Results

1. Examinations of fecal samples by the formalin-ether concentration method

The collection of feces started from March 20, 2000 to January 23, 2001 and from December 3, 2001 to January 3, 2002. The fecal samples would be later used for evaluation of the sandwich ELISA for coproantigen detection. A total number of 216 fecal samples were screened for intestinal parasites by formalin-ether concentration method and for collecting L3 for antigen preparation. The examination showed that 20.37% of the samples were positive for *S. stercoralis* larvae. Eggs and cysts of other parasites were also found in other fecal samples (Table 1).

All of the fecal samples were subjected to agar plate culture to detect the remaining *S. stercoralis* infections missed by the formalin-ether concentration method. The examination showed 21.29% (46/216) and 26.85% (58/216) of the samples were positive for *S. stercoralis* larvae by formalin-ether concentration and agar plate culture methods, respectively. The results are shown in Table 2.

2. Densities of *S. stercoralis* larvae in fecal samples

All the stool samples that were positive for *S. stercoralis* larvae by formalin-ether concentration method were examined by Beaver's standard direct smear to determine the larval densities. The larval density of each sample would be analyzed against the OD of sandwich ELISA of the corresponding sample. The densities varied considerably ranging from 100 to 132,700 larvae/gram feces (Table 3). Most samples contained a few hundred to a few thousand larvae/gram feces.

Table 1. Number of stool samples positive for intestinal parasites by formalin-ether concentration method

Parasites	No. of positive (%)
Strongyloides stercoralis	44 (20.37)
Hookworm	14 (6.48)
Opisthorchis viverrini	17 (7.87)
Giardia lamblia	5 (2.31)
Trichuris trichiura	7 (3.24)
Taenia spp.	2 (0.93)
Ascaris lumbricoides	4 (1.85)
Entamoeba coli	5 (2.31)
Mixed infections*	12 (5.56)
Negative for all parasites	106 (49.07)
Total	216 (100)

* *S. stercoralis* (Ss) + *O. viverrini* (Ov) = 1; Ss + *Fasciola* spp. = 1; Ss + Ov + hookworm (HW) = 1; *Taenia* spp. (T) + *E. coli* (Ec) = 1; Ec + *E. nana* = 2; *T. trichiura* (Tt) + *A. lumbricoides* (Al) = 1; T + HW = 1; *Entamoeba histolytica* + Tt = 1; *G. lamblia* + HW = 1; Tt + HW = 1; Al + Tt + Ec = 1.

Table 2. Stool examinations by agar plate culture as compared to formalin-ether concentration method

Parasite	Agar plate culture	Formalin-ether concentration
S. stercoralis[1]	58*	46*
Hookworm[2]	17	17
Mixed infections[3]	1	1
Negative[4]	140	152
Total	216	216

[1]Positive for *S. stercoralis* only or *S. stercoralis* plus other parasites by formalin-ether concentration method

[2]Positive for hookworm only or hookworm plus other parasites by formalin-ether concentration method

[3]Mixed infections between *S. stercoralis* and hookworm by agar plate culture or formalin-ether concentration method

[4]Negative for *S. stercoralis* and hookworm but some were positive for other parasites (see Table 1)

*Statistically different ($\chi^2 = 7.2$; $p < 0.05$)

Table 3. Densities of *S. stercoralis* larvae examined by Beaver method

Densities (larvae/gram)	No. of samples
<100	8
101-1,000	20
1,001-10,000	13
10,001-100,000	2
>100,000	1

3. Collection of adult worms, L3 and L1 for antigen preparation

L3 were initially obtained from filter paper slant method. However, the larvae obtained seemed to be contaminated with fecal debris when loose, diarrheic or watery stool samples were used. Later the modified agar plate culture as described in the Materials and Methods was used and it gave a cleaner preparation of L3. The total number of L3 collected from 36 cultures of positive stool samples was 4,173,200.

Adult worms were collected from agar plates 2 days after culture. Female worms were distributed into wells of a 24-well plate containing NSS to culture for L1 as described in Materials and Methods. The results of the cultures for L1 are shown in Table 4. The total number of L1 produced by 8,510 female worms was 86,185 and the ratio between adult female worms and L1 varied from 1:5 to 1:18.

4. Determination of protein content of *S. stercoralis* antigens

The amounts of proteins extracted from L3 varied considerably between different preparations ranging from 0.104 mg/1 X 10^5 larvae to 0.737 mg/1 X 10^5 larvae (Table 5). Extraction of proteins from adult worms was performed only once and the amount of proteins obtained from 4,490 adult female worms was 0.454 mg. Determination of the amount of proteins extracted from L1 was not possible because there was only a small number of the larvae.

