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Véronique Marius, Serge Bernard, J.P. Raynaud, P. Pery, G. Luffau, Non  
Renseigné, B. Naudin, J.C. Leroy, C. Carrat, R. Lhortolary

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## **DICTYOCAULUS VIVIPARUS IN CALVES : QUANTITATION OF ANTIBODY ACTIVITIES IN SERA AND RESPIRATORY SECRETIONS BY IMMUNO-ENZYMATIC ANALYSIS**

Véronique MARIUS<sup>1</sup>, S. BERNARD<sup>2</sup>, J.P. RAYNAUD<sup>3</sup>, P. PERY<sup>2</sup> and G. LUFFAU<sup>2</sup>

with technical assistance of G.WILLIAM<sup>3</sup>, B. NAUDIN<sup>3</sup>, J.C. LEROY<sup>3</sup>, C. CARRAT<sup>2</sup> and R. LHORTOLARY<sup>2</sup>

<sup>1</sup> Laboratoire National de Pathologie aviaire, BP 9 - 22440 Ploufragan

<sup>2</sup> INRA, Station de Recherches de Virologie et d'Immunologie, 78850 Thiverval-Grignon

<sup>3</sup> Station de Recherches et de Développement Vétérinaire et Nutrition Animale - 37400 Amboise

### **Résumé**

*Dictyocaulus viviparus* CHEZ LE VEAU : DETERMINATION QUANTITATIVE DE L'ACTIVITE ANTICORPS DANS LE SERUM ET LES SECRETIONS RESPIRATOIRES PAR ANALYSE IMMUNO-ENZYMATIQUE. — La cinétique des anticorps réagissant avec un antigène préparé à partir de vers adultes de *Dictyocaulus viviparus* a été suivie par une technique immuno-enzymatique (Elisa), dans le sérum et les sécrétions nasales de veaux ayant subi une infestation mixte avec *D. viviparus* et des strongles digestifs. Les anticorps ont été également recherchés dans les lavages broncho-pulmonaires effectués à l'abattage de ces veaux, 53 jours après l'infestation.

Dans le sérum et les sécrétions nasales, les anticorps apparaissent au cours de la cinquième semaine.

L'activité anticorps des sérums est due en totalité aux IgM et aux IgG, alors que des anticorps IgA existent à l'état de traces dans les sécrétions nasales et en concentrations notables dans les lavages broncho-pulmonaires.

After a first infection or after vaccination, calves become resistant to further infections with *Dictyocaulus viviparus* (Jarrett *et al.*, 1959 ; Jarrett and Sharp, 1963 ; Michel, 1962).

Infected calves produce precipitating (Michel and Mackenzie, 1965 ; Swietlikowsky, 1969), hemagglutinating (Tewari *et al.*, 1972), or complement fixing antibodies (Weber, 1958 ; Jarrett *et al.*, 1959 ; Cornwell and Michel, 1960), but no correlation was found between antibody titers and protection.

The protection can be transferred by serum, but very large amounts are needed (Jarrett *et*

*al.*, 1955). Importance of serum antibodies in the protection mechanism is therefore questionable.

In this disease, host-parasite relationships are very close in lungs. A challenge infection of immune calves is followed by inhibition of parasite growth (Michel, 1954), decrease of worm burden (Michel and Mackenzie, 1965) and development of lymphoid nodules in lungs (Jarrett and Sharp, 1963 ; Pirie *et al.*, 1969).

The mechanisms of such reactions had certainly an immunological basis and we investigated the antibody secretion in parasitised

lungs through an immunoenzymatic assay (Elisa) to demonstrate that a local immune response was really operating during infection.

## Materials and methods

### Infections

Eight calves (Française Frisonne pie noire) were used. They were three months old (70 kg) at the beginning of the experiments and were untreated controls of an experiment designed to test the efficiency of a new anti-*Dictyocaulus viviparus* substance in polyparasitised calves (Raynaud, unpublished results).

One calf was left uninfected, whereas seven were first infected with *Dictyocaulus viviparus* and then with intestinal nematodes (*Trichostrongylus* spp. 49%, *Cooperia oncophora* 41%, *Ostertagia ostertagi* 10% and *Oesophagostomum radiatum*) 6 days later (table 1).

### Parasitic counts

#### Larvae :

They were measured according to Raynaud (1970) by using potassium iodomercurate solutions and estimated as number of larvae per gram of faeces.

Intensity of the infections was estimated according to Raynaud (1975).

#### Adult worms :

Adult *Dictyocaulus viviparus* were collected after dissection of infected lungs and incubation of lung sections for 16 hours in water at 37°C.

