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SELECTION AND CHARACTERIZATION OF A PRECOCIOUS LINE OF *Eimeria intestinalis*, AN INTESTINAL RABBIT COCCIDIUM.

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INTRODUCTION

In spite of the many studies carried out on poultry, no attempt to immunize chickens with killed coccidia or material derived from these parasites has been able, until now, to protect the animals from subsequent infection. Only inoculation with live coccidia provides protection (LONG and ROSE, 1982; ROSE, 1982; 1986; ROSE and LONG, 1980). In addition, the use of coccidia strains with attenuated pathogenicity has been the object of several studies (JEFFERS, 1986).

One of the ways to obtain an attenuated strain is to obtain a precocious line, generally by selecting the first oocysts excreted, during successive passages in the host animal (JEFFERS, 1975; JOHNSON et all, 1986; Mc DONALD and BALLINGALL, 1983 a, b; Mc DONALD et all, 1982; 1986; SHIRLEY et all, 1984; SHIRLEY and BELLATTI, 1984).

In the rabbit, *Eimeria intestinalis* is without doubt one of the most pathological coccidia (CATCHPOLE and NORTON, 1975; COUDERT, 1976; 1979; LICOIS and COUDERT 1982; LICOIS et all, 1978 a, b; PEETERS et all, 1984). Although *E. intestinalis* isn't the most common, PEETERS et all (1981) as well as ZUNDEL et all (1980) found it in over 21% of the battery-reared animals. Moreover, we have already demonstrated that *E. intestinalis* has considerable immunogenicity (LICOIS and COUDERT, 1980 a).

We therefore felt that it is of interest to obtain a precocious line of this species and characterize it.

MATERIALS AND METHODS

1 - Animals

Six to seven week old New Zealand rabbits (INRA strain A 1077) were used. They were obtained free of coccidia from the Station de Pathologie Aviaire et de Parasitologie de l'INRA de TOURS and reared like SPF animals (SCHELLENBERG, 1976; COUDERT et all, 1979).

2 - *E. Intestinalis*

The method used to obtain the parental strain (EiO) and the precocious line (EiP) is indicated in table 1.

* Original strain. Beginning with a mixture of *E. intestinalis* and *E. magna* isolated in 1975 from the caecal content of a rabbit striken with coccidiosis, we formed 5 clones in 1985 by inoculating 5 animals with 1 oocyst of *E. intestinalis* each. Multiplication of the mixture of the 5 clones thereby obtained in another animal provided the normal or original strain (EiO). In fact, for each multiplication, the animals were sacrificed 10 days after infestation. This corresponds to the excretion peak in this species (COUDERT and LICOIS, 1988). The strains were isolated from the caecal content.

* Precocious strain. It was derived from the original strain after only six passages. The oocysts were recovered in the caecum during the last multiplication, 6 days after infestation.

3 - Excretion Curve and Rate of Multiplication

Two separate rooms comprising 6 cages each contained 12 animals divided into 3 identical lots. There were 4 animals per lot and 2 animals per cage. In one room, the rabbits were inoculated with the EiP strain with doses of 50, 500 and 5000 sporulated oocysts corresponding to each lot. The rabbits in the other room were inoculated with the same doses of the EiO strain.

The excreta were harvested every day between post-inoculation day 5 and 14. The coccidia counts were made according to a method previously described by LICOIS and COUDERT (1980 b).

4 - Morphological Characteristics and Sporulation Time of the Oocysts.

These two criteria are among the most simple to use in poultry for the diagnosis of the different species of *Eimeria* (NORTON and CHARD, 1983).

* The measurements of the sporulated oocysts were determined for both the EiP and EiO strains according to the technique described by COUDERT et all (1979) in order to check whether selection for precociousness does not change the size of the oocysts.