Table 4. The ratio between adult female worms and L1 of *S. stercoralis* after overnight culture of adult worms

No. of adult females	No. of L1	Ratio
580	5,900	1:10
60	320	1:5
920	12,000	1:13
920	5,500	1:6
630	11,500	1:18
5,400	50,965	1:9

Table 5. Amounts of proteins extracted from L3 of *S. stercoralis* as determined by the BCA protein assay kit

No. of L3	Amount of proteins per $1X10^5$ larvae (mg)	Amount of total proteins (mg)
767,300	0.104	0.804
500,000	0.296	1.482
483,500	0.476	2.301
442,800	0.472	2.09
402,800	0.737	2.964
478,000	0.474	2.265

5. Demonstration of the 41-kDa protein in L1, L3 and adult worms of *S. stercoralis*

The 41-kDa protein of L3 was used to immunize mice to produce mAbs, which would be used to detect L1 and/or adult antigens in the feces. It is therefore necessary to show that antibodies to the 41-kDa protein would detect the protein in L1 and adult worms. Western blot analysis in Figure 1 showed that the serum from the mouse immunized with partially purified 41-kDa protein reacted strongly with the 41-kDa protein of L3. It also reacted with the 41-kDa protein of L1 and adult worms. In addition, the serum reacted weakly with 23 and 26-kDa proteins of L3. The result suggested that mAbs that would be produced against the 41-kDa protein should be able to detect this protein in the feces of strongyloidiasis patients.

6. Production of mAbs

In the first attempt to produce mAbs to *S. stercoralis*, spleen cells were from a mouse immunized with the 41-kDa protein. Upon screening by ELISA using L3 extract as antigens, only one of the culture supernatants from wells with growing hybridomas was positive. The hybridoma cells from this well were cloned twice by limiting dilution which resulted in four hybridoma clones, namely 4H5, 4H12, 3B5, and 3F11. The culture supernatants of these clones apparently reacted with the 41-kDa protein of L3 by Western blot analysis (Figure 2). They also recognized the 73-kDa protein. However, these mAbs could not be isotyped because they gave positive reactions with antibodies of all isotypes. It was found later that all of these mAbs