Adult intestinal worms were estimated by counting both free and encysted parasites in the intestine. Here again, intensity of the

infections was appreciated according to Raynaud (1975).

### Serum and nasal secretion samples

Blood and nasal secretion samples were collected weekly during seven weeks post infection. Pulmonary washings were obtained at slaughter.

Nasal secretions were obtained through tampons which were inserted in nostrils of calves for 15 minutes and then squeezed out in a syringe. The fluids were centrifuged immediately (700 g, 15 min) and stored at -20° C until use. Samples containing blood were discarded.

For bronchopulmonary secretions, 150 ml of phosphate buffered saline were injected with a respiratory probe (Delamotte, Paris, France). Washing fluids were centrifuged and stored at -20° C. The collected fluids had variable volumes from one animal to another. The true secreted volumes were estimated from the optical density at 280 nm of the samples by assuming that protein concentration of the washings is a characteristic of the species. Antibody titers were expressed for such corrected volumes.

### Antigen

Adult *Dictyocaulus viviparus* were ground and delipidated according to Benex (1974). 25 mg of dried powder were diluted in one ml of a 0.05 M pH 7.8 phosphate buffer.

### Anti-bovine immunoglobulin antibodies

Two anti-bovine immunoglobulin antisera were produced in rabbits by hyperimmunising them with either bovine IgG or with bovine  $\alpha$  chains (this last serum was a gift from Dr D. Leveux, INRA, Theix). Antibodies were purified by immuno-absorption on respective polymerized antigens and elution with 0.1 M glycine-H Cl buffer (Avrameas and Ternynck, 1969).

Purified antibodies were conjugated with alkaline phosphatase (Sigma type VII, St-Louis, U.S.A.) with 0.2% glutaraldehyde (Merck, Darmstadt, G.R.) according to Engvall and Perlmann (1972).

Specificity assays of the two conjugates were performed by fixing them on purified bovine IgG, IgM or IgA sensitized microtitration plates. The anti-IgG conjugate is called

Table 1. - Infections of calves

D <sub>0</sub>	D <sub>0</sub> + 6	D <sub>0</sub> + 53
↑	↑	↑
<i>D. viviparus</i>	Intestinal nematodes	Slaughtering
3500 L <sup>3</sup> /Al*	100000 L <sup>3</sup> /Al	

Al\* = Animal

MG since it can bind to either IgM or IgG, whereas the anti-IgA conjugate is called a since it recognizes only IgA molecules.

*Antibody titration*

An immunoenzymatic technique was used, the optimal conditions of which were determined in preliminary experiments.

100 µl of the 100 fold diluted antigen in 0.1 M carbonate bicarbonate buffer pH 9.6 were used to sensitize microtiter plastic plates (ISVC 96 flow laboratories, Asnières, France) for 3 h at 37°C.

All subsequent washings and dilutions were performed with phosphate buffered saline containing 0.05% Tween 20 (Merck, Darmstadt, G.R.) and 0.02% natrium azide.

Broncho-pulmonary washings were used directly, whereas sera were diluted 100 fold and nasal secretions 10 fold.

100 µl of diluted samples were added to each well for 3 hours at 37°C.

After washings the linked anti-*Dictyocaulus viviparus* antibodies were used for binding the diluted conjugates (100 µl) for 3 hours at 37°C.

Enzymatic reactions were performed with the ditris salt of p-nitrophenyl phosphate (Sigma chem Co. St. Louis, Mo) in 5 × 10<sup>-2</sup> M carbonate, 10<sup>-3</sup> M MgCl<sub>2</sub>, pH 9.8 buffer.

The enzymatic reactions were stopped after one hour (unless stated in the text) with 4 N NaOH and the yellow colour was read at 400 nm.

**Results**

*Parasitological studies*

*Dictyocaulus viviparus* infections.

L<sub>1</sub> larvae were eliminated from day 30 post infection in three out of seven calves. On day

31 post infection, all calves were eliminating L<sub>1</sub> larvae (fig. 1).

This elimination peaked one or two weeks later and began to decrease on day 43 post infection (37th day in calf 33, 49th day in calf 48) and became non existent on day 53 post infection.

At slaughter, adult worm loads were low (Table 2).

Intestinal nematode infections.

From the egg counts in faeces and from the worm loads at autopsy (Table 3a and 3b), one can deduce that the level of infection is either high or intermediate (calves 2 and 73).

