and $1.2x10^6$ trypanosomes/ml from 10°C incubation, Fig. 5) was obtained on day 42 p.i.

Table 1: The evaluation of actual numbers of trypanosomes (± SEM) during the study.

Days	*Experiment 1* Groups			*Experiment 2* Groups			*Experiment 3* Groups			*Experiment 4 (20°C)* Groups			*Experiment 4 (10°C)* Groups		
	A	B	C	E	F	G	H	I	J	K	L	M	K'	L'	M'
7	3.74± 0.41	5.29 ±0.24	3.91± 0.45	0.14± 0.04	0.13± 0.04	0.14± 0.14	0.08 ±0.03	0.13± 0.04	6.44± 0.09	0	0.05± 0.05	4.39± 0.21	0.12± 0.05	0.2±0 .06	4.53± 0.27
14	5.21± 0.16	6.01± 0.21	5.3±0 .2	0.33± 0.09	0.31± 0.12	6.34± 0.16	0.17 ±0.05	0.36± 0.05	7.04± 0.05	0	0.02± 0.01	4.92± 0.16	0.10± 0.04	0.24± 0.08	5.04± 0.16
21	5.72± 0.11	6.32± 0.11	5.75± 0.2	0.19± 0.08	0.12± 0.06	6.74± 0.17	0.03 ±0.01	0.89± 0.03	7.8±0. 04	0	0	5.31± 0.14	0.01± 0.01	0.39± 0.02	5.57± 0.02
28	6.32± 0.23	6.549 ±0.24	6.29± 0.1	0.07± 0.03	0.01± 0.01	7.18± 0.15	0	0.31± 0.012	8.23± 0.04	0	0	5.81± 0.14	0	0.08± 0.03	6.02± 0.14
35	6.56± 0.19	6.63± 0.20	6.36± 0.18	0	0	7.64± 0.14	0	0	8.45± 0.02	0	0	6.52± 0.15	0	0	6.52± 0.14
42	6.5±0 .15	6.6±0 .22	6.31± 0.11	0	0	7.56± 0.13	0	0	0.81± 0.14	0	0	7.04± 0.13	0	0	7.07± 0.09
49	6.22± 0.15	6.45± 0.09	6.29± 0.11												
56	6.06± 0.21	6.2±0 .16	6.0±0 .24												
63	5.71± 0.08	5.93± 0.11	5.51± 0.15												
70	5.38± 0.11	5.44± 0.19	5.1±0 .2												

Log 10 mean ± SEM

Table 2. The incubation of live trypanosomes in immune plasma at different temperatures and their effect in lysing live trypanosomes as well as the infectivity of *Trypanosoma danilewskyi* strain FCc 1 in immunized fish.

Plasma of fish from		20°C		10°C		5°C	
Experiment 1	**Time** (hrs)	fish inoculated / infected	Lysis %	fish inoculated/infected	Lysis %	fish inoculated/ infected	Lysis %
Plasma B	1	10/3	92.3 ± 10.6	10/5	88.2±12.7	10/10	20.2±10.6
	2 hrs	10/3	94.0±8.7	10/4	90.7±8.7	10/10	31.6±13.5
Plasma C	1 hr	10/0	95.0±12.6	10/5	91.6±16.5	10/10	31.6 ±13.5
	2	10/0	97.0±10.3	10/3	93.1±13.8	10/10	38.9±9.5
Plasma D	1	10/10	3.8±1.1	10/10	2.35±0.4	ND	0
	2	10/10	6.1±0.9	10/10	3.69±0.25	ND	0
PSG (control)	1	ND	4.0±1.2	ND	1.65±0.5	ND	0
	2	ND	5.5±1.0	ND	2.09±0.3	ND	0

