Braz. J. vet. Res. anim. Sci.
São Paulo, v.31, n.3/4, p.225-32, 1994.

EVALUATION OF AN OIL EMULSIFIED VACCINE AGAINST BOVINE COLIBACILLOSIS USING SEMI-PURIFIED K99-F41 ADHESINS*

AVALIAÇÃO DE UMA VACINA OLEOSA CONTRA A COLIBACILOSE BOVINA UTILIZANDO OS ANTÍGENOS K99-F41 SEMIPURIFICADOS

Marcina GARCIA[1]; Domingos da Silva LEITE[2]; Tomomasa YANO[3]; Antonio Fernando Pestana de CASTRO[4]; Maria Aparecida Moreira SHENK[5]

SUMMARY

The K99 and F41 fimbrial antigens were extracted from ***Escherichia coli*** B41 strain by heating, and semi-purified by precipitation with ammonium sulfate and treatment with sodium desoxicolate (DOC). The semi-purified antigens K99 and F41 were used to prepare an oil emulsified vaccine against bovine colibacillosis. The vaccine was made up containing in each dose 1,500, 750 or 380 HU (Haemagglutinating Units). The test of the vaccine efficiency was measured by gel immunodiffusion and ELISA test. Challenge of calves from vaccinated and control cows was carried out with the virulent ***Escherichia coli*** B41. Our findings suggested that 750 HU vaccine, induces better production of anti-K99-F41 antibodies in vaccinated cows, and the transference of these antibodies to calves through the colostrum, gave full protection to challenge. Furthermore no adverse effects were observed in any of the vaccinated cows.

UNITERMS: ***Escherichia coli***; Bovidae; Vaccines

INTRODUCTION

Among the bacterial diarrheal diseases which affect young calves, neonatal colibacillosis is undoubtedly the most important, leading to considerable economic losses due to high morbidity and mortality[19].

The mechanism of enteropathogenicity of these bovine colibacilli is based on two main virulence factors: a) production of a thermostable (STa) enterotoxin and b) production of proteic fimbrial antigens called adhesins, of colonization factors (CFs), of adherence antigens, etc., wich enable the bacteria to adhere to specific receptors of the gut epithelial cells. As a consequence of the adherence, these CFs render enterotoxigenic ***Escherichia coli*** (ETEC) to colonize the small intestine reaching 10^9-10^{12} viable ETEC cells/gram of stools and increasing therefore at the gut level the amount of STa enterotoxin production. This enterotoxin, although being low MW (< 2.000 d) and thermostable, activates the guanilate cyclase system which leads to an increase in cyclic GMP, and this complex mechanism causes loss of electrolytes from the gut cells towards intestinal lumem and provoking severe osmotic diarrhea[3,5].

Nevertheless, clinical observations reported that usually calves which had been suckled by immunized cows were protected against the disease[15]. Conversely, colostrum-deprived calves often developed diarrheic symptoms caused by ETEC. Further studies on the Kco antigen described by ORSKOV et al.[12] (1975), and later named K99 demonstrated that this protection was due to antibodies against the K99 antigen present in the colostrum due to natural infection or vaccination[1,16].

Former vaccines against bovine colibacillosis had been bacterins observed to fail in many cases[2,8,11]. Since the K99 antigen is plasmid encoded, it was soon reported that the prolonged store of the vaccinal strains could lead to the loss of plasmid and no further production of the K99 CF. It is important to note that STa induces no protection since it is not immunogenic.

Presently, mainly in developed countries, puridied K99 vaccines produced by recombinant DNA techniques are widely used[19]. However, in developing countries, these vaccines are available but cost is very high and most farmers cannot afford the current prices. This is the present situation in Brazil where $K99^+$ strains have been used as bacterins against bovine colibacillosis. There are several disadvantages with regard to this policy such as: 1) need of controlling each batch of vaccine as to the presence and amount of K99

- Biólogo - Universidade Estadual de Campinas, São Paulo, Brasil
- Doutor - Instituto de Biologia, Universidade Estadual de Campinas, São Paulo, Brasil
- Professor Associado - Instituto de Biologia, Universidade Estadual de Campinas, São Paulo, Brasil
- Professor - Instituto de Biologia, Universidade Estadual de Campinas, São Paulo, Brasil
- Doutor em Medicina Veterinária - Centro Nacional de Gado de Corte, EMBRAPA, Campo Grande, MS, Brasil

This work was supported by a grant-in-aid for Scientific research from the FINEP and FAPESP.

antigen; 2) since whole bacterial cells are used in the preparation of the bacterin, adverse effects of endotoxin may occur, including abortion by the vaccinated cows; 3) because of the presence of antibodies against O, H, K, besides K99 antigens, the use of serum agglutination to evaluate the potency of a batch of vaccine is not very reliable since anti-O antibodies may interfere with the evaluation and it is well known that they are not protective[17].

