Veterinary Parasitology
Short communication

Title: Immunoblot analysis of specific antigen bands predictable for *Dirofilaria immitis* infection in cats

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Abstract

Serial sera from four mongrel cats experimentally inoculated with infectious larvae of *Dirofilaria immitis* were analyzed by immunoblot patterns against a phosphate buffered saline-extract of *D. immitis*. Antigen-specific protein bands detected indicate that the low molecular weight bands of 36, 32, 22, 19 and 14 kDa, are predictable for positive adult worm-infection, suggesting diagnostic usefulness for adult *D. immitis* infection in cats.

Key word: cat, diagnosis, *Dirofilaria immitis*, immunoblotting, specific protein band

1. Introduction

Recently, an increase number of infection cases in cats have been reported (Atkins, 1999, DeFrancesco et al., 2001, Roncalli et al.,1998). To address this problem, we attempted to identify specific bands in an immunoblotting test for the diagnosis of contemporary active *D. immitis* infection in cats by examining the serial sera of experimentally infected cats against the filarial worm antigen.

2. Materials and methods

The serial sera of cats with *D. immitis* infection examined in present study were used for the sera of experimentally infected cats preserved in previous study (Hayasaki et al., 2003). Briefly, five mongrel healthy female cats, age between 1 to 5 years and weight between 2.5 to 3.1 kg, were used and confirmed to be free of *D. immitis* infection by Knott test for microfilaria, physiological examination, X-ray examination, ultrasonography, and antibody-based enzyme-linked immunosorbent

assay (ELISA). Four out of five cats (Cats 1 to 4) were experimentally inoculated with 105, 100, 123 or 104 infective larvae (L_3) of *D. immitis*, respectively, according to the procedure described previously (Hayasaki, 1982), that the L_3 were collected from the proboscises of *Aedes togoi* mosquitoes at days 10 to 14 after a taking blood meal from a *D. immitis*-infected dog, and suspended in saline to inject subcutaneously into the shoulder region of these cats. Cat 5 served as uninfected control. They were kept in mosquito-free laboratory animal room throughout the experiment, and bled every 10 days from the day before the infection until Day 240 postinfection (PI). The sera were stored at −80 ℃ until used.

Immunoblotting was performed for all the sera collected from four infected cats. The cat sera were examined against a phosphate buffered saline (PBS) extract of adult *D. immitis* worm antigen. The antigen was fractionated by electrophoresis in a minislab gel containing of 12.5% acrylamide and 0.1% SDS, followed by transferring onto a nitrocellulose membrane for a western blot analysis (Kaneko et al., 1990). The nitrocellulose membrane was reacted with the cat sera followed by the reaction with peroxidase-conjugated goat anti-cat IgG (H+L chain) (Cappel Lab., Inc. Malvern, PA) at a dilution of 1:1,000. The bands on the nitrocellulose membrane were developed in a substrate solution containing 0.5 mg/ml 4-chloro-1-naphthol and 0.015% H_2O_2 in Tris-buffered saline.

Results and discussion

Despite that the diagnostic guideline for feline heartworm infection had been reviewed by Atkins (1999) and Atkins et al. (2000), a more accurate serological test is still considered necessary for diagnosing *D. immitis* infection in cats. Immunoblotting may be significant for a better

diagnostic technique with a high specificity for this purpose. For validating the specificities of the positive bands in immunoblotting, serial sera of cats experimentally infected with *D. immitis* should be used as the standard sera for monitoring the presence or absence of the bands throughout the course of the infection. However, in our literature search, we could not find any report that detailed the time-course changes of the immunoblot specific bands pattern of sera from cats experimentally infected with *D. immitis* L_3, especially during the pre-patent and the patent period of the infection. In our study, although only four cats were inoculated with *D. immitis* L_3, the presence of specific bands for *D. immitis* adult worm infection was well evident.