*Seric antibodies*

About antibody titers, the expression : high,

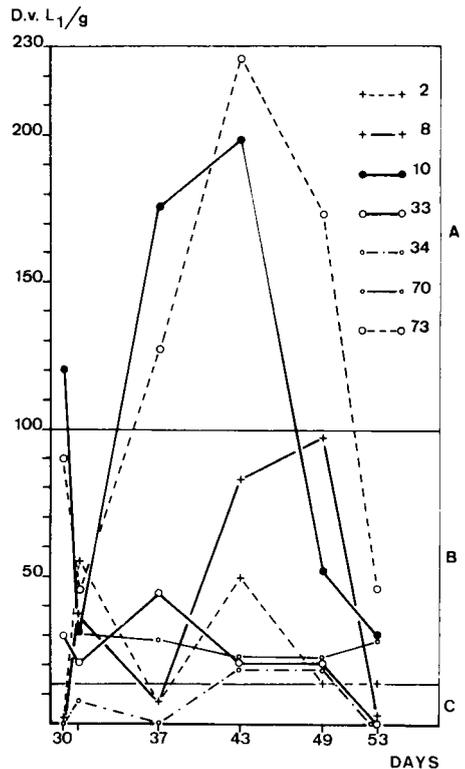


Fig. 1 : Fecal elimination of L<sub>1</sub> *Dictyocaulus viviparus* larvae in different calves. A, B and C are related to the level of quantitative coproscopies. A = high level ; B = intermediate level ; C = low level ; 2, 8, 10, 33, 34, 70, 73 : identification of calves.

Table 2. - *Dictyocaulus viviparus* in the lungs

	Calf number						
	2	8	10	33	34	70	73
<i>D. viviparus</i>	53	147	141	29	0	61	109

Table 3a. - Intestinal nematode eggs in the faeces

	Calf number						
	2	8	10	33	34	70	73
D + 30	.. 90	.. 400	.. 800	..1,400	..450	.. 950	..250
D + 31	..800	..1,500	.. 850	..1,400	..350	..1,050	..500
D + 37	..200	..2,700	..1,150	..1,200	..800	.. 800	..200
D + 43	..450	..1,700	.. 650	..1,750	..650	..1,200	..250

Results are expressed as eggs/g of faeces without differentiation between parasites

.. low level (0-50)	.. intermediate level (50-500)	.. high level (500-2,500)	.. very high level ( < 2,500)
------------------------	-----------------------------------	------------------------------	----------------------------------

Table 3b. - Intestinal nematodes in intestine of calves

	Calf number						
	2	8	10	33	34	70	73
parasites	5,320	9,920	6,210	19,440	0	11,000	4,900

low, intermediate level which is given here, has no absolute value but is only related to these experiments.

With MG conjugate (fig. 2), antibodies were detected during the 5th week post infection (4th week for calf 33).

The antibody titers were immediately high in calves 2, 10, 33. These titers increased till day 49 post infection, but in calves 3 and 10 the titers remained stable during the last week.

A conjugate did not detect antibodies in the sera. This absence was not the result of an experimental artifact since the conjugate detected IgA antibodies in pulmonary washings.

#### Nasal antibodies

Conjugate MG (fig. 3) detected antibodies during the 4th week post infection in several calves, and during the following week for calves 34 and 70.

Calves 2, 8, 33 had very intense antibody productions, but their titers decreased rapidly

whereas other calves showed increasing responses.

Only traces of antibodies were detected with the A conjugate.

#### Antibodies in broncho-pulmonary washings (Table 4).

Antibody titers varied from one calf to another but IgA antibodies accounted for part of antibody activity in all animals.

#### Discussion

Elisa enabled us to detect antibodies during the fourth week post infection with our antigen which had been prepared from adult *Dictyocaulus viviparus*.

Labelled conjugates had already been used for the titration of serum antibodies in sheep infected with *Dictyocaulus filaria*. Movsesijan (1971) and Movsesijan and Lalic (1971) sho-

Table 4. - Antibodies in broncho-pulmonary washings (53 days post infection)

Calf number	Sample without mucus		Elisa with A conjugate		Elisa with MG conjugate	
	volume (ml)	O.D. (280 nm)	O.D. (400 nm)	ratio IgA <sup>a</sup>	O.D. (400 nm)	ratio IgG + IgM <sup>b</sup>
2	24	3.48	0.43	4.0	0.99	3.3
8	68	1.62	0.38	2.6	0.59	1.5
10	52	1.45	0.38	4.0	1.05	4.1
33	33	3.50	0.70	4.7	1.58	3.8
34	60	1.21	0.36	3.8	1.00	3.8
70	31	3.70	0.40	2.6	0.48	1.0
73	52	1.46	0.50	5.0	0.64	2.3
Control	11	1.01	0.01	1.0	0.04	1.0