Besides the K99 antigen, some ETEC strains also carry the F41[10] antigen previously described as part of the K99 CF. It has been reported that immunization of the cow with only the K99 antigen does not fully protect calves against infection by ETEC strains harbouring $K99^+$-$F41^+$ alone.

Based upon these data the present study had the following objectives:

1- Production of an oil emulsified vaccine containing semi-purified K99-F41 antigens.

2- Standardization of the doses of vaccines to be inoculated into pregnant cows.

3- Standardization and use of an enzymatic immunoassay (ELISA) for the detection of anti-K99-F41 antibodies in bovine sera.

4- Test of vaccine efficiency through the measurement of serological conversion of vaccinated cows and challenge of their calves with virulent $K99^+$-$F41^+$ strain.

MATERIAL AND METHODS

Bacterial strain - Enterotoxigenic ***Escherichia coli*** (ETEC) strain B41 (kindly supplied by MOON[9] 1981, NADC, Ames, Iowa, USA), serogroup 0101:$K99^+$-$F41^+$, producing STa enterotoxin was used as a reference strain for this study.

Mannose-resistant micro hemagglutination (MRMH) test - This test was carried out with cell-free K99-F41 semi purified antigens on 96-well microplates as previously described by PARRY; PORTER[14] (1978). One percent of horse and guinea pig red blood cells (RBC) (1%) in phosphate buffered saline (PBS) containing 1% D-mannose, was used for the determination of the hemagglutinating units of K99 and F41 antigens. One hemagglutinating unit (HU) was defined as the reciprocal of the highest dilution of K99 and F41 antigens which were still able to hemagglutinate horse and guinea pig RBC, respectivelly.

Semipurification of K99-F41 antigens - B41 strain was grown in batches of 5 liters of Minca medium[4] in a microfermentor (New Brunswick Scientific Co.Inc. USA) at 37°C for 18 h with vigorous aeration (15 psi). The extraction and semipurification of K99-F41 antigens were carried out as previously described by us[21]. Briefly, B41 aerated cultures were centrifuged at 10,000 rpm for 20 minutes at 4°C. Sedimented bacterial cells were resuspended in PBS, containing 1M NaCl. This suspension was heated at 60°C for 30 minutes with intermittent shaking, as reported by STIRM et al.[18] (1967). The suspension was centrifuged under the same conditions and to the supernatant was added $(NH_4)_2 SO_4$ at 60% saturation. The mixture was centrifuged and the precipitate resuspended with 0.05M PBS, pH 7.2, followed by exhaustive dialysis against the same buffer.

The dialysate was then treated during 72h with 0.05M PBS, pH 7.2, containing 0.5% sodium desoxicolate (DOC). Afterwards the sample was centrifuged and the DOC-soluble material was dialyzed again using the same PBS in order to eliminate the DOC substance. The amount of semi-purified K99 and F41 antigens was thereafter measured by the number of HU, using horse and guinea pig RBC in the MRMH test for K99 and F41 antigens, respectively.

Sodium Dodecyl Sulphate - Polyacrilamide gel electrophoresis (SDS-PAGE) - This test was carried out in 0.1% SDS as described by LAEMMLI[7] (1970) using 12% acrylamide. Volumes of 20µl of the semi-purified K99-F41 antigens (2mg protein/ml) were applied to the gel. The gel was stained with Coomassie blue and the molecular weights (MW) of the semi-purified K99 and F41 antigens were determined by using as standards the low MW kit (Pharmacia).

Antisera preparation - For this purpose adult albino rabbits were used. Aliquots of 1 ml semi-purified K99 and F41 antigens (100µg/ml/animal) emulsified in equal volumes of Freund's complete adjuvant (Difco Lab) were injected subcutaneously. An equal booster was given 3 weeks later and the animals were bled after a further 2 week period. The obtained serum was distributed in 3ml aliquots and stored at -20°C until use.

Vaccine preparation - The aqueous phase (Preparation A), of the vaccine was made up of 98% K99 and F41 semi-purified antigens in adequate amounts so that each dose (4ml) of the final vaccine contained one of the following concentration of K99 and F41: 1,500 HU, 750 HU or 380 HU. To complete this phase, merthiolate solution was added (2%). The oily phase (Preparation B) had the following composition: mineral oil 82.3%, Arlacel 16.3% and Tween 80 1.4%. In order to prepare the final emulsified vaccine, 40% preparation A was carefully emulsified in 60% preparation B (V/V). The homogenicity of the emulsion was checked by phase-contrast microscopy and stability in water.

Immunization of pregnant cows - This experiment was carried out in the National Center of Beef Cattle (EMBRAPA, city of Campo Grande, MS, Brazil).