DISCUSSION

About 25 % mortality occurred in juvenile carp, *Cyprinus carpio*, less than 5 months old, when infected with *Trypanosoma danilewskyi* strain FCc 1. This percentage of mortality is lower than reported earlier by Lom (1979) as 10-100 % (x= 48 %) in < 1 year old in carp (2-4 cm in length) depending on the trypanosome strain and fish-age class. The same author indicated that the mortality was not more than 33 % in carps of the same age but 7-9 cm in length, which indicates an increased resistance in fish bigger in size although of same age. The inoculum size is also important because when 100,000 trypanosomes/fish were inoculated in group G, showed 25 % mortality which is significantly higher than the mortality (10 %) occurring in group A (50,000 trypanosomes/fish). The earlier studies of Woo (1981 a) describes 60 % mortality in goldfish, *Carassius auratus* (8 cm), when inoculated with bigger size of inoculum (380,000) trypanosomes/fish) as compared to a 100 time smaller size of inoculum (3,800 trypanosomes/fish), the mortality was only 10 %. It is clear that a lower dose of inoculum takes longer time to produce infection, meanwhile the immune response of the fish gets enough time to develop and eliminates the parasites before they could produce peak of infection.

The fish recovering from the initial infection were protected from re-infection due to the presence of trypanocidal antibodies in their blood. Furthermore this protective immunity was not sterile until at least 195 days p.i. These results confirm the previous studies of Woo (1981 a) during the experimental infection of *Trypanosoma danilewskyi* in goldfish. Jones and Woo (1991) also reported such

protective immunity against *Cryptobia salmositica* in rainbow trout, *Oncorhynchus mykiss* and *Trypanosoma danilewskyi* in goldfish. From the present study the protective immunity (both *in vivo* and *in vitro*) is evident against *T. danilewskyi* strain FCc 1 in juvenile carp. It has been suggested that the presence of protective immunity against haemoflagellates for several months (195 days) was due to the presence of humoral and may be cellular immune responses.

Passive immunity can be acquired in naïve fish by the inoculation of immune plasma. When immune plasma B and C were inoculated in naïve carps, fish were protected from the re-infection showing the presence of passively acquired protective immunity. The juvenile carp remained protected from infection challenges of *T. danilewskyi* (Ahmed, 1994). Passively immunized carp with IgM purified from serum of recovered carp remained protected from re-infection of *T. danilewskyi* and the anti-parasite antibody level remained high for several months (Overath *et al.*, 1999). Woo (1999) successfully protected susceptible fish (Salmonid) from *Cryptobia salmositica* with monoclonal antibody IgG3. Similarly, Vein *et al.* (1975) described life-long protection in mice recovered from *Trypanosoma musculi* infection. This protection was due to persistence of *T. musculi* in the vasa recta of the kidney of the recovered mice that were responsible to stimulate the immune response for the maintenance of protection (Oliver and Vein, 1985).

The immune plasma B & C had similar trypanosome neutralizing effects when incubated at 20°C for 1 or 2 hours, but their effectiveness was slightly reduced at 10°C and was even ineffective at 5°C. The presence of lytic antibodies in immune plasma of recovered carp confirms the findings of Woo (1981 a) and Overath *et al.* (1999) who found similar plasma effects during the infections of *Trypanosoma danilewskyi*. Rijker *et al.* (1980) described kidney and spleen as the major organs for the production of plaque-forming cells (PFC) in carp, *Cyprinus carpio*, when immunized with sheep red blood cells (SRBC) at ambient temperature (12-24°C). Rainbow trout, *Oncorhynchus mykiss*, when kept at 20°C, were resistant to *Cryptobia salmositica* infection, while the infection removal was delayed at 5°C. In goldfish, *Carassius auratus*, at 20°C the infection of *Trypanosoma danilewskyi* was decreased quickly as compared to 10°C (Woo *et al.*, 1983). Carp, kept at 8°C, were inoculated with *Trypanoplasma borreli*: a rapid increase in parasitemia took place, but when incubated at 20°C on day 55 post infection, a sharp decrease in parasitemia was seen. This explains the temperature dependent immune response in fish which was not active enough at 8°C during the infection (Steinhagen *et al.*, 1989, 1990).