The parasitological results of the five cats were reported previously (Hayasaki et al., 2003). Briefly, only one (Cat 1) out of four inoculated cats showed microfilaremia from Day 201 PI onwards till the end of the experimental period. At necropsy, in Cat 1, 10 live adult worms (3 male and 7 female) were recovered from the right ventricle and pulmonary arteries, and 2 fragmented dead worms were recovered from the peripheral parts of the pulmonary arteries. In Cat 2, only 1 live male worm in the right ventricle and 1 fragmented dead worm in the peripheral pulmonary arteries were recovered. No live or dead adult worms of *D. immitis* were detected in Cats 3, 4, and also in Cat 5, although dead worm or its vestige was confirmed in the histo-pathological section of peripheral pulmonary arteries of Cats 3 and 4, respectively, by necropsy examination.

Based on the immunoblot band patterns, the experimental cats could be divided into two groups; with 2 cats (Cats 1 and 2) that harbored the adult worm(s), and the other 2 cats (Cats 3 and 4) that were not harbored it at the end of the experiment. Therefore, our results reflect the host humoral immune response of cats in *D. immitis* infection, albeit the number of cats used in this study was small. Cat 5 was not performed

immunoblot analysis because we previously confirmed no infection by monitoring the antibody production to *D. immitis* (Hayasaki et al., 2003).

Immunoblot analysis of the sera of experimentally infected cats showed common specific antigen bands of molecular weights of 36, 32, 22, 19 and 14 kDa, as comparatively low molecular weight bands (Table 1, Fig. 1). The sera of Cat 1 showed all the five specific bands throughout the fifth stage of worm development as juvenile and matured adult worm stages, after the fourth molting period (after about 60-70 days PI) of migrating *D. immtis* larvae (Hayasaki, 1982) , although these were initially present at various timings. Particularly the 32, 22 and 14 kDa bands were observed from Days 100 and 80 PI, respectively and persisted until the end of the experimental period. Cat 2 showed four out of five bands, particularly the 32, 22 and 14 kDa bands were observed from Days 110, 160 and 150 PI, respectively, and persisted until the end of the experiment. In both of Cats 1 and 2, the 19 kDa band observed from Day 170 PI and Day 160 PI, respectively, and persisted until the end of the experiment. These may indicate that the 19 kDa band reflect a patent period of infection as a phase of matured adult stage. On the contrary, in Cat 3, the 32 and 22 kDa bands were observed as early as from Days 70 and 80 PI, respectively, which was just after the fourth molting period theoretically, and persisted for about 4 months until Day 190 PI. However, the both bands disappeared after Day 200 PI, and in Cat 4, some of the aforementioned five specific bands was not observed. These results indicate that the migrating *D. immitis* larvae in Cats 3 and 4 might be dead at the juvenile adult stage or the fourth larval stage, respectively.

Such a presumption may be possibly supported by the time-course changes of ELISA-antibody titers to *D. immitis*, reported previously (Hayasaki, et al., 2003), briefly showing that the ELISA titer in Cat 1 persisted in a higher level from Days 70 PI through the juvenile stage and matured adult stage until the end of the experiment, and in Cat 2 the

titer persisted in a relatively medium level until Day 150 PI and then increased into a higher level from Day 160 PI to the end of the experiment. On the contrary, in Cat 3 the higher titer persisted from Day 50 PI to Day 180 PI, and similarly in Cat 4 it persisted from Days 10 to 100 PI, thereafter, the titers of both cats, however, decreased into low levels. The titer in Cat 5 as the control was consistently persisted in negative level throughout the experiment. Therefore, it considered that these time-course changes of antibody titers relatively coincided with the appearance and disappearance of the immunoblot bands determined in present study, particularly those in Cats 1, 2 and 3.

These findings indicate that the appearance of these low molecular weight bands of 39, 32, 22, 19 and 14 kDa may be predictable for the presence of fifth stage worm.