* In the rabbit, a detailed study of the sporogony of E. stiedai was performed by COUDERT et all (1973) and that of E. perforans by COUDERT et all, (1979). We carried out a similar study of the sporogony of the EiP and EiO strains and followed the change in this evolution at 3 different temperatures : 18.0 ± 0.5 °C, 22.0 ± 0.5 °C and 26.0 ± 0.5 °C. In order to complete the morphological observation, the evolution of sporulation as a function of time was determined by inoculating the rabbits with aliquot samples at 4 hour intervals, between hour 49 and hour 73, of the oocyst suspension allowed to sporulate at 22 °C. The control inoculum corresponding to 100% sporulation was obtained at hour 85.

The inoculums were formed by taking the assumed percentage of sporulation into account according to the morphology so as to inoculate 5000 sporulated oocysts from the EiP strain per animal and 500 from the EiO strain. These doses were chosen since, especially for the EiO strain, the excretion increases in proportion to the inoculum, but only within a range of 1 to 1000 inoculated oocysts (COUDERT and LICOIS, 1988).

5 - Pathogenicity and Immunogenicity

Two experiments were carried out in order to measure the pathogenicity :

* The first experiment compared the EiP strain with the EiO strain. Eight rabbits per dose were infested with 50, 500, 5000 and 50000 oocysts for each strain. Eight non-inoculated animals were used as controls. The pathogenicity was determined by measuring the weight gain in the animals, with individual weighings carried out twice a week.

* In the second experiment, we compared the evolution of weight gain of rabbits only inoculated with doses of $4.6 * 10^3$, $15.5 * 10^3$, $93 * 10^3$, $880 * 10^3$ and $2440 * 10^3$ of the EiP strain. There were 18 animals per dose and a control group of 18 non-inoculated animals. The animals were individually weighed twice a week. Moreover, the oocyst excretion was evaluated over two periods going from day 4 to 10 and then day 10 to 14 after infestation.

* The immunogenicity was evaluated by reinoculating the animals in the first experiment 14 days after the first infestation. Five thousand oocysts from the strain that wasn't used the first time were used for the second inoculation. The rabbits were weighed twice a week and the oocyst excretion was determined between day 6 to 11 after the second inoculation for the precocious strain and between day 9 to 14 for the original strain.

RESULTS

1 - Excretion Curve and Rate of Multiplication

Figure 1 provides the results for the daily oocyst output from the EiP line as compared with those from the original strain.

Excretion with the EiP strain begins on day 6 instead of day 9 with the EiO strain. However, the oocysts are really only detected with the dose of 5000 inoculated oocysts. With the EiO strain, several oocysts may be excreted as of day 8 after infestation with high doses (5000).

With the EiP line, the excretion peak is at day 7 with all doses. As of day 11, oocysts are no longer detected with the doses used. With the EiO strain, the higher the dose of inoculum, the later the peak. This is certainly due to the fact that the animals can fall ill with a few hundred E. intestinalis oocysts (refer to pathogenicity). This may lead to a slowing down of the transit (FIORAMONTI et all, 1981) and a delay in the excretion of the feces. In the absence of illness (dose 50), the excretion peak is on day 10.

The total oocyst output (table 2) is considerably reduced in rabbits inoculated with the EiP strain. This reduction varies by a factor of 1000 to 10000. Whereas total excretion reaches a peak at about 3 to $7 * 10^9$ oocysts excreted with more than 1000 oocysts inoculated from the EiO strain, these figures are attained only with doses of over $5 * 10^5$ oocysts inoculated with the EiP strain (table 3).

2 - Morphology and Sporogony

About 150 oocysts from each strain were measured. The mean values for these measurements are given in table 4. Although the 1µ difference, whether for the length or width is highly significant ($P < 0.001$), these values agree with those already cited by CHEISSIN (1948), PEETERS et all (1981) and PELLERDY (1954). The more or less pear or lozenge-shape of the oocyst from the EiP line is strictly identical to that of the EiO line. The micropyle is

clearly visible. However, noteworthy differences are found in the sporulated sporocysts.

