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Passive immunity in cattle against enterotoxigenic *Escherichia coli*: serologic evaluation of a bacterin containing K99 and F41 fimbriae in colostrum of vaccinated females and calf serum

[*Imunidade passiva contra* Escherichia coli *enterotoxigênica: avaliação sorológica de uma bacterina contendo as fímbrias K99 e F41 no colostro de fêmeas vacinadas e no soro de bezerros*]

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ABSTRACT

A bacterin from enterotoxigenic *Escherichia coli* (ETEC), containing fimbriae K99 and F41, was produced and its capacity to induce anti-K99 and anti-F41 antibodies in colostrum of vaccinated cows and in calf serum, and the persistence of these antibodies in neonates were determined. Three experiments were performed on two commercial farms. In all experiments animals were allotted randomly to the blocks, each block consisting of two pregnant females (a vaccinated one and a control one) and their respective calves. In experiment A (farm 1), comprised of 18 blocks, the animals received a vaccine dose 30 days before delivery. In experiment B (farm 1), consisted of 26 blocks, the animals received two vaccine doses (60 and 30 days before delivery). In experiment C (farm 2), consisted of 22 blocks, the animals received two vaccine doses (60 and 30 days before delivery). In experiments A and B pregnant cows and heifers were used and colostrum and serum from 24- to 36-hour-old calves were collected. In experiment C, pregnant embryo-recipient heifers were used and colostrum and sera from calves at 7, 14, 28 and 42 days of age were collected. Anti-K99 and anti-F41 antibodies were detected by ELISA using purified K99 and F41 fimbrial antigens. In experiment A no difference between treated and control groups was observed for the concentration of anti-K99 and anti-F41 antibodies in colostrum and calf serum. In experiment B a difference ($P<0.001$) was observed for colostrum of vaccinated females and for serum of their calves. In experiment C, difference between vaccinated and control animals was observed for colostrum and calf serum at 7, 14, 28 ($P<0.001$ in all cases) and 42 days of age ($P= 0.003$). The results showed the efficiency of the bacterin to induce detectable humoral immune response.

Keywords: calf, enterotoxigenic *Escherichia coli*, vaccine, diarrhea, K99, F41

RESUMO

Produziu-se uma bacterina de Escherichia coli *enterotoxigênica (ETEC) contendo as fímbrias K99 e F41 e avaliaram-se a capacidade de indução de anticorpos anti-K99 e anti F-41 no colostro de vacas vacinadas e no soro de bezerros e a persistência dos anticorpos nos neonatos. Três experimentos foram realizados em duas fazendas comerciais. Os animais foram aleatoriamente alocados em blocos, de duas fêmeas prenhes (uma vacinada e outra controle) e seus respectivos bezerros. No experimento A (fazenda 1), com 18 blocos, os animais receberam uma dose da vacina, 30 dias antes do parto. No experimento B (fazenda 1), com 26 blocos, os animais receberam duas doses de vacina, aos 60 e 30 dias antes do parto. No experimento C (fazenda 2), com 22 blocos, os animais receberam o mesmo esquema de vacinação do experimento B. Nos experimentos A e B foram coletados colostro das parturientes e soro dos bezerros entre 24 e 36 horas de vida. No experimento C, foram usadas novilhas receptoras de embriões e*

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coletados colostro e soro dos bezerros aos 7, 14, 28 e 42 dias de idade. Anticorpos anti-K99 e anti-F41 foram detectados por ELISA utilizando antígenos K99 e F41 purificados. No experimento A não foi observada diferença entre o grupo vacinado e o controle quanto à detecção de anticorpos. No experimento B foi observada diferença (P<0,001) entre o colostro de fêmeas vacinadas e o soro de seus bezerros. No C houve diferença entre o grupo vacinado e o controle para o colostro e o soro dos bezerros aos 7, 14, 28 (P<0,001) e 42 dias de idade (P= 0,003). A bacterina utilizada foi eficiente para a indução de resposta imune humoral detectável.