Eighty-nine cows about 7-months pregnant were used. Fifty-four (Lot.54) were vaccinated with 2 doses of 1,500 HU, 8 and 2 weeks before delivery. The 12 cows (Lot. 12) were divided into 4 groups (A, B, C and D) of 3 animals each. Group A was vaccinated with 2 doses containing 1,500 HU; Group B with 2 doses of 750 HU; and group C with 2 doses of 380 HU. Pregnant cows of the group D and the remaining 23 cows (Lot. 23) were not vaccinated (negative controls).

All animais were vaccinated in the neck by subcutaneous route and sera from pregnant cows were collected at the times of vaccinations and at delivery. Blood samples were also collected from calves 2 days after suckling of colostrum. All collected sera were stored in small vials (2m) at -20°C until use.

Serological tests - Sera from vaccinated and unvaccinated cows and their respective calves were examined by gel immunodiffusion (GID) and ELISA tests. The GID was carried out according to OUCHTERLONY[13] (1958). Holes were punched out in the gel (Noble Agar 1%) in a standardized pattern. Twenty microliters of two-fold dilutions (1:2-1:64) of each serum were dropped into the peripheral well whereas the same amount of semi-purified K99-F41 antigens was applied to the central hole. The slides were incubated into a wet chamber at room temperature for 24h, washed with saline, dried and stained with Coomassie blue.

The ELISA test was carried out in 96-flat bottom well microplates (Hemobag). The method recommended by VOLLER et al.[20] (1976) was adapted. Semi-purified K99-F41 antigen (2µg/ml) was immobilized in each well, the plates being incubated at 37°C for 1h. PBS, pH 7.2 containing 0.05% Tween 20 (PBS/T) containing 1% BSA was added to the wells, to block free bounds still available, and the plates were incubated at 37°C for 1h. Between any phase of the reaction the wells of microplates were washed 3 times for 2 minutes with PBS/T. This procedure was repeated in all tests during the phases of antigen addition, sera and conjugate.

Block titration of sera and conjugate showed that the better dilutions were 1:1,500 and 1:10,000 respectively and the reactions could be read visually. Because the amount of sera to be examined, all were examined at 1:1,500 dilution and the results expressed in relation to the positive and negative control present in each microplate.

Challenge - The calves from groups A, B and C which had suckled the colostrum were inoculated by oral route with 200 ml ***Escherichia coli*** B41 ($K99^+$-$F41^+$-STa^+) containing 10^9 CFU/ml. Calves from group D were deprived suckling the colostrum and challenged just after birth, being maintained there after with artificial colostrum. Blood collection was performed 18h after birth. Stools were collected from all calves and after culturing on MacConkey medium, 5 lac^+ colonies were subcultured and examined for the detection of ETEC ($K99^+F41^+STa^+$) strains from each plate.

RESULTS

The growth of ***Escherichia coli*** strain B41 in Minca medium, under aeration, was assayed by the MRMH test using horse and guinea pig RBC for titration of K99 and F41 antigens respectively. Titres in these tests were 1/512 for horse (K99) and 1/256 for guinea pig (F41) RBC, whose data were considered appropriate for use in the semi-purification of the CFs K99 and F41.

Upon taking into consideration the initial volume of crude K99-F41 antigens, the specific activity, and the MRMH titres, the yield of the semi-purification procedure used was 66% (data not shown). Also, it was verified in the PAGE-SDS that the dialysate of the DOC soluble fraction showed only two proteic bands whose relative mobilities were near 18,000 and 30,000 daltons, suggesting that they corresponded to K99 and F41 respectively. This DOC-soluble preparation when evaluated by the GID test showed specific precipitation lines only with K99-F41 antisera. No reaction was observed with anti-K88 and anti-F42 immune sera.

Sera collected from pregnant cows (Lot. 54 and Lot. 23) and respective calves were examined by GID test, using semi-purified K99-F41 antigens, and sera diluted from 1:2 to 1:64 (Fig. 1). Sera from cows of Lot. 54 before first vaccine inoculation, sera from cow controls (Lot. 23) and respectives calves did not present positive reaction in GID test (data not shown).

All sera, as mentioned above, and sera of Lot. 12, were assayed by the ELISA test using a single dilution (1:1,500). Those which showed an intense yellow color as compared to the positive control, were considered positive. The negative control was used in the ELISA test at 1:20 dilution. Those sera which showed less yellowish color, than the negative control serum up to the 1:20 dilution, were considered negative for K99 and F41 antibodies as tested by ELISA (Fig. 2). A comparison of GID and ELISA tests is shown in Fig. 3, demonstrating that the ELISA performed better than GID for the detection of K99-F41 antibodies in the evaluation of our vaccine against bovine colibacillosis.