On the other hand, higher molecular weight bands of more than 50 kDa of the immunoblot were conspicuous during the pre-patent period, such as at Days 60-130 PI in Cat 1, Days 160 PI and thereafter in Cat 2, Days 70-150 PI in Cat 3, and Days 20-100 PI in Cat 4, respectively. An appearance and disappearance of the higher molecular weight bands also relatively coincided with these time-course changes of antibody titers above mentioned, particularly those in Cats 3 and 4. It considered that the appearance of these high molecular weight bands may reflect the existence of live worm(s) even they were larval stage or adult stages. Therefore, the appearance and disappearance of these high molecular weight bands may be predictable for the live and death of the worm of these stages. It should be necessary to verify whether these high molecular weight bands are due to infection or are non-specific. The immunoblotting indicated that, before the *D. immitis* inoculation, only one band of 138 kDa in Cat 1 and also one band of 138 kDa in Cats 3 and 4 were detected, respectively, and no bands were detected from Cat 2, while the 19 kinds of bands between 50-145 kDa could be counted after the

inoculation (data not shown). These findings indicate that most of high molecular bands are due to the infection.

The other report also described that high molecular weight specific antigen protein bands of 75-200 kDa had been implicated as the major host immune response during the pre-patent period, while those of the low molecular weight bands of 15-38 kDa were considered significant during the patent period as expressed by the presence of microfilaremia (Boto et al., 1984). Despite that this report was based on *D. immitis* infection in dogs, our observation concurred with their suggestion. The usefulness of low molecular weight specific bands produced using excretory-secretory (ES) products of *D. immitis* for diagnosis had been proposed. An antigen band of 18 kDa derived from the ES proteins collected *in vitro* from adult worms of *D. immitis* was observed to react strongly with the sera of human patients with pulmonary dirofilariosis (Akao et al., 1991). In addition, the ES proteins of 20-23 kDa collected *in vitro* during the molting from the third stage to the fourth stage larvae of *D. immitis* had been reported to be specific to the larval stage (Frank and Grieve, 1996). *D. immitis* adult worm somatic proteins of 19, 30 and 40 kDa and the ES proteins of 22 and 25 kDa were also reported to be specific to the adult worm stage (Pieto et al., 1997). Thus, the aforementioned reports support our proposal for the use of low molecular weight antigen bands for the specific diagnosis of *D. immitis* infection in cats.

In conclusion, we propose that, when the immunoblotting of cat serum showed some of low molecular weight specific bands of 36, 32, 22, 19 and 14 kDa together with the group of high molecular weight bands, it may suggest the prediction of positive adult worm infection as juvenile and matured adult stages of live worm(s), in contrast to this, when it showed only the group of high molecular weight bands even it was absent the low molecular weight bands, it suggest the prediction of the live larval worm

infection or the expressing only an exposure to *D. immitis* infection.

