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VACCINATION OF PREGNANT COWS WITH K99 ANTIGEN OF ENTEROTOXIGENIC *ESCHERICHIA COLI* AND PROTECTION BY COLOSTRUM IN NEWBORN CALVES

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Abstract—The immune response to the K99 was tested in 45 pregnant cows, subcutaneously vaccinated, for protecting the newborn calves. Serological tests were performed in the blood sera of all animals and in the milk and colostrum sera; hemogram, inhibition of the adhesion to the brush border and histological tests were performed. The calves from vaccinated cows survived the experimental infection after the suction of colostrum in spite of the fact that the calves from control dams died with diarrhea.

Key words: Colibacillosis, K99, dams vaccination, calves protection, colostrum

Résumé—La vaccination dirigée contre l'antigène K99 d'*Escherichia coli* a été étudiée chez 45 vaches gestantes immunisées par voie sous cutanée. Chez tous les animaux on a mesuré le titre des agglutinines anti K99 dans le sérocolostrum et le lait des vaches ainsi que dans le sang des veaux. On a également observé les modifications histologiques au niveau intestinal et mesuré l'inhibition de l'attachement aux entérocytes par les anticorps anti K99. Les veaux recevant le colostrum des vaches vaccinées ont survécu alors que les veaux recevant le colostrum de vaches non vaccinées ont eu la diarrhée et sont morts.

Mots-clefs: Colibacilloses, K99, vaccination des vaches, colostrum, protection des veaux

INTRODUCTION

The enterotoxigenic strains of *Escherichia coli* are able to induce enteric syndromes in newborn calves producing enterotoxins and colonizing at the enterocytes by K99 antigen or other Pili [1–6].

Epidemiological surveys in different countries show however that the incidence of enterotoxigenic strains can reach a high percentage [7–10]. The disease usually occurs in calves less than three days old it can be reproduced experimentally by administering the bacterial suspension *per os* to calves, lambs or pigs early after birth either to the conventional or gnotobiotic animals [11–13].

The resistance to the infection develops rapidly with age and the enterocytes of calves one week old are resistant probably because E.T.E.C. is not able to colonize [14].

The first defensive action of the newborn comes from the colostrum. The secretion of many cows lacks the antibodies capable of inhibiting the bacterial colonization. The antigen K99 could constitute a logical basis for the preparation of a vaccine for administration to pregnant females.

The aim of this study was to test the immunological capacity of K99 by vaccination of pregnant cows and also to verify the degree of colostral protection in newborn calves.

MATERIALS AND METHODS

Bacterial strains

The enterotoxigenic strains of *Escherichia coli* B41 (O101:K^-:H^-, K99, F41) were used for the preparation of the antigen and *Escherichia coli* B44 (O9:K30:H^-, K99, F41) for the experimental infection of calves [15].

Antigen

The colonizing antigen K99 was obtained from the enterotoxigenic *Escherichia coli* B41 by means of chemical detachment of the Pili according to a process of partial purification [16–18] and was injected at a protein concentration of 15 mg in 10 ml of saline solution [19] with the somatic residual antigens associated with the incomplete Freund adjuvant [20].

Animals

Forty-nine pregnant cows were used, 6–8 years old, clinically healthy, belonging to a 200 head herd and fed silage corn, grain and concentrates.

Vaccination

Forty-five pregnant cows were vaccinated subcutaneous 6 and 4 weeks before calving; 4 pregnant cows were maintained as controls, they received a broth culture lacking bacteria [21, 22].

Experimental infection of calves

Escherichia coli B44, grown for 18 h at 37°C in Minca-Is [23] was administered to calves 5–10 h old after the first suckling of colostrum. The infective dose of 4×10^{10} colonies forming units/ml, was given orally to calves born. A rectal swab was obtained from each calf before exposure to the challenge [13].

Collection of blood and colostrum samples

The blood samples were obtained from cows at the time of vaccination and then 15 and 25 days later, within 24 h of calving and 7 days *post partum*. The colostrum was collected before the suction by the calf and 7 days after calving.

The blood samples were obtained from calves after the suction of colostrum at 12–24 h after birth and at 7 days old.

Clinical control of the animals

All the subjects underwent a direct clinical control, when samples of blood and colostrum were taken from the cows and the calves daily. The blood samples were examined for the hemogram according to the usual hematological techniques.

