IMMUNITY AND PROTECTION ELICITED BY A RECOMBINANT VACCINE AGAINST ENTEROTOXIGENIC *E. coli*.

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SUMMARY

We studied the effectiveness of a recombinant vaccine capable of protecting piglets from enterotoxigenic *E. coli* during lactation and after weaning. Following this purpose, we vaccinated some pregnant sows with the recombinant vaccine VACOLI ^{TM} (Heber Biotec S.A, P.O.Box 6162, Havana, CUBA), composed of enterotoxigenic *E. coli* K88ab and K99 recombinant antigens, plus an oil adjuvant. Before weaning piglets were reimmunized to extend protection, and serum samples were tested by an enzyme-linked immunoassay in solid phase to determine the antibody levels against K88ab and K99. The control of *E. coli* infections was performed by plating stool samples in selective media. There were no death recorded in piglets from vaccinated sows by collibacillosis during lactation, while in piglets from the control sows mortality was of 21%. The morbility and the number of deaths by other causes in the vaccinated group were significantly lower than in the control group. Total protection achieved was 93%. After weaning, mortality caused by *E. coli* was 0.37% in the vaccinated group of piglets and 21.7% in the control group. The total protection in this period was 98%.

RESUMEN

Para estudiar la efectividad de una vacuna recombinante capaz de proteger a cerdos contra la *E. coli* enterotóxica durante la lactancia y después del destete, se realizó la vacunación de cerdas gestantes con la vacuna recombinante VACOLI (Heber Biotec S.A., Apartado 6162, Habana, CUBA), compuesta por los antígenos recombinantes de *E. coli* K88ab y K99 en adyuvante oleoso. Se realizó además la inmunización de los lechones antes del destete para extender su protección y sus muestras de suero se analizaron para determinar los niveles de anticuerpos antiK88ab y antiK99 mediante un ensayo inmunoenzimático en fase sólida. El aislamiento e identificación de las cepas de *E. coli* provenientes de cerditos diarreicos fue realizado mediante los métodos bacteriológicos estandáres. Durante la lactancia no se produjeron muertes por colibacilosis en los cerditos provenientes de cerdas vacunadas, mientras que en los provenientes de las cerdas controles la mortalidad fue del 21%. La morbilidad y el número de muertes por otras causas dentro del grupo vacunado se comportaron significativamente bajas con respecto al grupo control. La protección total alcanzada fue del 93%. La mortalidad causada por *E. coli* después del destete en los cerditos del grupo vacunado fue de un 0.37% y 21.7% en los cerditos del grupo control. La protección total en esta etapa fue del 98%.

INTRODUCTION

Escherichia coli enterotoxicosis is a commonly found swine disease causing diarrhea in piglets in different countries. The pathogenesis of bacterial-enteric infections and host-parasite interactions have been extensively studied in order to develop vaccines to protect piglets from diarrheas, through their dams' colostrum. Knowing the *E. coli* action mechanism in the intestinal tract (1-4), the main interest has been focused on antiadhesine vaccines which keep bacteria from penetrating into the mucous epithelium of the small intestine(5.6).

Several recombinant DNA (rDNA) vaccines against *E. coli* enterotoxicosis used worldwide have proven success (NOBI VAC PORCOLI, INTERVET, Holland; Suvaxyn, SOLVAY, USA, etc.). They are produced by cloning genes responsible for the synthesis of immunoprotector antigens (adhesines, fimbriae) in a new host cell to obtain the recombinant antigens (7).

Enterotoxic *E. coli* strains causing diarrhea in neonatal pigs produce fimbriae known as K88, K99, and 987P (3, 8, 9, 10). The greatest advantage of a subunit vaccine compared with bacterins is the elimination of cellular components that do not contribute to the elicitation of a protective response; e.g: endotoxines that induce shock, vascular permeability and abortion in pregnant females. In this work we show the results from VACOLI vaccine assessment. VACOLI has recombinant K88ab and K99 antigens as the active constituents, plus an oil adjuvant. Administration of the vaccine to the dams in two doses during the gestation period led to production of adhesin-specific antibodies that were transferred to the piglets in colostrum and milk, thus neutralizing bacteria in the challenge experiment ($2LD_{50}$) with enterotoxigenic K88ab and K99 bacteria (11). After vaccination the cumulative probability of healthy piglets from vaccinated sows was significantly greater than that in piglets from control sows. The morbility due to diarrhea in piglets born from vaccinated and control groups was 2% and 21.5% respectively. Fifteen days after weaning, morbility in vaccinated and control groups was 1.73% and 29% respectively. Evaluation of vaccine efficacy was based on a comparison of morbility rates, the mortality by *E. coli* and the antibody titers in the sows' serum and colostrum of the vaccinated vs the control group, before and after vaccination. Vaccine protection was 93% and 98% before and after weaning, respectively.