a : Ratio IgA :  $\frac{\text{IgA antibody titer/ml of sample (calf number X)}}{\text{IgA antibody titer/ml of sample (control calf)}} = \frac{\text{O.D. 400 nm A conjugate) calf number X}}{\text{O.D. 400 nm A conjugate) control calf}} \times$  (volume) (O.D. 280 nm) control calf

b : Ratio IgG + IgM :  $\frac{\text{O.D. 400 nm (MG conjugate) calf number X}}{\text{O.D. 400 nm (MG conjugate) control calf}} \times \frac{\text{(volume) (O.D. 280 nm) control calf}}{\text{(volume) (O.D. 280 nm) calf number X}}$

Table 5. - Immune responses and infection

Calf number	Fecal elimination of <i>D. viviparus</i>	<i>D. viviparus</i> counts in respiratory tract	Fecal elimination of intestinal nematodes	Nematode counts in intestinal tract	Serum antibody levels	Antibody level in nasal secretions	Antibody level in broncho-pulmonary secretions	
							IgA	IgG + IgM
2	++	+	++	++	++	++	++	++
8	++ (+)	++ (+)	++ (+)	++	+	++	++	+
10	++ (+)	++	++	++	++ (+)	++	++	++
33	++	+	++	++	++	++	++	++
34	+	0	++ (+)	0	+	++	++	++
70	++ (+)	+	++	++	+	++	++	0
73	++ (+)	++	++	++	+	++	++	++

wed with immunofluorescence or radio-immunoassays that antibodies reacting with immature stages of *Dictyocaulus filaria* were present in sera of sheep 6 weeks after infection.

Complement fixing antibodies were evidenced in cattle infected with *Dictyocaulus viviparus*. Weber (1958) and Cornwell (1963), with an antigen which was very similar to ours, found such antibodies 12-13 days post infection. Jarrett *et al.*, (1959) who used a polysaccharide antigen (heated adult worm antigen) was unable to show complement fixing antibodies before the 30-35th day post infection. The antigen nature is then an important factor when considering the kinetics of antibody production.

In the experiments we report here, antibodies increased till the end of the 5th week post infection.

In nasal secretions, we first noted an antibody production during the 4th week post infection. This seemed rather a long time since

after Poulain *et al.*, (1976) reported that anti-*Nippostrongylus brasiliensis* antibodies were present in intestinal mucosa of rats on day 6 post infection, i.e. very rapidly after the worms reached the intestine.

In broncho-pulmonary washing at slaughter, antibodies were detected. In order to take into account the variability of collected volumes, we measured antibody titers in relation to the true secreted volume. This seems more valuable than to compare antibody titers in the collected volumes (Voisin *et al.*, 1973). Nevertheless we assumed that the protein concentration of such washings was invariable for further studies, we must add a labelled compound to the washing buffer in order to know the exact dilution of the washing fluid.

IgA antibodies were readily found in broncho-pulmonary washings, whereas there were only traces in nasal secretions and they did not appear at all in sera.

Low IgA titers are rather unusual in local secretions. These results can be explained in the following ways :

- 1) IgA are degraded between collection and

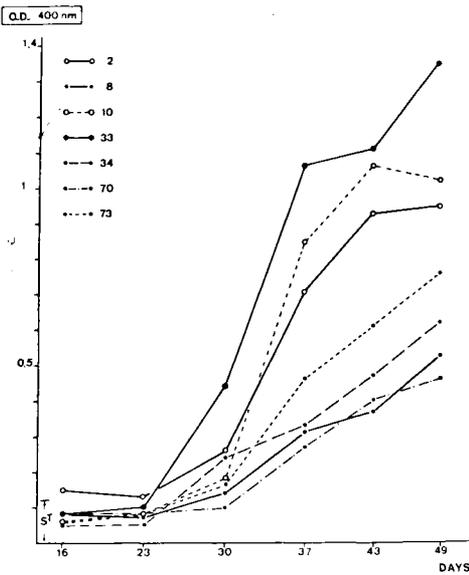


Fig. 2 : Serum antibodies of calves infected with *Dictyocaulus viviparus* and intestinal nematodes (one curve/calf).  
St = serum antibodies in the control calf.