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Explanation of Figures

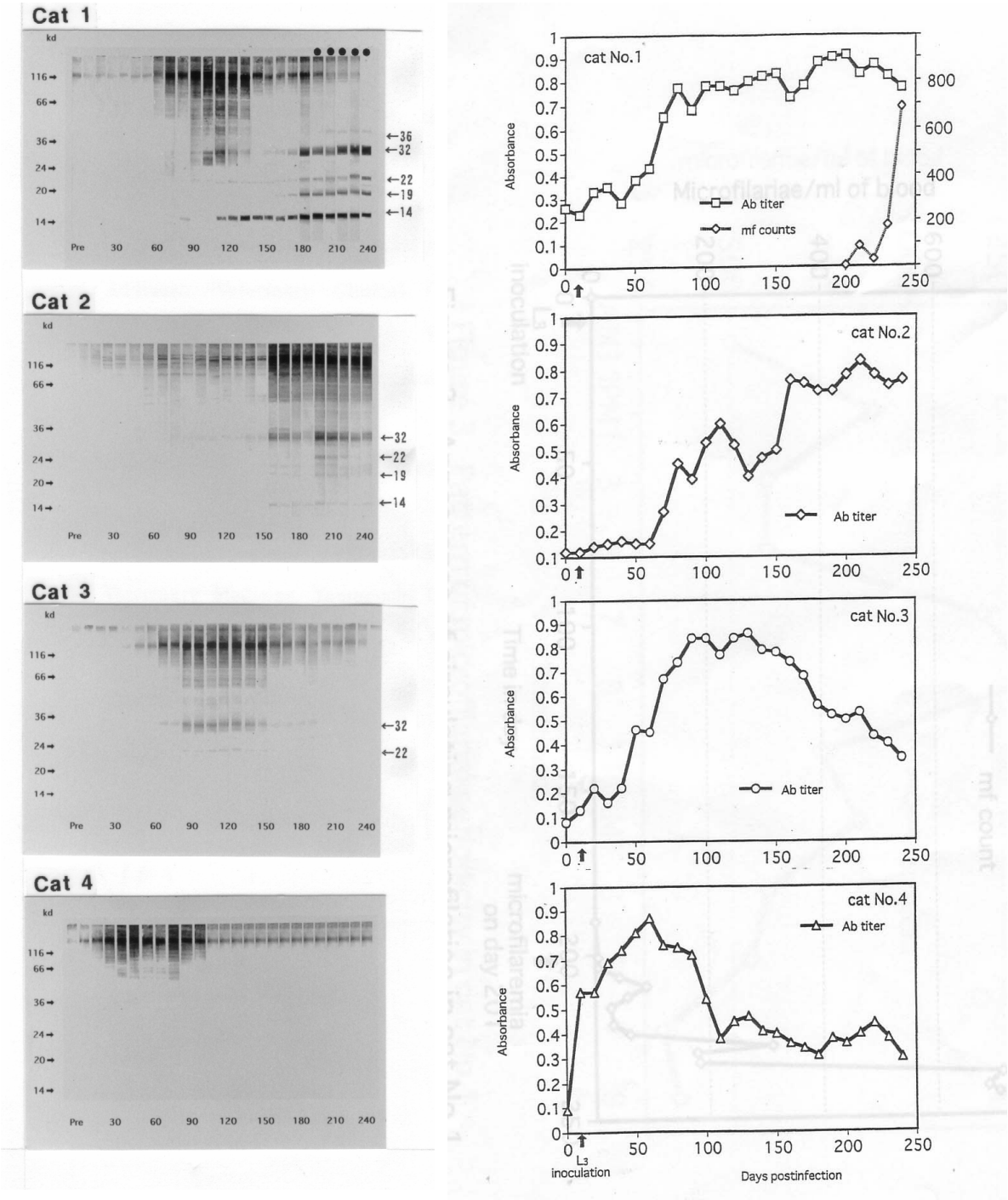

Figure 1. Immunoblot patterns of Cats 1-4 throughout the experimental infection with *D. immitis*.

●:microfilaremia in Cat 1.

Table 1
Immunoblot patterns of specific bands in four cats experimentally infected with *D. immitis*

	(kDa)	Days after infection																							
		0	10	20	30	40	50	60	70	80	90	100	110	120	130	140	150	160	170	180	190	200	210	220	230	240
Cat 1	36												+	+	+								+	+	+	+
	32											+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	22											+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	19																		+	+	+	+	+	+	+	+
	14									+			+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Mf[a]																					+	+	+	+	+
Cat 2	36																						+	+	+	+
	32												+		+	+	+	+	+	+	+	+	+	+	+	+
	22																	+	+	+		+	+	+	+	+
	19																	+	+	+		+	+	+	+	
	14																+	+	+	+	+	+	+	+	+	+
	Mf																									
Cat 3	36										+	+	+	+	+	+										
	32								+	+	+	+	+	+	+	+	+	+	+	+	+					
	22									+	+	+	+	+	+	+	+	+	+	+	+					
	19																									
	14												+		+	+	+									
	Mf																									
Cat 4	36																									
	32																									
	22																									
	19																									
	14																									
	Mf																									

kDa, kilo Dalton, Mf, circulating microfilaria.
[a] Circulating microfilariae were detected in only Cat 1 with 10 adult worms (3 males and 7 females) recovered.