Serological tests

Agglutination tests on the colostrum and milk serum, obtained by microbiological digestion and centrifugation, and on the blood serum samples were performed in microtiter plates using as antigen both *Escherichia coli* B41 and its K99$^-$ mutant [18, 24].

Brush border inhibition test

The test, employing epitelial cells from the small intestine of a newborn calf, was performed with *Escherichia coli* B41 in the presence of milk and colostrum serum. An immune serum anti K99 was utilized as a positive control [18, 25, 26].

Twenty enterocytes were examined for every test. The inhibition index was expressed in percent and the value was 100 only if none of the bacteria were attached to the epithelial cells. The immune serum anti K99 gave a value of 70%.

Histology

Intestinal fragments were collected from the small intestine, immersed in formalin, dehydrated, enclosed in paraffin and stained for histological control [13, 27].

Statistical analysis

The results were prepared for the analysis of the variant in relation to a single criterium of classification [28].

RESULTS

The vaccinated cows, including the controls, did not have local reactions or general changes. The gestation and parturition were normal, followed by birth at the end of term and the milk production was within the average of the herd.

Of the 45 calves born alive and viable from vaccinated mothers only 3 presented slight depression and water diarrhea in the second and third day of age.

The 4 control calves died, one at 24 h and the others at the third–fourth day with profuse diarrhea, serious dehydration with insufficient peripheral circulation (Table 1).

In the vaccinated and the control cows the hematomorphological examination showed hypochromic anemia and neutrophilic granulocytosis during the whole period of observation, let alone a decrease, statistically significant, of the eosinophils in the third and fourth samples (Tables 2 and 3).

In the calves from vaccinated cows the hemogram performed within 24 h from birth revealed anemia and neutrophilia; further checks showed the persistence of the anemia and the inversion of the granulocyte/lymphocyte relationship (Table 4). The results of the serological tests with K99 antigen and the acapsular mutant are shown in Tables 5 and 6, Figs 1 and 2.

Table 1. Clinical observations of calves after the challenge with *Escherichia coli* B44

	Diarrhea	Feces	Depression	Fever	Mortality
Newborn calves from vaccinated dams	45/3[a]	1[b]	B[c]	45/3	45/3
Control calves	4/4	3	A	4/0	4/4

[a]Calves tested/calves sick or dead.
[b]Identified according to the consistence: 1: soft feces, 2: fluid feces, 3: watery feces.
[c]The different gravities are identified with B: low, M: medium, A: high.

Table 2. Vaccinated cows hemogram

Parameters	I° $\bar{x}$	I° S	II° $\bar{x}$	II° S	III° $\bar{x}$	III° S	IV° $\bar{x}$	IV° S	M.S.D. 0.05	M.S.D. 0.01
Ht (%)	34.70	2.07	32.70	3.09	36.30	2.15	34.20	3.01	n.s.	n.s.
PVC (g/dl)	6.58	0.50	8.00	0.70	8.80	0.70	8.40	0.60	n.s.	n.s.
RBC (10^6/mm^3)	5.509	0.321	6.260	0.421	7.030	0.418	6.050	0.329	n.s.	n.s.
WBC (10^3/mm^3)	5.227	0.415	5.270	0.308	6.025	0.207	6.160	0.503	n.s.	n.s.
Lymphocytes (%)	54.00	9.18	41.60	12.30	57.10	8.21	56.00	11.04	n.s.	n.s.
Monocytes (%)	—	—	—	—	—	—	—	—	—	—
Neutrophils (%)	38.00	11.07	51.90	7.80	37.30	7.50	41.90	9.30	n.s.	n.s.
Eosinophils (%)	7.20	2.01	6.00	2.05	5.50	1.80	2.50	0.80	2.9	4.5
Basophils (%)	0.50	1.00	0.60	0.50	0.10	0.30	0.50	0.50	n.s.	n.s.

$\bar{x}$ = Arithmetic mean.
S = Standard deviation.
M.S.D. = Minimum significant difference.