Palavras-chave: bezerro, diarréia, Escherichia coli *enterotoxigênica, K99, F41, vacina*

INTRODUCTION

Neonatal diarrhea in calves is a syndrome which frequently occurs in many countries worldwide and it is an important cause of economic losses (Barragry, 1997). Enterotoxigenic *Escherichia coli* (ETEC) is one of the main causative agents of diarrhea in calves of up to two weeks of age, and it can still be pathogenic for calves of up to 6 weeks when associated with other agents such as rotavirus (Runnels et al., 1986). ETEC pathogenesis is determined by bacterial adhesion to the small intestine and by the induction of intestinal hypersecretion which are promoted by fimbriae and enterotoxins, respectively (Nataro and Kaper, 1998). Fimbriae K99 and F41 are frequently found in calf ETEC, with the simultaneous expression of both fimbriae in the same strain being commonly observed (Smyth et al., 1994).

In ruminants, immunity to ETEC infections is promoted by colostral anti-fimbrial antibodies, mainly immunoglobulin G (IgG), which inhibit the adhesion of this bacterium to the gastrointestinal tract by blockade of the fimbria-receptor interaction (Nagy and Fekete, 1999). Different vaccines have been developed using ETEC strains producing K99 and F41. These vaccines may consist of bacterins (Acres et al., 1979; Pugh and Wells, 1985; Contrepois et al., 1985; Cornaglia et al., 1992), crude K99 and F41 extracts (Nagy, 1980) or purified fimbriae (Acres et al., 1979; Nagy et al., 1990; Yano et al., 1995). In these studies, the most extensively employed method for the evaluation of these vaccines was post-vaccinal serologic analysis. However, some important aspects of the immunization of newborn calves against ETEC, such as the persistence of passive antibodies after ingestion of colostrum, response of heifers to vaccination and natural immunity of female animals before vaccination, are rarely discussed.

The objective of the present study was to determine by ELISA the passive transfer of anti-K99 and anti-F41 antibodies to calves, the persistence of these antibodies in neonates after vaccination of pregnant females (cows and heifers) with a bacterin from ETEC containing K99 and F41 fimbriae.

MATERIALS AND METHODS

The reference ETEC strain B41 (O101:K99:F41), kindly provided by Professor A.F. Pestana de Castro, Department of Microbiology, USP, was used to bacterin production and to obtain the K99 purified antigen used in the serologic tests. The ETEC strain ATCC 31616 (obtained from American Type of Culture Collection) was used to obtain purified F41 antigen. In all experiments, both ETEC strains were grown to confluency in Minca agar (Guinee et al., 1977) under aerobic conditions at 37^{o}C for 18 to 20 hours.

The bacterin was produced according to the method of Acres et al. (1979) with some modifications. Bacteria were collected from the flasks by washing with phosphate-buffered saline (PBS), pH 7.2, and centrifugation at 3.000xg for 15min at 4^{o}C. To inactivate the bacteria, formaldehyde was added to the suspension to a final concentration of 0.5%. One volume of a 10% solution of aluminum and potassium sulfate was added to one volume of the suspension as adjuvant. The pH was adjusted to 7.2 and the bacterial suspension to a final concentration of 3×10^{10} bacteria/dose of vaccine (3ml). The presence of K99 and F41 antigens in the cultures used for bacterin production was determined by the serum agglutination test (Guinee et al., 1977), using specific anti-K99 and anti-F41 hyperimmune sera produced in rabbits according to Edwards and Ewing (1972). After inactivation

for 24 hours, aliquots of the bacterial suspension were inoculated into thioglycollate broth and blood agar, incubated at 37ºC and media were observed for 72 hours. One dose of bacterin was injected subcutaneously into three guinea pigs to determine innocuity. These animals were examined daily during seven days for the detection of adverse effects.