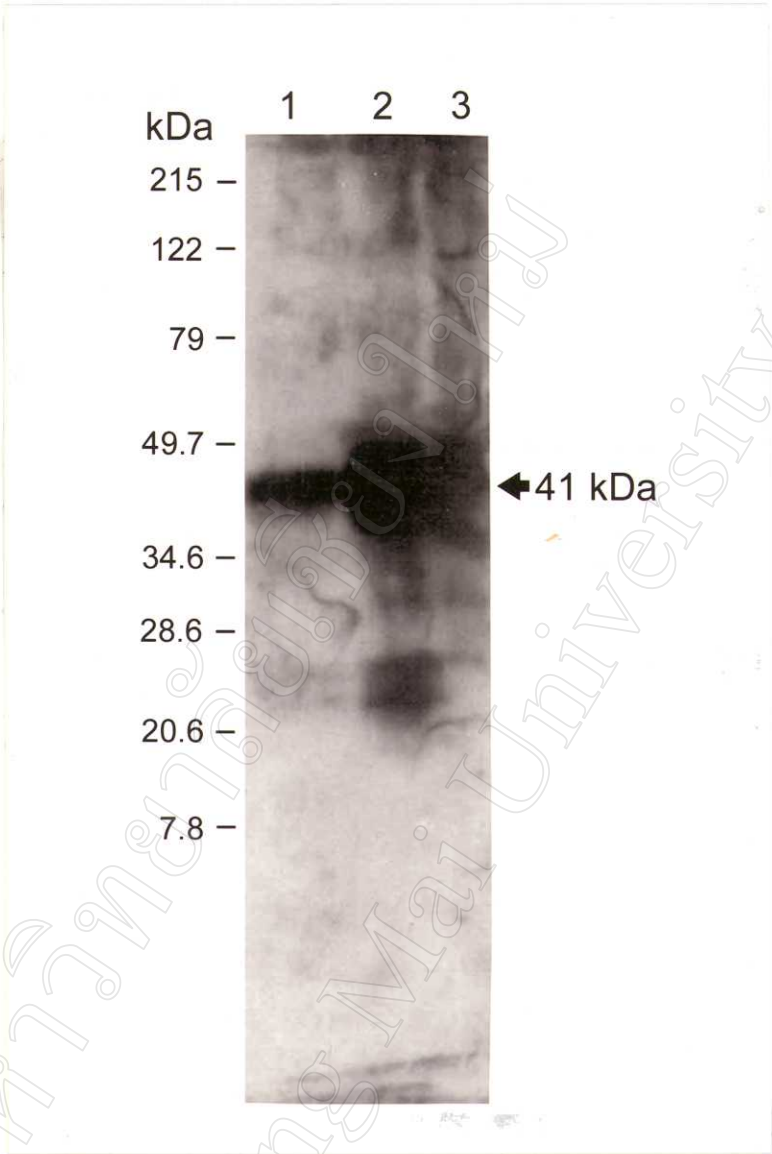

Figure 1. Western blot analysis of proteins of *S. stercoralis*. Twenty micrograms of proteins of adult worms (lane1) and L3 (lane 2) and proteins equivalent to 5,600 L1 (lane 3) were separated by SDS-PAGE (8-20% gradient gel) and transferred to nitrocellulose paper. The blot was reacted with mouse antiserum against 41-kDa protein. The binding of antibodies was detected by chemiluminescent method as described in Materials and Methods. Position of molecular weight markers (kDa) are shown on the left.

Figure 2. Western blot analysis of the mAbs from the first fusion. Strips of L3 blot were probed with NMS (lane 1), immunized mouse serum against 41-kDa protein (lane 2) and culture supernatants of hybridoma clones 3B5, 3F1, 4H12 and 4H5 (lane 3-6, respectively). Position of molecular weight markers (kDa) are shown on the left.

bound nonspecifically to the surface of ELISA plate resulting in false positive results from the beginning.

Another two fusion experiments were carried out to produce mAbs from spleen cells of mice immunized with the 41-kDa protein of *S. stercoralis* L3. There were three and four wells of hybridomas that produced antibodies to L3 antigens when tested by ELISA. However, none of them showed positive reaction by Western blot analysis.

In the fourth fusion experiment, spleen cells that had been used were from the mouse immunized with crude whole extract of *S. stercoralis* L3. Almost all culture supernatants (47 out of 48) gave positive ELISA which given the absorbance ranging from 0.956-1.314. When these supernatants were analyzed by Western blotting, it was found that only four of them showed reactivities to proteins of L3. Showed reactivity against 66-kDa; 22- and 38-kDa; 200-kDa and 34-, 39- and 68-kDa proteins, respectively (Figure 3). Unfortunately, the hybridomas producing these antibodies grew very slowly and eventually died.

To overcome the slow growth of hybridomas, the hybridoma growth supplement (BM Condimed H1) was used in the last fusion experiment. BM Condimed H1 contains a complex mixture of growth factors and cytokines that can support the growth of hybridomas after fusion and during cloning.

Hybridomas from 6 wells with high ELISA OD against L3 antigens were chosen for cloning by limiting dilution. However, only four hybridomas were derived from each parenteral line. They were designated as clones 20, 56, 64 and 83. The mAbs obtained from clones 20, 56 and 65 were of IgM isotype whereas that of clone 83 was of IgG1 isotype.