The oocyst from the original strain has 4 identical sporocysts each with 2 sporozoites and a residual sporocystic body. Each of the sporozoites encloses a refringent globule. The 4 sporocysts are not identical in the precocious strain. Two of them have a large refringent globule whose diameter is about double that of the parent strain although it is not possible to distinguish the sporozoites. The two other sporocysts also differ from those from the initial strain. No refringent globule is observed.

The differences between the 2 strains are manifested during sporulation. Although the sporulation of the wild strain is similar to that described for E. stiedai (COUDERT et all, 1973) and the oocysts from both strains are perfectly identical when just excreted, after several hours of sporulation, a type of vacuolization in the center of the sporoplasme is observed in the EiP line. This seems to be the source of the refringent globule that is clearly detected at the "outset" stage and that is found in the subsequent stages, divided into two and associated with 2 bodies (sporocysts) out of 4 until the "sporulated" stage.

The sporulation time at 18, 22 and 26 °C is indicated in table 5. The sporogony evolves almost at the same speed in both strains. The "initial" stage is relatively easy to identify by microscope and appears after 52, 33 and 24 hours at 18, 22 and 26 °C respectively. However, the "sporulated" stage is much more difficult to recognize. One of the criteria retained is the division of a granular although homogenous material in 2 parts inside each sporocyst corresponding to the sporozoites inside of which a refringent globule can be observed. Therefore, we estimated that the "sporulated" stage begins at about hour 50 (1% of the oocyst population) and ends at about hour 70 (100% of the oocyst population) with the original strain at 22 °C. However, with the EiP line, we noted that the refringent globules appear at the first stages of segmentation. It is therefore almost impossible to determine when there is actually a "sporulated" stage. For this reason, we tried to check the sporulated or infesting stage of the oocysts by carrying out inoculations of rabbits at regular intervals from the oocyst suspension allowed to sporulate at 22 °C. The results of these inoculations are provided in figure 2.

In both strains, with respect to an inoculation of an entirely sporulated oocyst population (hour 85), 1% of the oocysts are infesting at about hour 63, 50% between hour 73 and 75 and 100% of the population is sporulated after hour 80.

3 - Pathogenicity

With the EiP line, whatever the dose inoculated, there is little difference in comparison with the control group. This is also true with the 50 oocyst dose inoculated from the EiO strain. However, the pathogenicity is high from 500 oocysts onwards confirming the acute nature of coccidiosis by E. intestinalis (COUDERT, 1976; LICOIS et all, 1978 a, b; LICOIS and COUDERT, 1982; PEETERS et all, 1984). Mortality is induced by 5000 oocysts but curiously, there is less mortality with 50000 oocyts. With the EiP line, it is necessary to have very high doses of inoculum (over $8 * 10^5$) in order to observe any consequences on the weight gain (figure 3). Even though lesions with EiO arise from 500 oocysts, it is necessary to inoculate over $5 * 10^5$ with EiP in order to obtain the same results. In both cases, the localization of the macroscopic lesions is strictly identical, that is, they affect the ileum and to a lesser degree the jejunum.

4 - Immunogenicity

Inoculation of 50, 500 and 5000 oocysts from the EiO strain provides very effective protection to the animals against subsequent infestation with the precocious line whether the degree of protection is evaluated by oocyst excretion (table 6) or weight gain (figure 4). This confirms the highly immunizing nature of E. intestinalis as published elsewhere (COUDERT and LICOIS, 1988; LICOIS and COUDERT, 1980). However, the same doses of inoculum from the precocious strain do not seem to protect from the original parent strain although a slight reduction as a function of the dose is observed in oocyst excretion. Complementary tests currently being analysed seem to indicate that protection is acquired with primary infestations superior to 10 000 oocysts from the EiP line.