For post-vaccinal serologic evaluation, three experiments were conducted on two different commercial farms. Farm 1 has a herd of Holstein animals and pregnant cows and heifers were used in the experiments. Farm 2 also has a herd of Holstein cows and a program of embryo transfer. In the experiments carried out on farm 2, embryo-recipient heifers, Holstein/Zebu bred, pregnant with Holstein calves, were used. On both farms the pregnant females used for the experiments were divided into random blocks, each block consisting of two gestational age-matched females, a vaccinated one (treatment group) and a control one, and their respective calves. In the experiment A (farm 1) it was determined the levels of anti-K99 and anti-F41 antibodies in colostrum and calf serum employing only one dose of bacterin. Forty-four pregnant females (33 cows and 11 heifers) were divided into 22 random blocks, according to the date of parturition. Animals from the treatment group received one vaccine dose subcutaneously in the scapular region 30 days before parturition. Colostrum from all studied females was collected at parturition and sera were collected from treatment and control calves at 24 to 36 hours of age. In the experiment B (farm 1) it was determined the levels of anti-K99 and anti-F41 antibodies in colostrum and calf serum employing two vaccine doses 60 and 30 days before parturition. Sixty-two pregnant females (48 cows and 14 heifers) were divided into 31 random blocks, according to the date of parturition. Colostrum from all studied females was collected at parturition and sera were collected from treatment and control calves at 24 to 36 hours of age. In the experiment C (farm 2) it was determined the persistence of passive antibodies transmitted to calves by pregnant females receiving two vaccine doses 60 and 30 days before parturition. Forty-six heifers were divided into random blocks (23 in the treatment group and 23 in the control group), according to the date of parturition. Colostrum was collected at parturition and sera were collected from all calves at 7, 14, 28 and 42 days of age. Calf serum samples were aliquoted and stored at -20ºC for later analyses. The collected colostrum was ultracentrifuged at 100.000 x g for two hours, as described by Haggard (1982), to obtain colostral serum, and aliquots were stored at -20ºC. Before specific analysis of anti-K99 and anti-F41 antibodies, the total antibody concentration was determined in calf sera from all experiments, using zinc sulfate turbidity, according to the method of Pfiffer et al. (1977). All animal groups that showed failure of transfer of passive immunity (antibody concentration ≤16mg/ml) were excluded from the study.

With the objective to determine the causative agents of diarrhea throughout experiment C, calves were monitored for the presence of diarrhea. Feces were collected from each animal presenting diarrhea for the detection of enterotoxigenic *Escherichia coli* by isolation, identification and detection of fimbriae (Guinee et al., 1977) of *Salmonella* sp. by isolation and identification (Quinn et al., 1994) of rotavirus by electropherotyping (Ludert et al., 1991) and of *Cryptosporidium* sp. by modified Ziehl-Neelsen staining (Henriksen and Pohlenz, 1981).

In order to measure anti-K99 and anti-F41 antibodies two indirect ELISA tests were standardized, one using K99 antigen (K99-ELISA) and the other using F41 antigen (F41-ELISA). The purification of K99 fimbriae was carried out as follows: ETEC B41 cultures were centrifuged at 3000x g for 15min at 4ºC and the pellet was resuspended in 0.05 M phosphate buffer (PB), pH 7.5, containing 1 M NaCl. K99 was extracted by heating the suspension in a water bath at 65ºC for 25min under occasional shaking and the extract was fractionated with ammonium sulfate at 45% saturation. The pellet was dialyzed against PB, treated with 0.5% (w/v) sodium deoxycholate (DOC), incubated at 4ºC for 48 hours, and then centrifuged at 10.000 x g for 30 minutes. The pellet was ressuspended in 0.05M PB containing 2M urea, equilibrated by dialysis against the same buffer and submitted to ultracentrifugation for two hours, at 4ºC and 130,000 x g. The supernatant was collected and submitted to other ultracentrifugation step, at 4ºC and 243,000 x g, for four hours. The pellet obtained was ressuspended in 0.5ml of 0.05M PB and used as K99 purified antigen. To obtain purified F41, the fimbriae extraction, ammonium