As to the correlation of vaccine does given in HU our findings suggest that 750 HU induced better production of anti-K99-F41 antibodies in vaccinated cows and the respective trans-

TABLE 1

Results obtained in the agar gel immunodiffusion (GID) test and ELISA test, with sera of pregnant cows from groups A, B and C vaccinated with 1,500 HU, 750 HU and 380 HU respectively, and their calves which suckled the colostrum and were challenged with B41 virulent strains 48h after birth. Cows from group D were not vaccinated and their calves were challenged 18h after birth, and afterwards maintained with artificial colostrum. Campo Grande-MS, Brazil, 1991.

	SERA COLLECTION								
GROUPS		GID test				ELISA test			
	N°	1st[a]	2ndb	3rdc	calves[d]	1st	2nd	3rd	calves
	2147[e]	0[i]	0	0	16[j]	-[k]	-	-	-
A	2277	0	0	0	0	-	-	-	-
	2279	0	0	4	64	-	-	+[l]	+
	0488[f]	0	64	32	16	-	+	+	+
B	0577	0	16	64	64	-	+	+	+
	1118	0	64	64	64	-	+	+	+
	1117[g]	0	0	64	64	-	-	+	+
C	1393	0	0	64	64	-	-	+	+
	3106	0	4	0	0	-	-	-	-
	1162[h]	0	0	0	M	-	-	-	M
D	1337	0	0	0	M	-	-	-	M
	3009	0	0	0	0	-	-	-	-

a Sera collected before the 1st dose of vaccine.
b Sera collected before the 2nd dose of vaccine.
c Sera collected just after delivery challenge with B41 strain performed within high after birth.
d Sera collected from the respectives calves.
e Group A: vaccinated with doses of 1,500 HU K99-F41 antigens.
f Group B: vaccinated with doses of 750 HU K99-F41 antigens.
g Group C: vaccinated with doses of 380 HU K99-F41 antigens.
h Group D: negative controls - unvaccinated cows.
i Negative reaction in the agar gel immunodiffusion (GID) test.
j Reciprocal of the last dilutions of sera which still gave a precipitation line in the GID test.
k Negative reaction in ELISA assay when the sera tested gave reaction equal or weaker than 1:20 dilution used for the negative control serum.
l Positive reaction in ELISA assay, when the serum under tested shown yellowish color equal to or intenser than the 1:1,500 dilution used for the positive control serum.
M Deaths of challenged calves.

ference of these antibodies to calves through the colostrum, as is shown in Tab. 1. With regard to group D it is important to note that 2 out of 3 calves born, died as a consequence of acute diarrhea (Tab. 1). Neither the cows of this group nor the respective calves showed positive reaction for K99-F41 antibodies either by GID or ELISA tests. On the other hand, calves from Lots A, B and C were fully protected to challenge even in case of the calf born from 2277 (group A) which did not show antibody transference through colostrum. Also, this cow did not answer to the vaccine injection by the production of humoral immune response as detected by GID and ELISA tests.

DISCUSSION

STIRM et al.[18] (1967) described methods for the extraction of K88 antigen. When used in the present study for the extraction of K99 and F41 antigens, these procedures showed a very good yield. KORHONEN et al.[6] (1980) reported that treatment of such preparations with sodium desoxicolate (DOC) precipitated some lipids and lipoproteins which render the final performance of the process very efficient and allow the recovery of 66% of K99 and F41 antigens present in the crude extract. Thus, the production of a semi-purified K99-F41 vaccine becomes feasible. In other words, starting from 5 litres of Minca Medium inoculated with strain B41 ($K99^+F41^+$), 300,000 HU of K99 and F41 were obtained, which corresponded to 200 doses of vaccine, containing 1,500 HU. Taking into consideration that 750 HU was the ideal dilution, 400 doses of this vaccine could be obtained, with better performance (Tab. 1). Finally, a vaccine made with 380 HU, though showing a dose-response of 21.1% of serological conversion it was observed to fully protect calves of the respective cows against the challenge and 2 out of 3 calves reflected that antibody transference through colostrum did occur, as detected by GID and ELISA tests. In conclusion, we might have 800 doses of vaccines from 5 litres of culture in Minca medium whose preparation is easy and very cheap.

Sixteen sera from the 63 calves born from vaccinated cows (1,500 HU) could not be tested since the sera were lost mainly due to contamination and/or broken vials during mailing. Also taking into consideration 3 calves born group A (1,500 HU) among the 50 remaining calves, 33 (66%) showed serological conversion transmitted through colostrum. Even considering that the GID tests is of limited sensitivity, it was noticed that 5 cows (data not shown) with a negative serological conversion after vaccination were able to transfer antibodies to their calves.