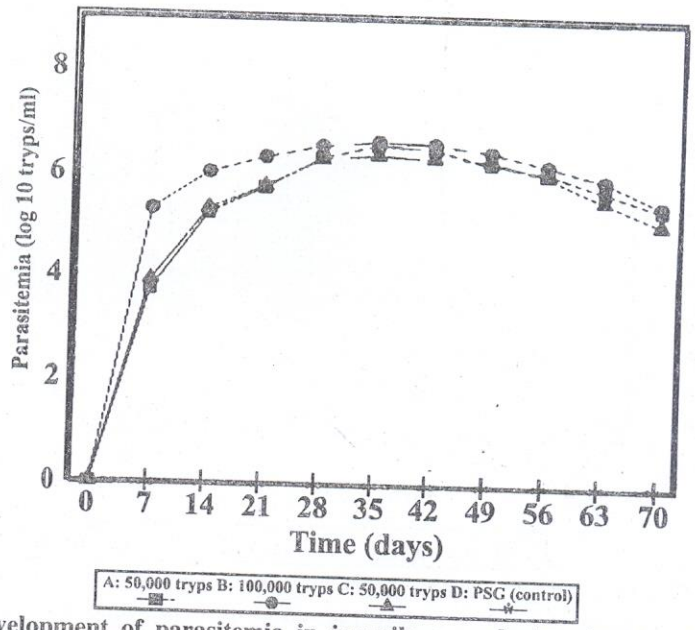

Fig. 1. Development of parasitemia in juvenile carp during the infection of *Trypanosoma danilewskyi* strain FCc 1 (experiment 1).

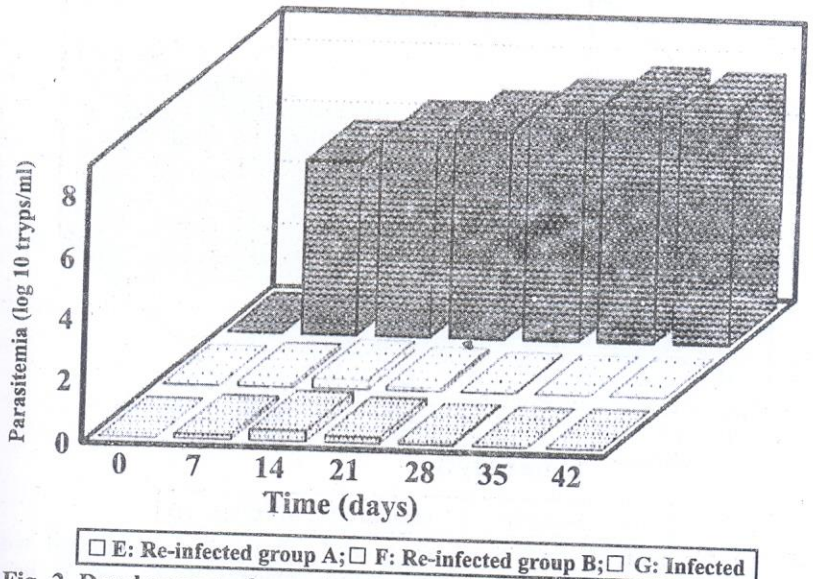

Fig. 2. Development of parasitemia in juvenile carp recovering from the initial infection of *Trypanosoma danilewskyi* strain FCc 1 and challenged at 195 days p.i (experiment 2).

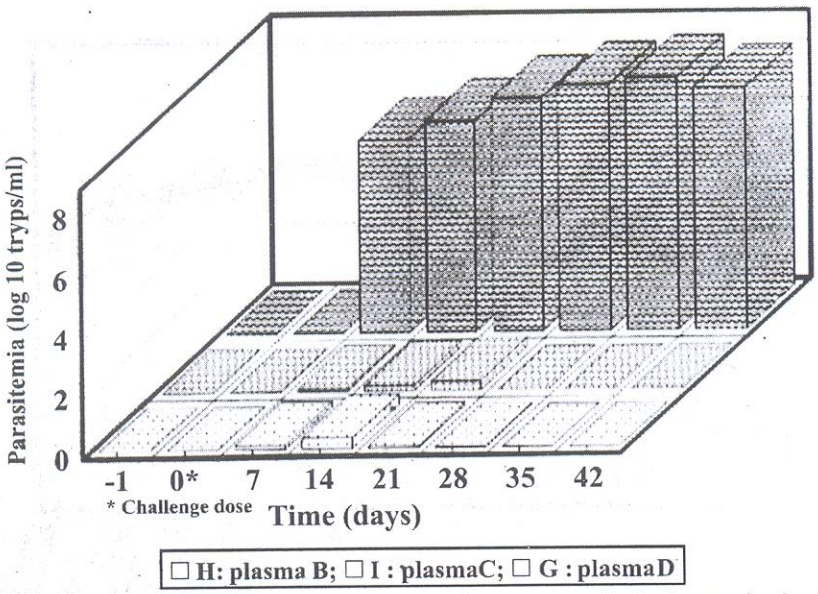

Fig. 3. Development of parasitemia in juvenile carp passively immunized with immune plasma B, C and plasma D and challenged with *Trypanosoma danilewskyi* strain FCc 1, 24 hours after immunization (experiment 3).