sulfate fractionation and DOC treatment was carried out as described for K99 antigen preparation. The purified F41 was obtained in the supernatant after DOC treatment. The protein concentration of both fimbriae were determined by the method of Lowry et al. (1951), and the purity was analyzed by silver-stained (Oakley et al., 1980) 12% SDS-PAGE (Laemmli, 1970). Immune reactivity of purified fimbriae was checked by western blotting using specific anti-K99 and anti-F41 sera. The ELISA tests were standardized by block titration of antigen, test sera and peroxidase-conjugated bovine anti-IgG[1]. The optimum antigen concentration was 173ng/well to K99 and 110ng/well to F41. The optimum test serum and conjugate dilutions were 1:50 and 1:3000, respectively. Antigen was then diluted in carbonate buffer, pH 9.6, and 100 µl/well was added to ELISA microplates[2] and incubated at 4°C for 12 hours. The plates were washed three times with PBS containing 0.05% Tween 20[3] (PBST) followed by the addition of blocking solution (5% powdered skim milk in PBS). After a 30-min incubation at 37°C the plates were washed three times with PBST. Colostral and calf sera were diluted in PBST containing 1% powdered skim milk and 100µl/well was added to the plates. All sera were tested in duplicate. After one hour of incubation at 37°C the plates were washed three times with PBST, 100µl of bovine anti-IgG conjugate was added and the plates were incubated for one hour at room temperature. The plates were then washed 5 times with PBST and 100µl *o*-phenylenediamine solution (0.4mg/ml)[1] containing hydrogen peroxide (0.4µl/ml)[2] in citric acid buffer, pH 5.0, was added to each well. After 15min of incubation the reactions were blocked by the addition of 30µl 4N sulfuric acid[2] and read in a plate reader[1] at 492nm. Results are reported as optical density (OD). Positive and negative controls were used in all tests to verify reproducibility of the test among different microplates and different test days.

Analysis of variance of the randomized blocks was used to compare control and vaccinated groups of experiments A, B and C. The decrease in antibody concentration in serum from calves at 7, 14, 28 and 42 days of age was analyzed by linear regression. The Student *t*-test was used to compare antibody concentration between the control groups of farms 1 and 2. An α error of less than 5% was considered to be significant.

RESULTS

The produced bacterin was shown to be sterile and harmless in the tests performed. None of the females from the three experimental groups showed adverse reactions to vaccine administration throughout the experiments.

After analysis of passive immunity transfer by zinc sulfate turbidity, 4 (18.2%), 5 (16.1%) and 1 (4.4%) animal blocks were excluded from experimental groups A, B and C, respectively, since they presented a low total antibody concentration. Thus, statistical analysis of group A was performed on 18 blocks, of group B on 26 blocks and of group C on 22 blocks.

In experiment A (one vaccine dose, 30 days before parturition), analysis of variance did not show any difference in the anti-K99 (Table 1) and anti-F41 (Table 2) antibody concentrations between colostrum from females of the treated group and colostrum from the control group ($P= 0.864$), or between serum from calves at 24 to 36 hours of age born from vaccinated and control dams ($P= 0.622$).

In experiment B (two vaccine doses, 60 and 30 days before parturition) the anti-K99 (Table 1) and anti-F41 (Table 2) antibody concentrations were higher ($P<0.001$) in colostrum from females of the treatment group and serum from calves at 24 to 36 hours of age born from vaccinated mothers ($P<0.001$) than control animals. Cows (n= 19) from the treatment group of this experiment showed higher anti-K99 and anti-F41 colostral antibody concentrations ($P<0.001$) than heifers from the same group (n= 7). However, these heifers still had a higher colostral concentration of antibodies anti-K99 and anti-F41 compared to the control group ($P= 0.003$).

[1] Sigma, USA.
[2] Costar, USA.
[3] Merck AG

Table 1. Anti-K99 antibody concentrations (DO 492nm) in colostrums and sera of calves from experiments A, B, and C

	Farm 1				Farm 2				
	Experiment A[a]		Experiment B[a]		Experiment C[a]				
	Colostrum[b]	Calf[c]	Colostrum[b]	Calf[c]	Colostrum[b]	7 days[d]	14 days[d]	28 days[d]	42 days[d]
Treatment group	0.582 (0.048)	0.477 (0.078)	0.992* (0.128)	0.926* (0.085)	1.213* (0.076)	1.097* (0.049)	0.931* (0.079)	0.829* (0.057)	0.692[+] (0.062)
Control group	0.539 (0.059)	0.470 (0.042)	0.593 (0.060)	0.532 (0.064)	0.822 (0.068)	0.800 (0.073)	0.725 (0.088)	0.642 (0.058)	0.588 (0.049)

[a] Mean (standard deviation); [b] collected at parturition; [c] Sera collected between 24 and 36 hours of life; [d] Sera of calves
* Differ significantly from control (P≤0,001)
[+] Differ significantly from control (P= 0,003).