As to the results of ELISA as compared with the GID test we observed overall that from 259 sera obtained from calves and from the pregnant cows in the 2nd and 3rd collections 101 (38.99%) were positive in the latter test, whereas in the

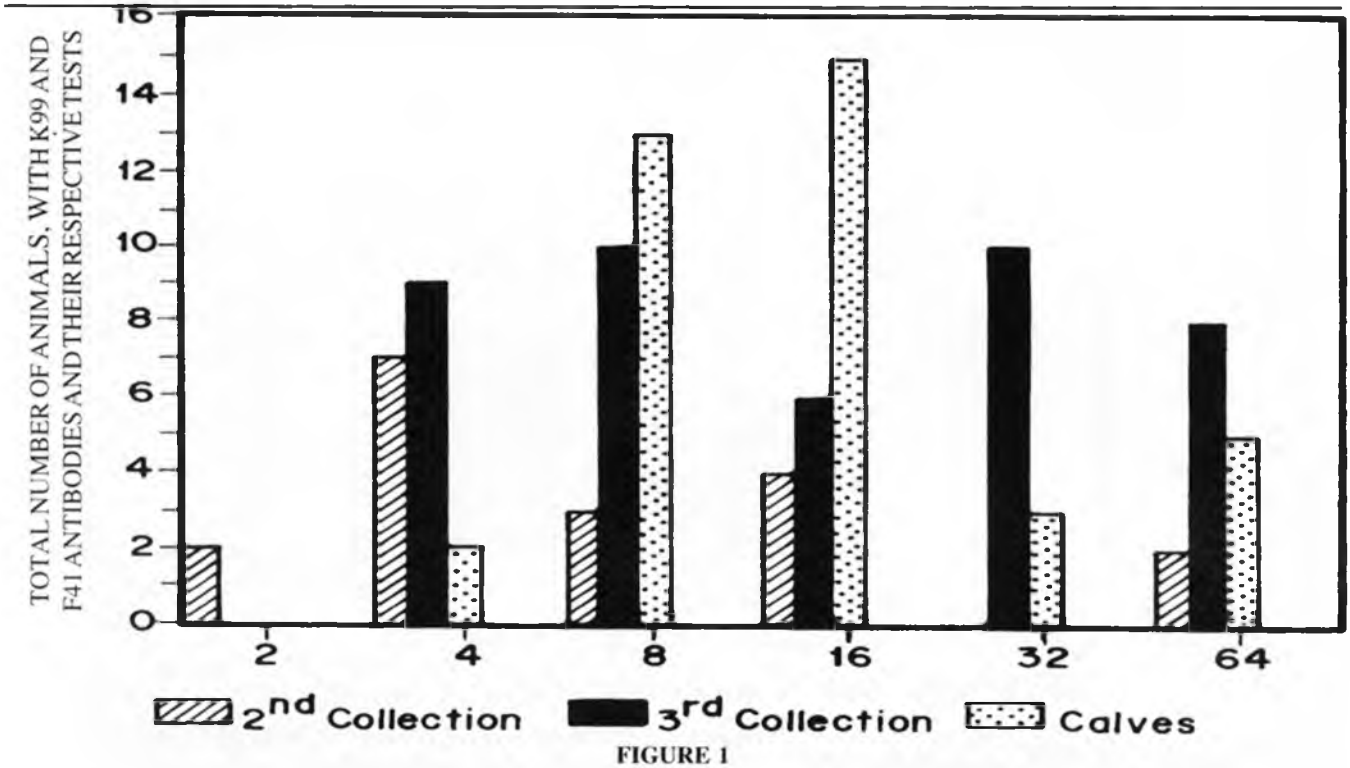

FIGURE 1

Results of the agar gel immunodiffusion (GID) test with sera of pregnant cows vaccinated with 1,500 HU and their respective calves, sera were two-fold diluted from 1:2 to 1:64. Positive results were obtained only with sera from cows bled in 2nd and 3rd collection as well as from calves which suckled the colostrum.