DISCUSSION

Selection of the first oocysts excreted after experimental infestation and serial passage in the animal made it possible to obtain a precocious line of E. intestinalis . As far as we know, this is the first line of this type obtained for a rabbit Eimeria

The characteristics of this strain, compared with the parent strain show that the prepatent period is considerably reduced. According to CHEISSIN (1948), it varies from 214 to 216 hours for a wild strain. This corresponds to our own observations. It drops to 144 hours for the EiP line, a 33% reduction, which is in general superior to the reduction in the prepatent periods observed for precocious lines of Eimeria in the chicken (table 7). Moreover, the number of passages required to produce a precocious line is relatively high (over 10 generations) in poultry. In the case of the EiP strain, only 6 passages were required and 48 hours were gained between the 5th and 6th generation. Besides the selection pressure exerted, this may also be the result of a mutation as most likely indicated by the morphological

anomalies of the sporocysts that we noted. The shape of the oocyst as well as the size correspond to descriptions of E. intestinalis. Moreover, the site of the lesions in the second part of the small intestine and the fact that only one inoculation with 50 oocysts of the original strain completely prevent the multiplication of the precocious strain, indicate that E. intestinalis is concerned. However, changes in the inner structure of the oocyst were never reported in Eimeria. Nervertheless, morphological changes in the inner part of the cycle were noted with E. tenella (JEFFERS, 1975).

As regards the rate of multiplation and the pathogenicity, the EiP line has characteristics in common with the precocious lines of coccidia in birds, that is, a considerable reduction in the oocyst output and a major reduction or disappearance of the pathogenic effects with doses that are usually dangerous for the animals (JEFFERS, 1975; JOHNSON et all, 1986; Mc DONALD and BALLINGALL, 1983 a, b; Mc DONALD et all, 1982; 1986; SHIRLEY and BELLATTI, 1984; SHIRLEY et all, 1984). Only BEDRNIK et all (1986) report that their precocious strain of E. tenella did not lose its multiplication power or virulence.

As regards the immunogenicity other tests are required in order to confirm the immunizing nature of the EiP strain. If such is the case, this line will prove to be a good model to study the mechanisms of acquired resistance with coccidosis. It is also necessary to study the internal development of the parasite in order to try to account for the important reduction in the production of oocysts.

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TABLE 1 . Selection of EiO and EiP strains.

DATE	STRAIN	DOSE OF OOCYSTS GIVEN	FIRST DETECTION OF OOCYSTS IN THE CAECUM (DAYS)
1975	Mixed strains of *E. intestinalis* and *E. magna*		
1985	Isolation of 5 clones of *E. intestinalis*	One oocyst of *E. intestinalis* per clone	8 1/2 (10)
24.01.1986	PA 1986.01 = PARENT STRAIN (EiO)	4000	8 1/2 (10)
9.12.1986	PA 1986.01/1	100000	8 1/2
13.01.1987	PA 1986.01/2	<10000	7 1/2
3.02.1987	PA 1986.01/3	<10000	8
17.02.1987	PA 1986.01/4	<10000	7 1/2
3.06.1987	PA 1986.01/5	<10000	7 1/2
16.06.1987	PA 1986.01/6 = PRECOCIOUS STRAIN (EiP)	10000	5 1/2 (6)

( ) Day of oocyst recovery for the constitution of the EiO and EiP inoculum.

TABLE 2 . Total oocyst output per animal between the 6th and the 14th day after infection of rabbits given 50, 500 or 5000 oocysts of EiP or Ei0 strains.

		NUMBERS OF OOCYSTS GIVEN		
		50	500	5000
OOCYSTS YIELD /RABBIT	EiP	$4,60.10^{4}$	$5,62.10^{5}$	$7,70.10^{6}$
	Ei0	$4,40.10^{8}$	$3,79.10^{9}$	$7,64.10^{9}$

TABLE 3 . Total mean oocyst output of animals inoculated with high numbers of oocysts of the EiP strain.