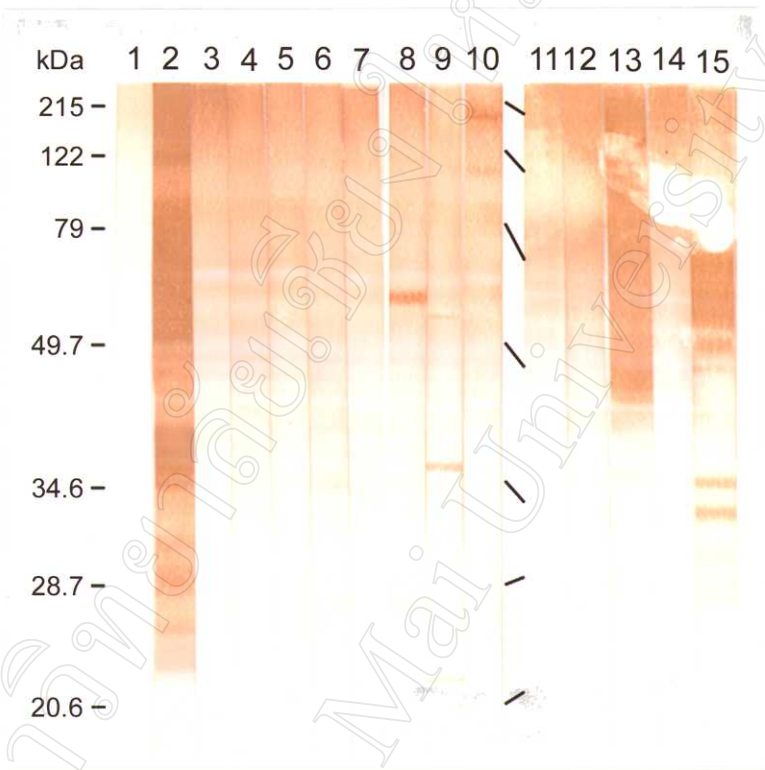

Figure 3. Western blot analysis of hybridoma supernatants from the fourth fusion. Strips of L3 antigens were probed with NMS 1:100 (lane 1), IMS 1:100 (lane 2), hybridoma culture supernatants (lanes 3-15). Note the reactivities at 66-kDa (lane 8), 22- and 38-kDa (lane 9), 200-kDa (lane 10) and 34-, 39-, and 68-kDa (lane 15). Position of molecular weight markers (kDa) are shown on the left.

The culture supernatants of parenteral lines clone 56 showed the specific band about 110-kDa but sometimes this clone showed more than three bands in this region. Clone 83 always showed a fuzzy band about 165-kDa. However, clones 20 and 65 did not show any specific bands when tested by Western blot analysis (Figure 4). Repeated Western blot experiments gave the same results. In contrast, the culture supernatants of these clones collected from different occasions consistently gave high ELISA absorbance ranging from 2.066 to 2.415.

Since the mAbs would be used to detect *S. stercoralis* antigens in the feces, it was necessary to show that the antibodies could react with antigens of L1 and adult parasites, which are normally present in the intestine of infected individuals. These mAbs were therefore tested on crude antigens of adults and L1 in comparison with those of L3. Crude antigens of L1 could be prepared only by boiling because there was a small number of L1 available. Boiled L3 and adult antigens were also used for comparison. As shown in Table 6, all of the mAbs could recognize antigens of all stages except for clone 20 that did not react with adult antigens. All antibodies seemed to give higher OD with unboiled antigens.

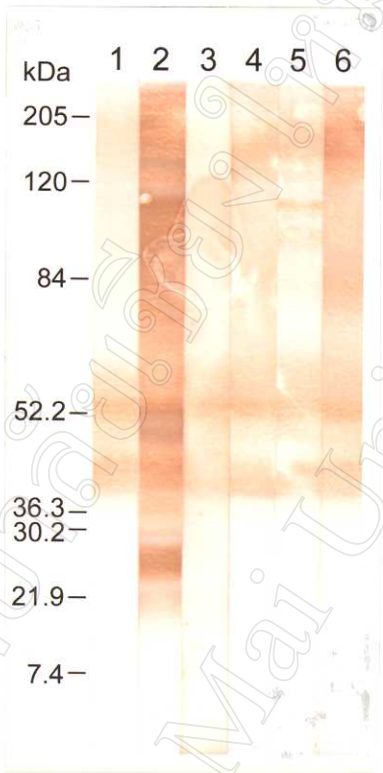

Figure 4. Western blot analysis of the culture supernatants from the seventh fusion. Strips of L3 blot were probed with NMS 1:200 (lane 1), immunized mouse serum against L3 antigen 1:200 (lane 2) and culture supernatants of hybridoma clones 20, 65, 56, and 83 (lanes 3-6, respectively). Position of molecular weight markers (kDa) are shown on the left.

Table 6. Optical densities of mAbs reacting with antigens of various stages of *S. stercoralis* by ELISA. Wells of ELISA plate were coated with antigens of L3, adults (0.25 μg/well) or L1. The amount of L1 antigens used to coat wells was arbitrary. All OD are average of duplicates

	L3 antigens		Adult antigens		L1 antigens
	Not boiled	boiled	Not boiled	Boiled	boiled
NMS 1:10,000	0.007	0.000	0.001	0.000	0.000
IMS 1: 10,000	1.819	1.291	0.827	0.378	0.602
NRS* 1:6,400	0.009	ND***	0.028	ND	0.088
IRS** 1:6,400	1.946	ND	1.220	ND	1.121
mAb 20	2.481	2.302	0.158	0.043	0.754
mAb 65	2.116	2.032	1.958	1.709	1.124
mAb 56	1.855	1.650	1.933	1.090	0.687
mAb 83	2.176	1.932	1.986	1.599	0.942
NH	0.105	0.034	0.085	0.057	0.034