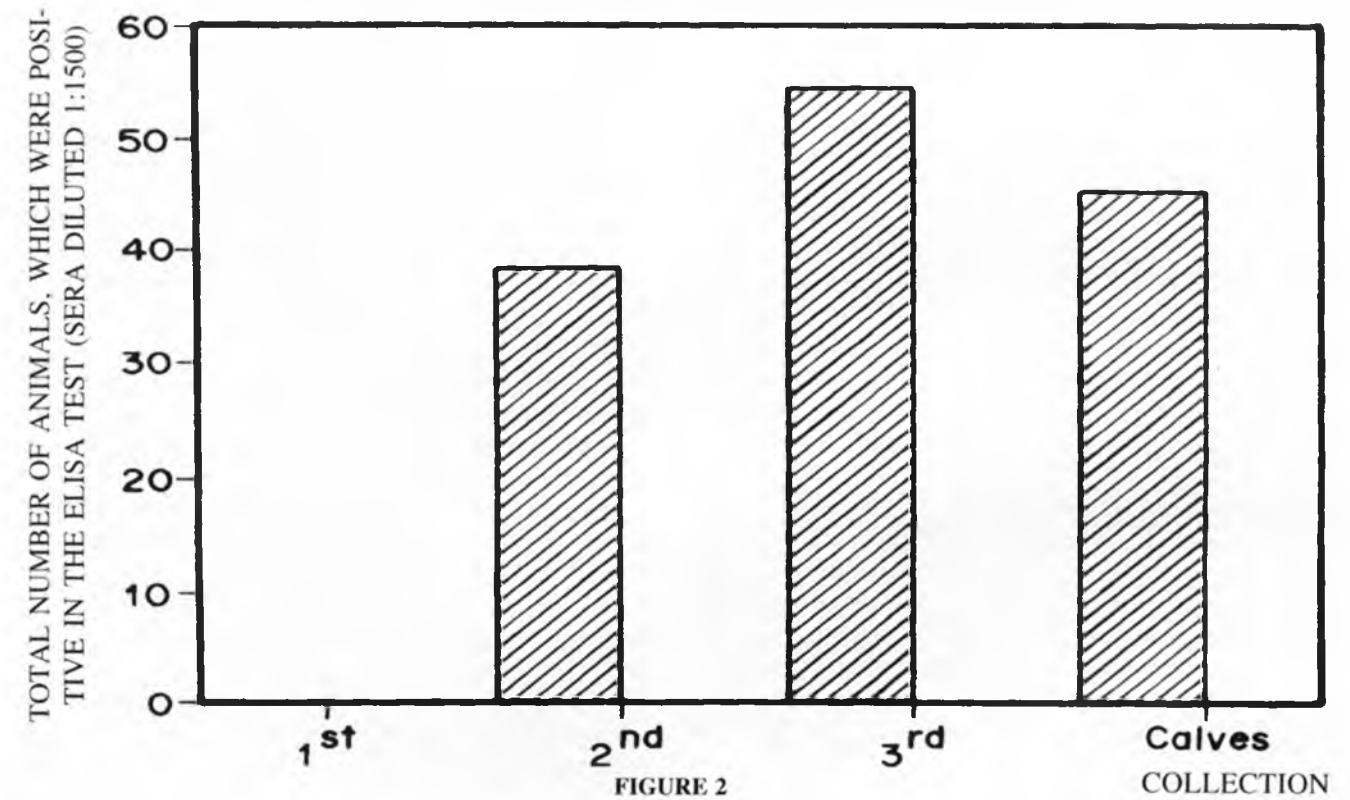

FIGURE 2

Results of serological conversion as assayed by ELISA test using sera diluted 1:1,500. (a) Sera collected before the 1st dose of vaccine (1,500 HU of K99 and F41 antigens), (b) Sera collected before the 2nd dose of vaccine (1,500 HU of K99 and F41 antigens), (c) Sera collected at delivrance time, (d) Sera collected from calves after suckling the colostrum.