	OOCYST OUTPUT		
	DAY 4 TO DAY 10	DAY 11 TO DAY 14	TOTAL
NUMBERS OF OOCYSTS GIVEN	$4,2.10^{3}$	$1,5.10^{7}$	$1,2.10^{6}$	$1,6.10^{7}$
	$1,6.10^{4}$	$1,7.10^{8}$	$1,0.10^{7}$	$1,8.10^{8}$
	$9,3.10^{4}$	$1,1.10^{9}$	$1,4.10^{8}$	$1,2.10^{9}$
	$8,8.10^{5}$	$3,8.10^{9}$	$8,5.10^{8}$	$4,6.10^{9}$
	$2,4.10^{6}$	$5,3.10^{9}$	$1,7.10^{9}$	$7,0.10^{9}$

TABLE 4 . Mean oocyst size (length and breadth, µ), of oocyst residuum and refractile globules of the two strains EiP and EiO : m ± sm and (range). A comparison with the data from different authors for E. intestinalis.

		Oocyst length	oocyst breadth	oocyst residuum diameter	refractile globule diameter
Personal results	EiO	26,09 ± 2,01	18,08 ± 1,10	6,07 ± 1,09	3,4 ± 0,6
		(21 - 31)	(16 - 20)	(3,1 - 7,8)	(2,5 - 4,5)
	EiP	27,10 ± 1,90	19,14 ± 1,04	6,60 ± 1,01	7,1 ± 0,6
		(22 - 31)	(16 - 21)	(4,6 - 8,5)	(5,4 - 8,5)
CHEISSIN (1948)		(27,1 - 32,2)	(16,9 - 19,8)	-	-
PELLERDY (1954)		27	18	-	-
		(23 - 30)	(15 - 20)	(3 - 5)	-
PEETERS (1981)		28,70 ± 1,82	18,40 ± 1,19	-	-
		(22,6 - 34,7)	(15,6 - 22,6)	-	-

TABLE 5 . Oocyst sporulation time (hours) at 18, 22 and 26°C for the EiP strain.

		TEMPERATURE OF SPORULATION		
		18°C	22°C	26°C
SPORULATION TIME	STAGE "OUTSET"	52	33	24
	STAGE "4"	60	39	28
	STAGE "8" : 1%	74	53	46
	STAGE "8" : 50%	95	61	-
	STAGE "8" : 100%	105	69	60

The "outset" stage corresponds to the beginning of the division of the sporoplasm; the stage "4" to the sporocyst formation and the stage "8" to the sporozoïte formation (sporulated oocysts). For the last stage, the sporulation time was recorded when 1%, 50% or 100% of sporulated oocysts were recognized.

TABLE 6 . Acquired immunity with EiP and Ei0 strains, determined by the total output of oocysts following a challenge inoculation with the strain not used for the primary inoculation (cross immunity). The second infestation was carried out, 14 days after the primary infestation, with 5000 oocysts given per animal.

			CHALLENGE INOCULATION (oocyst output / rabbit)	
			EiP	Ei0
PRIMARY INOCULATION (No. of oocysts inoculated)	EiP	50	ND*	$6,9.10^9$
		500	ND	$5,1.10^9$
		5000	ND	$1,4.10^9$
	Ei0	50	ε	ND
		500	ε	ND
		5000	ε	ND

* ND : Not Done

TABLE 7 . Reduction of the prepatent period of the precocious E.intestinalis line. A comparison with the reduction of prepatent periods of different strains of Eimeria from the chicken.

SPECIES	Prepatent period of the Parent Strain (hours)	Prepatent period of the Precocious Line (hours)	Reduction of prepatent period hours (%)	No. of passages with selection to obtain a Precocious Line	AUTHORS
E. intestinalis	216	144	72 (33,3)	6	personal data
E. maxima	120	107	13 (10,8)	15	Mc DONALD et all., 1986
E. necatrix	138	126	12 (8,6)	20	SHIRLEY, 1983
E. praecox	84	64	20 (23,8)	>10	SHIRLEY et all., 1984
E. mitis	93	67	26 (28,0)	14	Mc DONALD and BALLINGALL, 1983 a
E. brunetti	120	75	45 (37,5)	20	JOHNSON et all., 1986
		84	36 (30,0)	27	SHIRLEY et all., 1986
E. acervulina	89	70	19 (21,3)	14	Mc DONALD et all., 1982
		62	27 (30,3)	71	Mc DONALD and SHIRLEY, 1986
E. tenella	120	105	15 (12,5)	46	JEFFERS, 1975

Fig. 1 : Mean daily oocyst output from rabbits given 50 (□), 500 (+) and 5000 (◊) oocysts of the EiP line and 50 (Δ), 500 (X) and 5000 (▽) oocysts of the EiO strain.