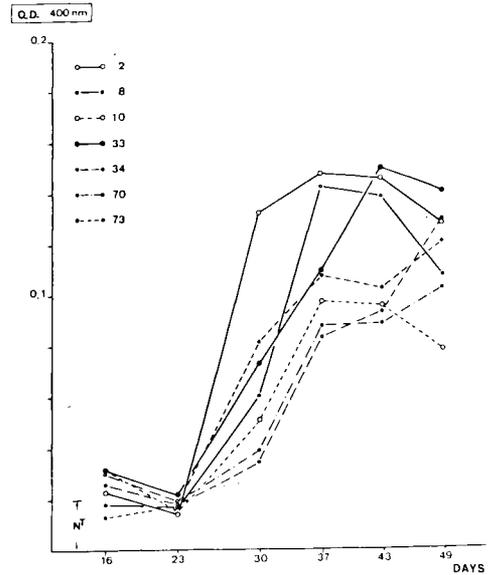


Fig. 3 : Antibodies in nasal secretions of calves infected with *Dictyocaulus viviparus* and intestinal nematodes (one curve/calf).  
Nt = antibodies in nasal secretion of the control calf.

freezing of the samples or IgA are eliminated with mucus during centrifugation.

2) IgA are diluted by transudation of plasma as a result of local inflammation.

3) IgA are not secreted as a result of lesions of epithelium. Indeed, in human patients suffering from either an acute inflammation (influenza : Bellanti *et al.*, 1965) or a chronic inflammation (overuse of tobacco : Reynolds and Newball, 1976), IgG are the main immunoglobulins of respiratory secretions. Charlton and Glanford (1977) have shown that pulmonary washings of mice which had received by nasal route homogenates of cells infected with parainfluenza contained early IgM and IgG antibodies whereas IgA antibodies are detected very late. IgA titers decreased also with the number of challenges. They suggested that this low and delayed IgA secretion together with lesions of lungs can be explained by an arrest of synthesis of the secretory component by the injured epithelial cells.

From the kinetics, the titers and the class of the antibodies were measured, and we can infer that at least part of locally detected antibodies are synthesized locally.

In calves 2, 10, 33, 8 the antibody titers in nasal secretions were already decreasing when antibodies were produced in the serum, and calf number 8 had high local and low seric antibody titers.

Since IgA are found locally when they are not present in serum, we cannot think that they are transudated from the systemic circulation.

Our calves had been polyparasitised and cross reactions are rather usual phenomenon between nematodes : Poynter (1963), Sinclair (1964), Capron *et al.*, (1968) and the antigen we used was not purified. We cannot therefore know if the antibodies we detected came from infection with *Dictyocaulus viviparus*. We cannot absorb antibodies reacting with other parasites, since we did not have antigens from each developmental stage and fluids of these parasites. Such absorption can withdraw antibodies which are induced by *Dictyo-*

*caulus viviparus* but react with other parasite. Indeed it seems that the antibodies we detected are directed against shared antigenic determinants (Marius, unpublished results).

Two mechanisms can be put forward to explain our results :

1) Local antibodies were induced locally by *Dictyocaulus viviparus* the developmental life-cycle of which had a respiratory phase whereas other parasites had no such phase.

2) Local IgA antibodies were secreted by IgA bearing cells which had been stimulated in the intestine and then migrated to the lungs.

Since we do not know the circulation of bovine lymphocytes we favour the first hypothesis.

Further experiments are needed to establish a correlation both in quantitative and in qualitative terms between the antibody production in nasal secretions and in broncho-pulmonary secretions.

We looked also for a correlation between immune responses and parasite eliminations.

Since poly-infections must have interfered with infection with *Dictyocaulus viviparus*, we selected calves which behaved similarly against intestinal nematodes (calves number 2, 73 and calves 8, 10, 33, 70).

Table 5 shows that in calves 2 and 73, 8 and 10 where the systemic response was low, larval elimination which was high at the beginning, decreased as local immune response gained a threshold. In calf number 33 which had both high local and high systemic responses, fecal elimination was low. However antibody titers cannot explain the behaviour of calves number 70 and 34 which showed low larval elimination. This may suggest that larval elimination is subject to complex regulation.

Further studies on calves, infected with *Dictyocaulus viviparus* alone, are required to know if this parasite can induce a local antibody production and to know the relationships between local antibodies and protection.

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### Summary

An enzyme-linked immunosorbent assay (Elisa) which is in detail described here has been adapted to trace antibody production in different fluids from infected calves.

Seven calves were infected both with *Dictyocaulus viviparus* and with several intestinal nematodes. Antibodies against an antigen prepared from *Dictyocaulus viviparus* adult worms were

first detected during the 5th week post-infection, both in serum and nasal secretions, and were also observed in bronchial washings of the same calves slaughtered 53 days post-infection.

IgA antibodies were never evident in the serum but were found in limited quantities in nasal secretions. In bronchial washings at slaughtering, a significant part of antibody activity is accounted for by the local IgA production.

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