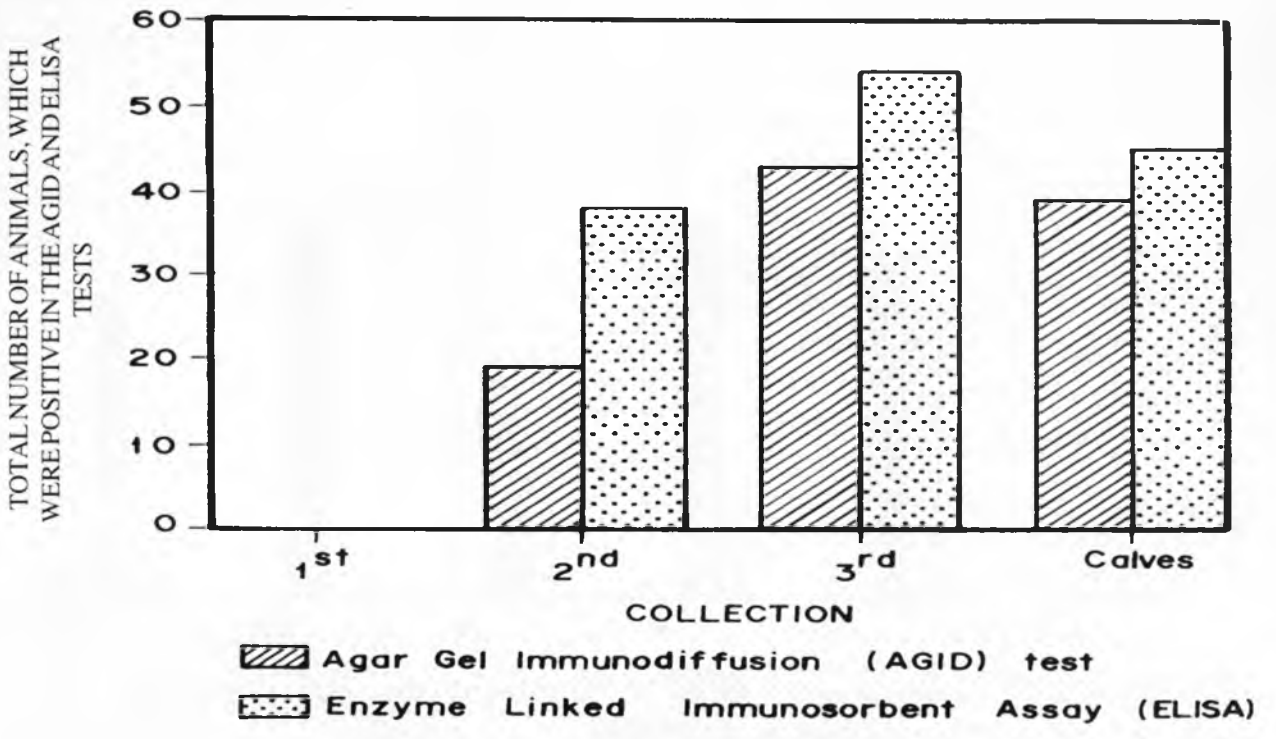

FIGURE 3

Comparison between the results obtained in the GID and ELISA tests with regard to the three collections of blood from vaccinated cows and their respective calves. It can be noticed the ELISA was more efficient than GID test as to the detection of K99 and F41 antibodies is concerned.

ELISA, 135 (52.73%) positive reactions were obtained $x^2 = 8.37$ ($p<0.01$). Furthermore we must consider that on the average, the sera dilutions in the ELISA test, were 70 times higher than those used in the GID. Finally, as is shown in Tab. 1, none of the vaccinated cows calves was susceptible to heavy challenge with virulent B41 strain ($K99^+ F41^+$). At this point we may consider that we have achieved an efficient and easy-to-standardize vaccine which is relatively cheap and with methods available to measure "in vitro", at least partially, its potency, specificity and serological conversion without interference of other antigens such as O, K and H which are not measured in our GID and ELISA tests.

On the order hand, it is important to call attention to the fact that none of the unvaccinated cows and their calves demonstrated the presence of K99 and F41 antibodies in their sera. Thus 2 out of 3 calves from Group D (control) died of acute diarrhea caused by challenge with B41 virulent strain, suggesting that vaccination with K99 and F41 antigens is strongly advisable for prophylaxis of bovine colibacillosis. There is no explanation for one of the calves from group D not dying due to diarrhea. One may assume that there were small amounts of antibodies present in the cows colostrum transfered to its calf and not detected by GID and ELISA tests. We cannot exclude, though there is no evidence to this concern, that our vaccine may also have induced some cell-mediated immunity, which by some mechanism not yet studied, might have interfered with the adhesion or enteropathogenicity of the B41 virulent strain.

Our finding also demonstrated that the vaccine made with semi-purified K99 and F41 antigens did not provoke any adverse reactions in the pregnant cow i.e no abortions, no noticeable general effects such as fever, malaise and so on, proving also to behave well as an easily prepared immunogen in homogeneous lots, efficient against bovine neonatal passive immunoprophylaxis.

ACKNOWLEDGEMENTS

We are grateful to all technician from EMBRAPA, MS, for excelent technical support. We thank Linda Headrick for reviewing the manuscript.

RESUMO

Os antígenos K99-F41 foram extraídos da amostra de ***Escherichia coli*** B41 por aquecimento e semi-purificados pela precipitação com sulfato de amônio e tratamento com desoxicolato de sódio (DOC). Os antígenos semi-purificados foram utilizados na produção de uma vacina oleosa contra a colibacilose bovina. Foram preparadas vacinas contendo em cada dose 1.500 HU (Unidades Hemaglutinantes), 750 HU e 380 HU. A eficiência da vacina foi avaliada através do ensaio de imunodifusão dupla, ensaio imunoenzimático (ELISA) e por um desafio, em que a amostra de ***Escherichia coli*** virulenta foi inoculada nos bezerros nascidos de vacas vacinadas e não vacinadas. Observamos que a vacina contendo 750 HU foi a que melhor induziu a produção de anticorpos nas vacas vacinadas, e que estes mostraram-se protetores, uma vez que os bezerros nascidos de vacas vacinadas e que mamaram o colostro, nada sofreram no desafio. Não se verificou nenhum efeito colateral nas vacas vacinadas.

UNITERMOS: Escherichiose; Bovinos; Vacinas; ***Escherichia coli***.

REFERENCES

01-ACRES, S.D. Enterotoxigenic **Escherichia coli** infections in newborn calves: A review. **Journal of Dairy Science**, v.68, p.229-56, 1985.

02-ACRES, S.D.; FORMAN, A.J.; KAPITANY, R.A. Antigen extinction profile in pregnant cows, using a K99 containing whole-cell bacterin to induce passive protection against enterotoxigenic colibacillosis of calves. **American Journal of Veterinary Research**, v.43, p.569-75, 1982.