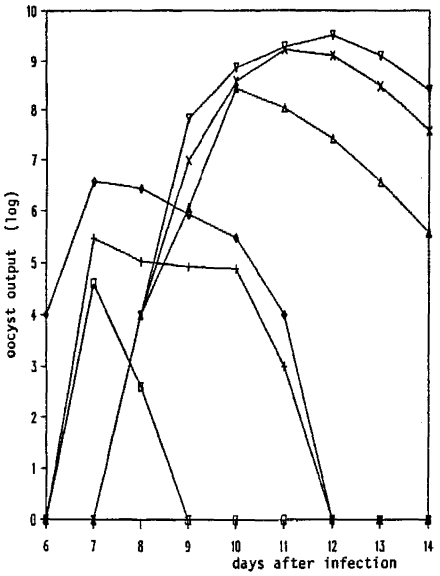
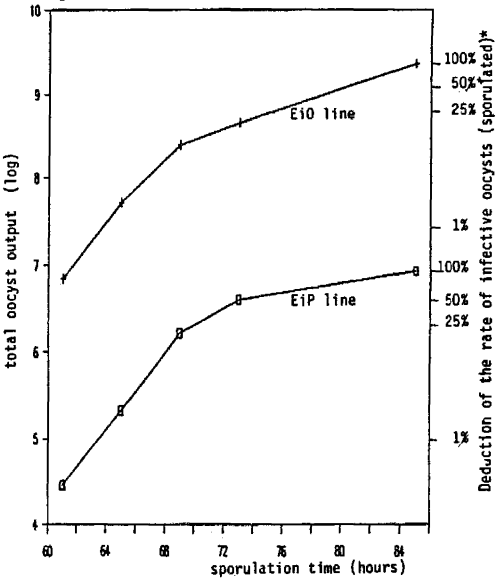


Fig. 2 : Total oocyst output assessed by inoculation at 4 h. intervals of sporulating oocysts as an indicator of the sporulation rate during sporogony (at 22°C). 500 oocysts of the EiO strain and 5000 oocysts of the EiP line were inoculated.



* After 85 hours of sporulation at 22°C, the number of sporulated oocysts does not change. So we have considered that 100% of oocysts were sporulated at the 85th hour.

Fig. 3 : Mean weight gain of uninfected controls (□) and of rabbits given $4,6x10^3$ (Δ), $1,6x10^4$ (+), $9,3x10^4$ (◊), $8,8x10^5$ (X) and $2,4x10^6$ (▽) oocysts of the EiP line.