Table 2. Anti-F41 antibody concentrations (DO 492 nm) in colostrums and sera of calves from experiments A, B, and C

	Farm 1				Farm 2				
	Experiment A[a]		Experiment B[a]		Experiment C[a]				
	Colostrum[b]	Calf[c]	Colostrum[b]	Calf[c]	Colostrum[b]	7 days[d]	14 days[d]	28 days[d]	42 days[d]
Treatment group	0.531 (0.089)	0.419 (0.064)	1.092* (0.077)	1.004* (0.093)	1.374* (0.113)	1.232* (0.088)	1.163* (0.062)	0.948* (0.084)	0.863[*] (0.083)
Control group	0.554 (0.039)	0.397 (0.080)	0.571 (0.062)	0.545 (0.055)	0.784 (0.053)	0.713 (0.066)	0.691 (0.048)	0.604 (0.078)	0.522 (0.065)

[a] Mean (standard deviation); [b] collected at parturition; [c] Sera collected between 24 and 36 hours of life; [d] Sera of calves
* Differ significantly from control (P≤0,001)

In experiment C (two vaccine doses, 60 and 30 days before parturition) the anti-K99 and anti-F41 antibody concentrations were higher in colostrum from heifers of the treatment group and in sera from their calves at seven, 14, 28 (P<0.001 for all cases) and at 42 days of age (P<0.001 to anti-F41 and P= 0.003, to anti-K99). Anti-K99 and anti-F41 antibody levels decreased from 7 to 42 days of age in a similar way for calves born from vaccinated and control mothers (Tables 1 and 2).

Tables 1 and 2 show the mean optical densities and standard deviations of anti-K99 and anti-F41 antibodies, respectively, for vaccinated and control groups from experiments A, B and C. All control animals from both farms had anti-K99 and anti-F41 colostral antibody levels detectable by K99-ELISA and F41-ELISA. Mean OD values for colostrum differed between the control groups of farm 1 (experiments A and B) and farm 2 (experiment C). The Student t-test revealed a higher (P<0.001) natural anti-K99 and anti-F41 antibody concentration in the control group of farm 2 (experiment C) than that observed in the control groups of farm 1 (experiments A and B).

In experiment C three calves (one from the vaccinated group and two from the control group) at 14, 32 and 35 days of age had diarrhea. Diarrhea occurred at different periods of time in these animals and none of the agents tested (ETEC, *Salmonella* sp., rotavirus or *Cryptosporidium* sp.) were detected in the feces of these animals during the episode of diarrhea.

DISCUSSION

The efficiency of vaccination against ETEC neonatal diarrhea in ruminants is based on the adequate transfer of passive antibodies through the colostrum. Since this is a factor that can be influenced by management conditions about 10% of calves may present lack of transfer of passive immunity under natural conditions (Besser and Gay, 1993). In the present study the measurement of total antibody in calf serum allowed the elimination of experimental blocks whose calves presented low total antibody concentrations. These animals showed low anti-K99 and anti-F41 antibody levels, as assayed by K99-ELISA and F41-ELISA, which may hinder statistical analysis. On farm 1, nine calves (9.5% of all calves) showed failure of passive immunity

transfer (FPT), whereas on farm 2, one calf (2.1% of all calves) presented FPT. It is generally expected that FPT occurs more frequently in calves born from heifers, mainly those which are used in embryo transfer programs (Tyler and Parish, 1995). However, this tendency was not observed in the present study. The difference in the frequency of FPT observed between farms 1 and 2 may be explained by the fact that farm 2, due to the embryo transfer program, possesses personnel specifically trained in the care of heifers and calves on the day of parturition which permitted the ingestion of an adequate amount of colostrum by the calves during the first 24 hours of life, thus reducing the occurrence of FPT. Therefore, in studies on vaccines which involve passive immunity in ruminants the selection of animals that do not present FPT contributes to a more accurate evaluation.