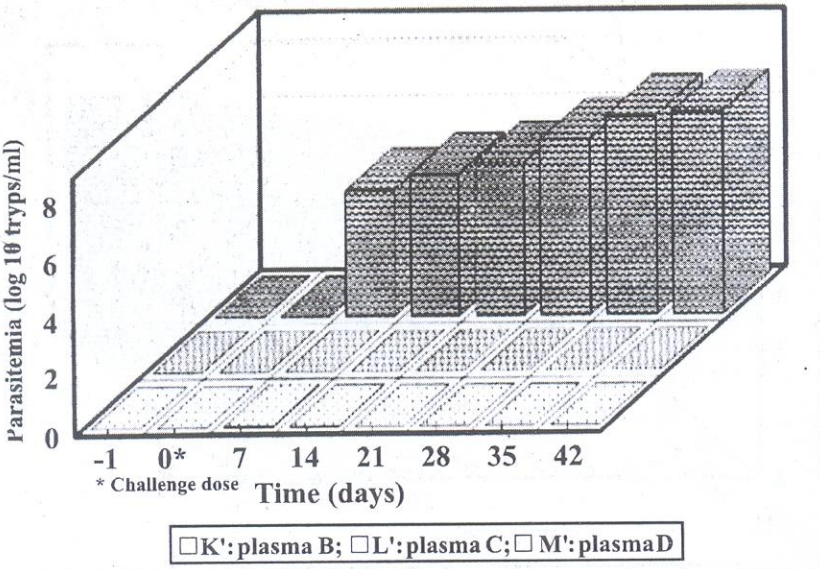

Fig. 4. Development of parasitemia in juvenile carp when inoculated with trypanosomes incubated at 20° C in immune plasma B, C and D from experiment 1 (experiment 4).

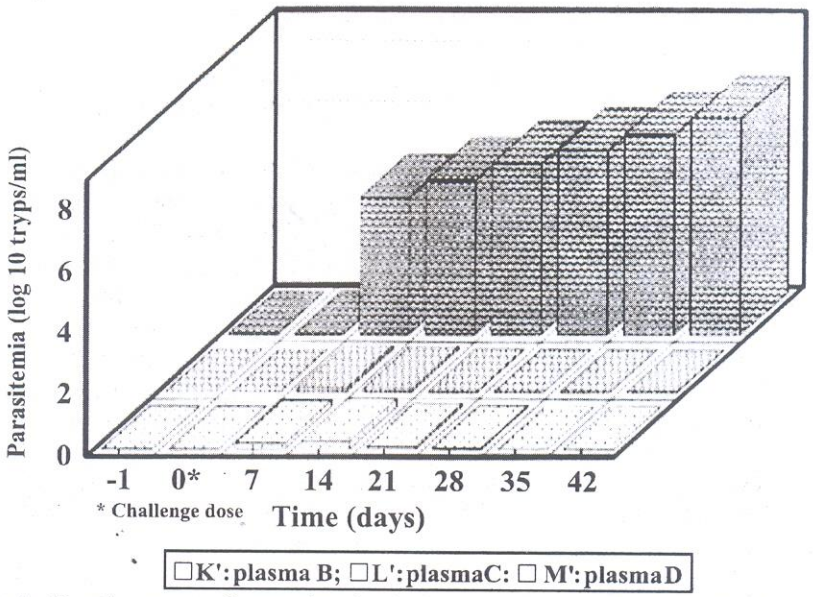

Fig. 5. Development of parasitemia in juvenile carp when inoculated with trypanosomes incubated at 10° C in immune plasma B, C and D from experiment 1 (experiment 4').

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