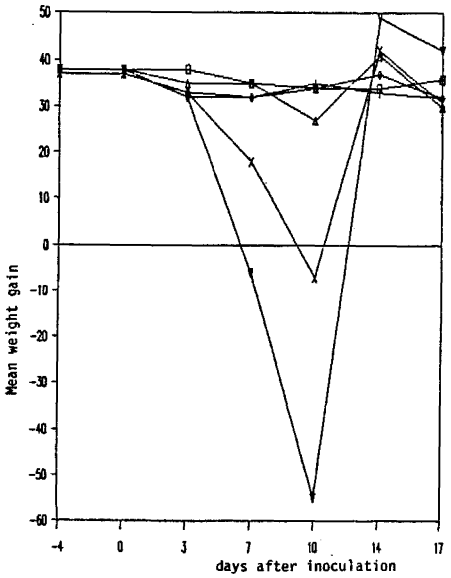
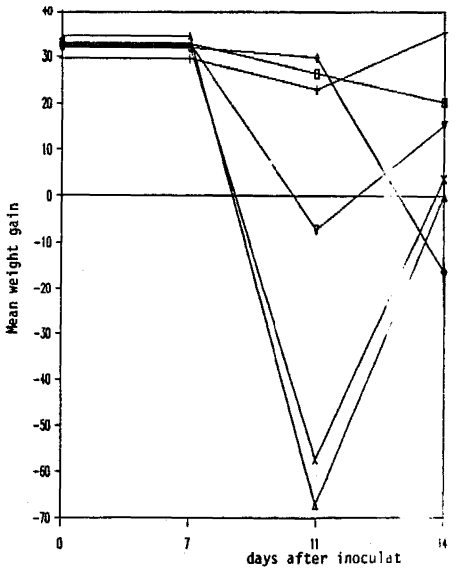


Fig. 4 : Mean weight gain 14 days after challenge of rabbits given 50 (□), 500 (+) and 5000 (◊) oocysts of the EiO strain or 50 (Δ), 500 (X) or 5000 (▽) oocysts of the EiP line with 5000 oocysts of either strain



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SUMMARY

SELECTION AND CHARACTERIZATION OF A PRECOCIOUS LINE OF EIMERIA INTESTINALIS, AN INTESTINAL RABBIT COCCIDIUM.

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A precocious line of Eimeria intestinalis was obtained by selection for early development of oocysts in rabbits and after six consecutive passages in animals. This line (EiP) was derived from a wild strain (EiO) isolated in 1975 from the caecal content of a rabbit with coccidiosis. The prepatent period of the EiP strain was reduced from 215 h to less than 144 h. In return, the oocyst sporulation time was the same for both lines. The excreted and unsporulated oocysts had exactly the same shape but microscopical examination of the sporulated oocysts showed a marked difference between EiP and EiO strains. A huge refractile globule was located in each of two sporocysts of the precocious line whilst no refractile globule was seen in the other two. The EiP line had a reproductive potential much lower (1000 times) than that of its parent strain EiO and its pathogenicity as judged by the weight gain, the mortality and the lesions which occured also in the jejunum and above all in the ileum, was substantially reduced. However, it seemed that immunogenicity was retained, at least with an inoculum exceeding 10^4 sporulated oocysts which was shown to be non pathogenic.

RESUME

OBTENTION ET CARACTERISATION D'UNE LIGNEE PRECOCE D'EIMERIA INTESTINALIS, COCCIDIE INTESTINALE DU LAPIN.

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Nous avons obtenu une lignée précoce d' Eimeria intestinalis en exerçant une pression de selection pour les premiers oocystes excrétés et six passages successifs chez l'animal hôte. Cette lignée (EiP) provient d'une souche sauvage (EiO) isolée en 1975 à partir du contenu caecal d'un lapin atteint de coccidiose. La période prépatente de la souche EiP est fortement diminuée et passe de 215 h à 144 h. En revanche, la durée de sporulation des oocystes est la même dans les 2 souches. Les oocystes excrétés donc non sporulés ont rigoureusement la même forme mais l'observation microscopique des oocystes sporulés révèle une différence importante entre les 2 lignées EiP et EiO. Un gros globule réfringent est visible, pour la souche précoce, dans chacun de 2 sporocystes alors que l'on ne distingue aucun globule réfringent dans les 2 autres. Le taux de multiplication de la lignée EiP est environ 1000 fois plus faible que celui de la souche initiale EiO. Son pouvoir pathogène, apprécié d'après le gain de poids, la mortalité et les lésions qui sont situées, comme pour la souche d'origine, au niveau du jejunum et surtout de l'ileon, est considérablement réduit. Cependant il semble que son pouvoir immunogène soit conservé, au moins avec un inoculum supérieur à 10^4 oocystes sporulés, dose totalement dénuée d'effet pathogène.