Enterotoxigenic *Escherichia coli* is considered to be an agent of worldwide distribution, with the hosts possibly having contact with this bacterium at different times (Nagy and Fekete, 1999). In the experiments carried out on farms 1 and 2, all control animals had anti-K99 and anti-F41 antibodies, as detected by K99-ELISA and F41-ELISA, demonstrating previous exposure of the study population to this agent.

The results obtained in experiment A show that, even in the presence of natural anti-K99 and anti-F41 antibodies in the study population, the administration of a single vaccine dose is not sufficient to induce an increase in colostral antibodies against these fimbriae. Subsequently, as shown by the results of experiment B, it was demonstrated that the administration of two vaccine doses (60 and 30 days before parturition) is necessary to increase the anti-K99 and anti-F41 antibody concentrations in colostrum and calf serum.

Neonatal calves are susceptible to ETEC infection until about the second week of life. From this time on the mucosa of the gastrointestinal tract ceases the expression of K99 and F41 fimbrial receptors (Teneberg et al., 1994). However, in mixed infections the susceptibility of the host may be extended until the sixth week of life (Runnels et al., 1986). To protect the neonatal gastrointestinal tract against ETEC adhesion, the presence of antibodies in the bowel during the period of susceptibility of the animal is necessary. Since the absorption of colostral antibodies by the gastrointestinal tract occurs only during the first 24 hours of life, ruminants are able, during the first 3 to 4 weeks of life, to secret into the gastrointestinal tract about 25 to 30% of antibodies absorbed from colostrum on the first day of life (Banks and McGuire, 1989). Therefore, vaccination of cows against ETEC should lead to a high concentration of passive anti-K99 and anti-F41 antibodies in calves on the first day of life and should also be able to maintain these concentrations until one month of life. Experiment C showed that calves born from mothers vaccinated with two doses had significantly higher anti-K99/F41 antibody concentrations than those observed in control calves throughout serologic follow-up (42 days of age), suggesting the efficiency of the bacterin produced and of the vaccination scheme used. The presence of anti-K99 and anti-F41 antibodies for a 42 days period could protect calves during the period of susceptibility to ETEC in mixed infections.

Although farms 1 and 2 used in this study have animals of high genetic and economic value and show good conditions of sanitary management, the colostrum from control animals of farm 2 showed higher anti-K99 and anti-F41 antibody concentrations than that from the control groups of farm 1. This difference may be due to the management of animals on these properties. On farm 1 new animals are not bought but cows are replaced by heifers from the herd, whereas on farm 2, due to the embryo transfer program, recipient heifers from different regions enter the herd. This epidemiologic condition may favor increased contact of heifers on farm 2 with ETEC K99/F41 strains and, therefore, a higher concentration of natural antibodies against these fimbriae.

One important aspect concerning the use of vaccines for the induction of passive immunity is that heifers produce colostrum with a lower antibody concentration compared to that of cows (Tyler and Parish, 1995). Although this fact was observed in experiment B (farm 1) for anti-K99 and anti-F41 antibodies, vaccination induced a significant increase in the concentration of these antibodies in heifer colostrum compared to the

control group. Thus, bacterin was also efficient for use in heifers.

None of the analyzed agents was detected in calves presenting diarrhea throughout the experiment. Although the agents analyzed in the present study (ETEC, *Salmonella* sp., rotavirus and *Cryptosporidium* sp.) are reported in the literature as being the most prevalent ones (Barragry, 1997), other agents, such as coronavirus, calycivirus and astrovirus, as well as non-infectious agents may have caused diarrhea.

The efficiency of ETEC B41 bacterin in inducing an increase in anti-K99 and anti-F41 antibody concentrations was demonstrated in the present study. The differences observed between farms 1 and 2 regarding anti-K99 and anti-F41 antibody concentrations and the frequency of failure of passive antibody transfer demonstrate that an adequate experimental design is required to evaluate vaccines against bovine ETEC, especially on commercial farms. Moreover, in spite of being more prone to failure of passive antibody transfer, calves from heifers, and mainly from recipient heifers, can be successfully immunized against ETEC if colostrum ingestion after birth is adequately monitored.

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