Table 3. Control cows hemogram

Parameters	I° $\bar{x}$	I° S	II° $\bar{x}$	II° S	III° $\bar{x}$	III° S	IV° $\bar{x}$	IV° S	M.S.D. 0.05	M.S.D. 0.01
Ht (%)	33.33	2.08	32.00	2.01	34.60	3.05	35.30	2.98	n.s.	n.s.
PVC (g/dl)	6.03	0.80	7.16	0.91	8.20	1.02	8.50	1.51	n.s.	n.s.
RBC (10^6/mm^3)	6.160	0.467	6.160	0.315	7.160	0.427	6.620	0.391	n.s.	n.s.
WBC (10^3/mm^3)	5.733	0.503	5.866	0.308	6.460	0.431	5.400	0.375	n.s.	n.s.
Lymphocytes (%)	50.30	10.69	39.60	7.50	51.30	6.90	44.00	8.21	n.s.	n.s.
Monocytes (%)	—	—	—	—	—	—	—	—	—	—
Neutrophils (%)	39.30	12.85	53.60	15.16	42.00	9.18	53.30	11.57	n.s.	n.s.
Eosinophils (%)	10.33	8.21	6.30	2.61	6.00	1.78	2.00	0.40	5.2	7.8
Basophils (%)	—	—	0.30	0.50	0.30	0.50	—	—	n.s.	n.s.

$\bar{x}$ = Arithmetic mean.
S = Standard deviation.
M.S.D. = Minimum significant difference.

Table 4. New born calves hemogram of vaccinated and control cows

Parameters	New born calves of vaccinated mothers: I° sample $\bar{x}$	I° sample S	II° sample $\bar{x}$	II° sample S	M.S.D. 0.05	M.S.D. 0.01	New born calves of control mothers, One sample $\bar{x}$	One sample S
Ht (%)	33.11	2.18	39.60	7.27	n.s.	n.s.	32.30	2.15
PCV (g/dl)	7.80	0.70	8.00	1.01	n.s.	n.s.	7.00	0.90
RBC (10^6/mm^3)	6.600	0.437	6.680	0.515	n.s.	n.s.	6.537	0.572
WBC (10^3/mm^3)	7.600	0.315	8.100	0.412	n.s.	n.s.	5.360	0.342
Lymphocytes (%)	27.30	5.87	47.60	6.91	10.7	15.9	28.30	7.28
Monocytes (%)	—	—	—	—	—	—	—	—
Neutrophils (%)	72.20	11.21	52.10	9.31	8.5	12.3	71.00	9.15
Eosinophils (%)	1.00	0.50	0.10	1.50	n.s.	n.s.	0.30	0.50
Basophils (%)	0.40	0.70	—	—	—	—	0.30	0.80

$\bar{x}$ = Arithmetic mean.
S = Standard deviation.
M.S.D. = Minimum significant difference.

The serum conversion, employing antigen K99, showed the titer to be higher in the second and third samples of the vaccinated cows, while in the control animals the values maintained constant for the experimental period.

No variation was observed in the antibody level with the acapsular mutant.

The colostrum antibody levels were higher than in milk. However the highest titers, 7 days from calving, found the level in milk coincided with the lower levels of the colostrum.

Table 5. Agglutination test with antigen K99 and acapsular mutant in comparison to serum samples drawn from the cows

	Vaccinated cows Samples									
	I°		II°		III°		IV°		M.D.S.	
Parameters	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	$\bar{x}$	S	0.05	0.01
K99	176.00	82.62	1440.00	843.27	1137.00	282.21	792.00	533.93	495.96	667.92
Acapsular mutant	31.00	20.24	66.00	54.20	51.00	22.60	45.00	26.35	n.s.	n.s.
	Control cows									
K99	213.33	92.37	266.66	92.37	426.66	184.75	160.00	138.56	n.s.	n.s.
Acapsular mutant	95.00	18.15	61.00	21.30	57.00	20.18	42.00	19.69	n.s.	n.s.

$\bar{x}$ = Arithmetic mean.
S = Standard deviation.
M.D.S. = Minimum significant difference.

Table 6. Agglutination test with antigen K99 and acapsular mutant in comparing colostrum and milk

	Vaccinated cows					
	Colostrum		Milk		M.D.S.	
Parameters	$\bar{x}$	S	$\bar{x}$	S	0.05	0.01
K99	1508.00	1032.00	19.00	11.97	861.66	931.79
Acapsular mutant	192.00	173.64	16.00	9.66	114.22	n.s.
	Control cows					
K99	213.33	92.37	36.66	37.85	n.s.	n.s.
Acapsular mutant	100.00	84.85	25.00	21.21	n.s.	n.s.