*NRS =normal rabbit serum

**IRS = serum from rabbit immunized with whole extracts of L3

***ND = not done

7. Sandwich ELISA

The sandwich ELISA could be used to detect *S. stercoralis* antigens in the feces of infected individuals. The sandwich ELISA was therefore evaluated whether it could detect the antigens intentionally added into the test. The capture antibodies in this study could be either the mAbs or rIgGαL3. The data in Table 7 showed that crude antigens of L3, adult and L1 could be detected by sandwich ELISA when mAb 65 was used as the capture antibody. Only L3 antigens were detected when the capture antibody was mAb 20. However, when the capture antibody was mAb 83, none of the antigens was detected by sandwich ELISA. Combination of the three mAbs as capture antibodies showed positive result with only L3 antigens.

On the other hand, using rIgGαL3 as capture antibodies showed that only crude antigens to L3 were detected when the detecting antibody was either biotinylated mAb 65 or biotinylated mAb 20 or the combination of these both antibodies (Table 8). In all cases crude antigens of adult or L1 could not be detected.

8. Coproantigen detection by sandwich ELISA

The sandwich ELISA was applied to the detection of *S. stercoralis* antigens in fecal specimens of infected individuals. In the first attempt the purified rIgGαL3 and the rIgGαL3-B were used as the capture and detecting antibodies, respectively. As can be seen from Figure 5 that the distribution of optical densities of 42 *S. stercoralis*-positive fecal samples was very similar to that of 40 negative fecal samples. The mean optical density of positive samples (0.0.123) was not statistically different from that of negative samples (0.091) ($p = 0.346$).

Table 7. Optical densities of sandwich ELISA using mAbs as capture antibodies. Wells of ELISA plate were coated with purified mAb (0.75 μg/well). Crude antigens of L3 (0.25 μg/well) and adult (0.25 μg/well) and L1 (arbitrary amount) were added, followed by rIgGαL3-B (0.5 μg/well), PO-anti-biotin mAb (1:2000) and its substrate. All OD are average of duplicates

Capture mAbs	L3 antigens	Adult antigens	L1 antigens	No antigens
mAb 83	0.205	0.167	0.131	0.143
mAb 65	0.366	0.357	0.234	0.125
mAb 20	0.665	0.175	0.142	0.143
combination	0.602	0.268	0.200	0.137

Table 8. Optical densities of sandwich ELISA using rIgGαL3 as capture antibodies. Wells of ELISA plate were coated with rIgGαL3 (0.5 μg/well). Crude antigens of L3 (0.25 μg/well), adult (0.25 μg/well) and L1 (arbitrary amount) were added, followed by biotinylated mAb (0.5 μg/well), PO-anti-biotin mAb (1:2000) and its substrate. All OD are average of duplicates

Detecting mAb	L3 antigens	Adult antigens	L1 antigens	No antigens
Biotinylated mAb 65	0.351	0.082	0.076	0.056
Biotinylated mAb 20	0.340	0.048	0.050	0.040
combination	0.442	0.091	0.114	0.089

The next attempt was the use of mAb 20 as the capture antibody and rIgGαL3-B as the detecting antibodies. In this experiment, pools of 42 positive and 40 negative fecal specimens were tested in stead of individual specimens. The data obtained from this experiment failed to show the difference between the optical densities of pooled positive and pooled negative fecal specimens (Table 9). In fact, pooled negative feces gave even higher optical densities. Lastly, using the rIgGαL3 as the capture antibodies and the biotinylated mAb (mAb 20 mixed with mAb 65) as the detecting antibodies also failed to detect *S. stercoralis* antigens in the feces of infected individuals. The optical density given by pooled positive feces was 0.144 whereas that given by pooled negative feces was 0.210.

The data described above showed that the sandwich ELISA could not detect *S. stercoralis* antigens in the feces of strongyloidiasis cases. The next experiment was performed to determine whether the sandwich ELISA would be able to detect *S. stercoralis* antigens in normal feces samples spiked with the antigens. Either L3 or adult antigens could be spiked to the feces samples since there was not enough L1 antigens to do these experiment. The results of this experiment are shown in Table 10. The data obtained from the experiment indicates that the sandwich ELISA could not distinguish spiked fecal supernatants from the unspiked ones, which conforms with the experiments done on fecal samples from normal and infected individuals.