03-FIELD, M.; GRAAF, L.H.; LAIRD, W.J.; SMITH, P.L. Heat-stable enterotoxin of ***Escherichia coli***:" in vitro" effects on guanilate cyclase activity, cyclic GMP concentration and ion transport in small intestine. **Proceedings of the National Academy of Sciences**, v.75, p.2800-4, 1970.

04-GUINÉE, P.A.M.; VELDKAMP, J.; JANSEN, W.H. Improved Minca medium for detection of K99 antigen in calf enterotoxigenic strains of ***Escherichia coli***. **Infection and Immunity**, v.15, p.676-8, 1977.

05-GYLES, C.L. Heat-labile and heat-stable forms of the enterotoxin from ***Escherichia coli*** strains enteropathogenic for pigs. **Annals of the NewYork Academy of Sciences**, v.176, p.314-22, 1971.

06-KORHONEN, T.K.; NURMIAHO, E.L.; RANTA, H.; SVANBORG-EDEN, C. New method for isolation of immunologically pure pili from ***Escherichia coli***. **Infection and Immunity**, v.27, p.569-75, 1980.

07-LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature**, v.227, p.680-5, 1970.

08-LOGAN, E.F.; PENHALE, W.J.; JONES, R.A. Changes in the serum immunoglobulins levels of colostrum-fed calves during the first 12 weeks post-partum. **Resear ch Veterinary Science**, v.14, p.394-7, 1973.

09-MOON, H.W. Protection against enteric colibacillosis in pigs suckling orally in vaccinated dams: Evident for pili as protective antigens. **American Journal of Veterinary Research**, v.42, p.173-7, 1981.

10-MORRIS, J.A.; THORNS, C.; WELLS, G.A.H.; SCOTT, A.C.; SOJKA, W.J. The production of F41 fimbriae by piglet strain of enterotoxigenic ***Escherichia coli*** that lack K88, K99 and 987P fimbriae. **Journal of General Microbiology**, v.129, p.2753-9, 1983.

11-MYERS, L.L. Enteric colibacillosis in calves: Immunogenicity and antigenicity of ***Escherichia coli*** antigens. **American Journal of Veterinary Research**, v.39, p.761-5, 1978.

12-ORSKOV, I.; ORSKOV, F.; SMITH, H.W.; SOJKA, W.J. The establishment of K99, a thermolabile, transmissible ***Escherichia coli*** K antigens, previously called "Kco" possessed by calf and lamb enteropathogenic strains. **Acta Pathologica Microbiologica Scandinavica**, v.83, p.31-6, 1975.

13-OUCHTERLONY, O. Diffusion in gel methods for immunological analysis. **Progress in Allergy** , v.5, p.1-78, 1958.

14-PARRY, S.H.; PORTER, P. Immunological aspects of cell membrane adhesion demonstrated by porcine enteropathogenic ***Escherichia coli***. **Immunology**, v.34, p.41-9, 1978.

15-SMITH, H.W.; HALLS, S. Studies on ***Escherichia coli*** enterotoxins. **Journal of Pathology and Bacteriology**, v.93, p.531-43, 1976.

16-SMITH, H.W.; HUGGINS, M.B. Experimental infection in calves, piglets and lambs with mixture of invasive and enteropathogenic strains of ***Escherichia coli***. **Journal of Medical Microbiology**, v.12, p.507-10, 1979.

17-SOJKA, W.J. **Escherichia coli in domestic animals and poultry**. Farnhan Royal, Commonwealth Agricultural Bureaux of Animal Health, 1965.

18-STIRM, S.; ORSKOV, F.; ORSKOV, I.I.; MANSA, B. Episomecarried surface antigen K88 of ***Escherichia coli***. II- Isolation and chemical analysis. **Journal of Bacteriology**, v.93, p.731-9, 1967.

19-TZIPORI, S. The relative importance of enteric pathogens affecting neonates of domestic animals. **Advances Comparative Medicine NewYork**, v.29, p.103-203, 1985.

20-VOLLER, A.; BIDWELL, D.E.; BARTLETT, A. Microplate enzyme immunoassay for the immunodiagnosis of virus infections. In: ROSE, N.R.; FRIEDMAN, H.; eds. **Manual of clinical immunology**. Washington, American Society for Microbiology, 1976. Cap. 69, p.506.

21-YANO, T.; LEITE, D.S.; CAMARGO, I.J.B.; PESTANA DE CASTRO, A.F. A probable new adhesive factor (F42) produced by enterotoxigenic ***Escherichia coli*** isolated from pigs. **Microbiology Immunological**, v.30, p.495-508, 1986.

Recebido para publicação em 09/09/93
Aprovado para publicação em 02/04/94