$\bar{x}$ = Arithmetic mean.
S = Standard deviation.
M.D.S. = Minimum significant difference.

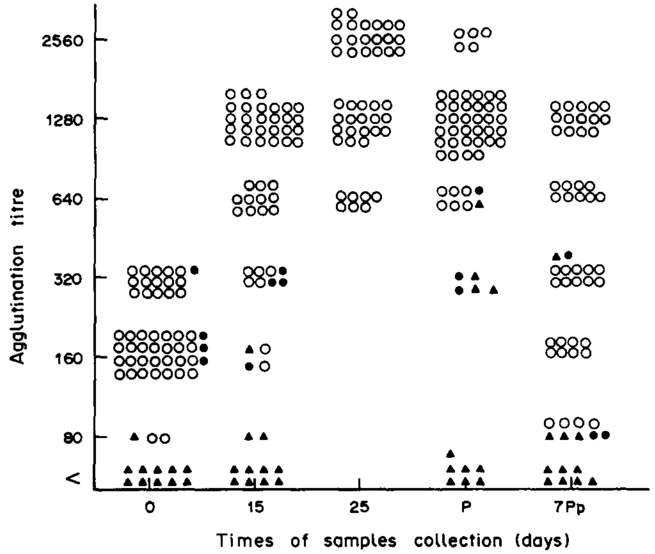

Fig. 1. Agglutination tests with K99 antigen against serum samples collected from pregnant cows in different times. ○, Vaccinated cows; ●, control cows; ▲, agglutination values obtained with acapsular mutant (pool of 4 sera); <, agglutination values < 80; P, partum; Pp, post partum.

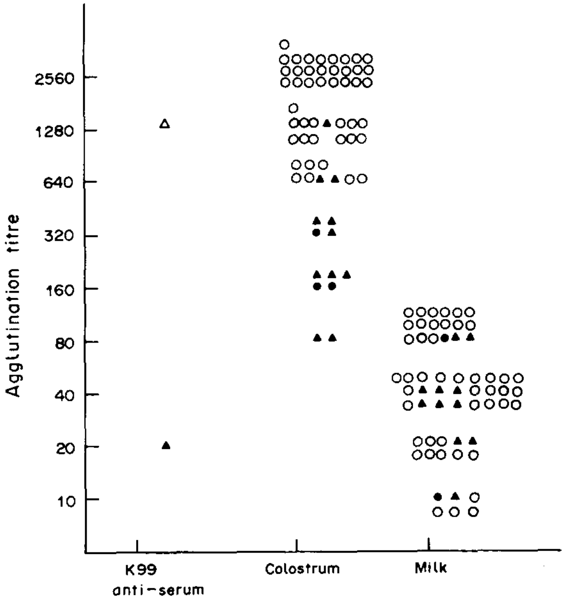

Fig. 2. Agglutination tests with K99 antigen against colostrum and milk. ▲, Agglutination value obtained with the acapsular mutant (pool of 4 sera); ●, agglutination value obtained with the control cows; ○, agglutination value obtained the vaccinated cows; △, agglutination value obtained K99 immune serum.

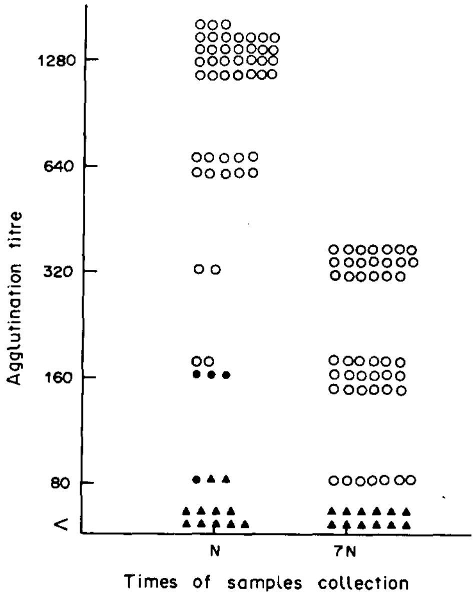

Fig. 3. Agglutination tests with K99 antigen against serum samples collected from calves after suction of colostrum and 7 days after birth. ○, Calves from vaccinated cows; ●, calves from control cows; ▲, agglutination value obtained with the acapsular mutant (pool of 4 sera); <, agglutination value <80; N, birth; 7N, 7 days after birth.