Table 9. Optical densities of sandwich ELISA tested on pooled positive and pooled negative feces. Wells of ELISA plate were coated with mAb 20 (0.75 μg/well). Pooled positive and pooled negative fecal supernatants prepared as described in Materials and Methods were incubated with coated wells followed by the addition of rIgGαL3-B, PO-anti-biotin mAb and substrate solution. Crude L3 antigens coated directly to wells were also included as controls. All OD are average of duplicates

	rIgGαL3-B	
	2 μg/well	1 μg/well
Crude L3 antigen added	0.874	0.714
No L3 antigen	0.245	0.142
Pooled positive feces	0.238	0.146
Pooled negative feces	0.364	0.316

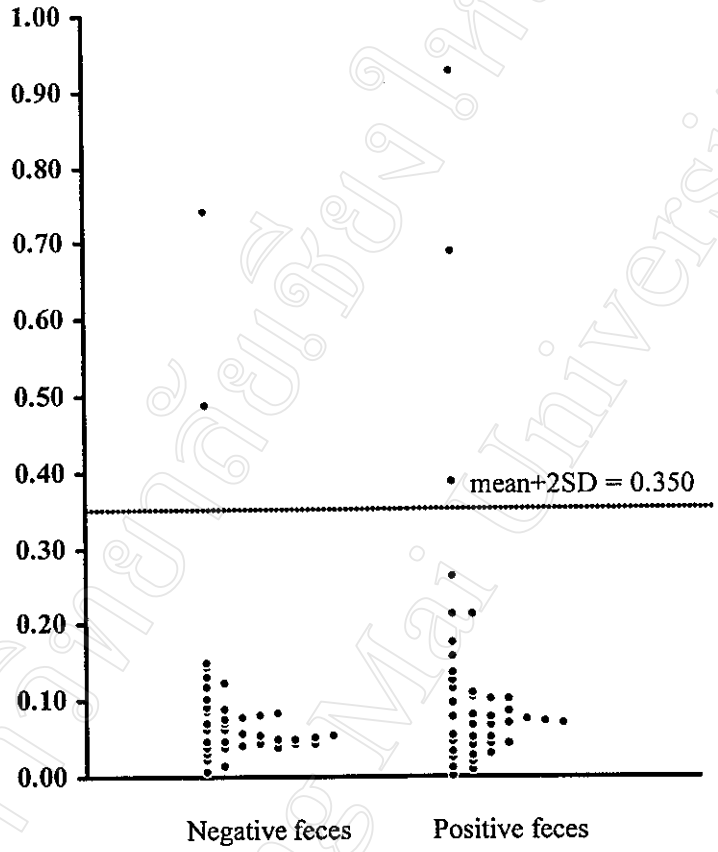

Figure 5. Optical densities of sandwich ELISA of 42 *S. stercoralis*-positive fecal samples and 40 negative fecal samples. Wells of ELISA plate were coated with rIgGαL3 (0.5 μg/well). Fecal supernatants prepared as described in Materials and Methods were incubated with the wells followed by the addition of rIgGαL3-B (1 μg/well), PO-anti-biotin mAb (1:2000) and substrate solution. The OD values were average of duplicates.

Table 10. Coproantigen sandwich ELISA of stool samples spiked with *S. stercoralis* antigens. Fecal supernatant of pooled normal feces were spiked with *S. stercoralis* antigens for detection by sandwich ELISA. Wells of ELISA plates were coated with 0.5 μg/well of rIgGαL3 (subtables A and C) or 0.5 μg/well of mAbs 20 and 65 (subtable B). Crude antigens or unspiked or spiked normal fecal supernatant were incubated with wells followed by biotinylated mAbs 20 and 65 (subtable A) or biotinylated rIgGαL3 (subtables B and C), PO-anti-biotin mAb and its substrate. All OD are average of duplicates

	A				B				C			
	0.5 μg/well		0.25 μg/well		0.5 μg/well		0.25 μg/well		0.5 μg/well		0.25 μg/well	
	Ag only*	Ag in NF**	Ag only	Ag in NF	Ag only	Ag in NF	Ag only	Ag in NF	Ag only	Ag in NF	Ag only	Ag in NF
L3	ND***	0.132	0.220	0.126	ND	0.230	0.449	0.166	ND	0.405	0.290	0.221
adult	ND	0.116	0.079	0.120	ND	0.066	0.176	0.120	ND	0.292	0.157	0.371
No Ag	ND	0.076	0.037	0.076	ND	0.162	0.096	0.162	ND	0.267	0.138	0.267

*crude antigens
**normal fecal supernatant spiked with antigens
***not done