Table 7. Agglutination test with antigen K99 and acapsular mutant in comparing serum samples drawn from calves after suction of colostrum and after 7 days from birth

	New born calves of vaccinated mothers					
	I° sample		II° sample		M.D.S.	
Parameters	$\bar{x}$	S	$\bar{x}$	S	0.05	0.01
K99	860.00	466.47	150.00	114.64	364.88	501.14
Acapsular mutant	35.00	37.80	30.00	23.29	n.s.	n.s.
	New born calves of control mothers					
K99	160.00	46.18	—	—	—	—
Acapsular mutant	15.60	5.77	—	—	—	—

$\bar{x}$ = Arithmetic mean.
S = Standard deviation.
M.S.D. = Minimum significant difference.

Table 8. Brush Border test inhibition by colostrum and milk against *Escherichia coli* B41

	Cows														
	1[a]	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	Vaccinated											Control			
Colostrum	15.5[b]	12	30.2	18.3	16.1	25.4	10.5	22.3	15.9	12.5	11.8	6.6	NE	20.1	7.3
Milk	15.0	5.5	4.7	13.1	10.5	9.2	2.5	11.6	5.8	2.0	16.2	2.1	NE	15.3	10.2
K99 Immune serum		70													

[a]Each number takes in a pool of colostrum or milk of 4 cows.
[b]Values expressed in %.
NE = Not done.

The calves, 12–24 h, from vaccinated cows had high antibody titers, higher than those registered in the control subjects (Table 7 and Fig. 3).

The capacity of milk and colostrum to inhibit, *in vitro*, the colonization of K99 antigen to enterocytes furnished the results expressed in percentages, inferior to 30 (Table 8).

The histological checks carried out on the control calves demonstrated marked exfoliation and shortening of the villi.

Furthermore from the feces and intestinal content of those animals was isolated *Escherichia coli* B44, which was irregularly present in the rectal swabs of calves from vaccinated cows.

DISCUSSION

The analysis of the results, in the cows vaccinated with K99 antigen, shows that the antibodies, significantly increased from the 25th day from the first inoculation until 24 h after calving. This was statistically significant and its drop is equally significant after 7 days.

The serological values of the colostrum and milk of the cows and blood serum of the calves show interesting and significant decreases of the antibody levels against K99 antigen, in the milk colostrum relation of the vaccinated cows, and in the sera of their calves checked when 7 days old.

The antibody levels for K99 antigen were determined using a heterologous serotype *Escherichia coli* B41, having in common with *Escherichia coli* B44 only the K99 antigen ensuring in this way the antibody specificity. As a further verification the acapsular mutant was also used.

The results of the colonization inhibition test at the enterocytes, even if low percentage, showed significant differences between colostrum and milk, and larger concentrations of specific antibodies in the colostrum according to the serologic test.

The fact that the calves from vaccinated cows resisted the challenge after suckling the colostrum and those of the controls did not, confirms the validity of the immunization. A further confirmation comes from the isolation of enterotoxigenic bacteria from the feces and intestinal content of the control calves and dysepithelization with shortening of the intestinal villi.

Therefore this result supports the validity of the vaccine, obtained from Pili, against the enterotoxigenic strains of *Escherichia coli* administered to pregnant females at 5 weeks before calving [21, 29, 30].

In similar conditions piglets and lambs suckling colostrum from vaccinated mothers resisted to the challenge [31–33].

In the cows the enterotoxigenic bacteria were injected differently, either as whole bacteria or as colonizing purified antigen or partially purified or associated to Rotavirus, each with different results [20–22, 29, 30, 34, 35].

It is important to note that there was a seroconversion against the acapsular mutant in all of the subjects of the experiment. This was presumably due to the high diffusion of *Escherichia coli* in the environment and due to the incomplete purification of K99 antigen in our vaccine that also contained the somatic antigen.

The seroconversion against antigen K99, also in non vaccinated cows with higher levels 24 h after calving and in control calves, which is spite of this immunity did not resist to the infection, justifies the existence of other colonizing antigens. It also confirms the hypothesis that not always the degree of protection corresponds to the level of specific antibody values present in the colostrum [1, 3, 25, 36–38].

Considering the results of this paper it is necessary in the future to establish the different antibodies of the colostrum and to find the lowest serological titer able to protect newborn calves.

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