MATERIALS AND METHODS

The vaccine (VACOLI ^{TM}, Heber Biotec, S.A. P.O. Box 6162, Havana, Cuba) was prepared by the thermic removal of recombinant pilus adhesins K88ab and K99 from the surface of genetically engineered strains of *E. coli* K12. Each 2 mL doses per pig of VACOLI vaccine contained 50 μg of K88ab and K99 adhesins in phosphate buffer with 0.1% formalin (final concentration) and formulated in equal quantities with mineral oil NF 55/ Span 80 adjuvant. VACOLI meets potency test in rabbits, innocuity test in mice and other typical tests for this kind of oily vaccines.

The vaccine was administered parenterally with two doses of 2 mL per pregnant sow to prevent the neonatal diarrhea and two doses of 1 mL per piglet to prevent the postweaning diarrhea.

Animals

Pregnant hybrid sows (crossing between Duroc and Dutch Landrace) having about 250 kg of weight, were used. For the selection of animals we began an epizoothiological study in all rearing units. In the selected unit ("Ingenio Viejo" in the province of Camagüey) several cases of diarrheas in neonatal and postweaned pigs were present. In the investigation, enterotoxic *E. coli* strains K88ab+ were found in a greater proportion than strain K99+ , which followed. Before Vaccination, we studied the antibody levels against K88ab and K99 in serum of sows and chose those having no antibodies in their blood. Two groups were selected at random: the control group (43 animals) and the vaccinated group. (41 animals).

Vaccination of pregnant sows

The group of vaccinated sows was immunized with the first dosis of the vaccine at week 8 of pregnancy, and a second dosis was given week 14 (15 days before parturition). The control group was not immunized. In both doses, 2 mL were applied intramuscularly on the animal's neck. Blood samples were drawn from both groups before the first immunization, and in week sixteen of pregnancy to determine antibody titers in serum at the beginning of the experiment and before parturition, respectively.

Piglets vaccination

Only piglets from vaccinated mothers were immunized. The first dosis was applied between the fifth and seventh days of birth. The second dosis was applied between the twenty first and twenty-third day after birth. Both doses consisted of 1mL of the vaccine, and were applied intramuscularly in the inner side of the thigh.

Enzyme-linked inmunoassay (ELISA)

For the detection of the antibodies against both antigens, serum samples were diluted in phosphate saline buffer (pH 7.2) with 0.05% skim milk, and 100 μL were applied to plates incubated with 50 ng of antigen in 0.05M carbonate buffer (pH 9.6) per well at 4°C overnight and then washed with 0.02% Tween 20 three times.

The plates were incubated at 37°C for 1h and then washed with 0.02% Tween 20 three times. The amount of antibodies binding to the antigen was measured colorimetrically by incubating the samples at 37°C for 1h with 100 μL of goat anti-pig IgG conjugated to peroxidase diluted 1:1000 in 0.05% skim milk in phosphate saline buffer. Then, the O-phenylenediamine dihydrochoride substrate was added. The reaction was stopped with 2.5 M H_2SO_4 and the optical density (OD) at 492 nm was determined in a plate reader (Multiskan Titertek MCC 340). The results were expressed in (OD) values at serum dilution of 1:500.

Sampling and clinical observations

All piglets between 0 and 35 days of age were observed daily for signs of diarrhea. Stools were collected from piglets with diarrhea and plated on Mac Conkey agar (Oxoid, UK) and blood agar base (Merck, Germany) containing 5% sheep blood. From each piglet, 5 to 10 colonies with the typical appearence of *E. coli* were randomly chosen. However, when typical *E. coli* colonies of different morphology existed on the same plate, at least one colony with each morphology was chosen for further characterization (12) . The *E. coli* enterotoxigenic K88ab+ and K99+ strains were classified by hybridization with DNA probes(13). Such tests went on until day fifteen after weaning, evaluating also morbility and mortality at that stage.

Statistical analysis

Results were compared by a hypothesis test (14).

RESULTS AND DISCUSSION

The development of the vaccine against *E. coli* enterotoxicosis was greatly stimulated by a thorough understanding of the disease pathogenesis mechanism. The technical development of the vaccine was also facilitated, since the genes being cloned directed the synthesis of surface structures that were transported to the cell surface in the recombinant strains . This

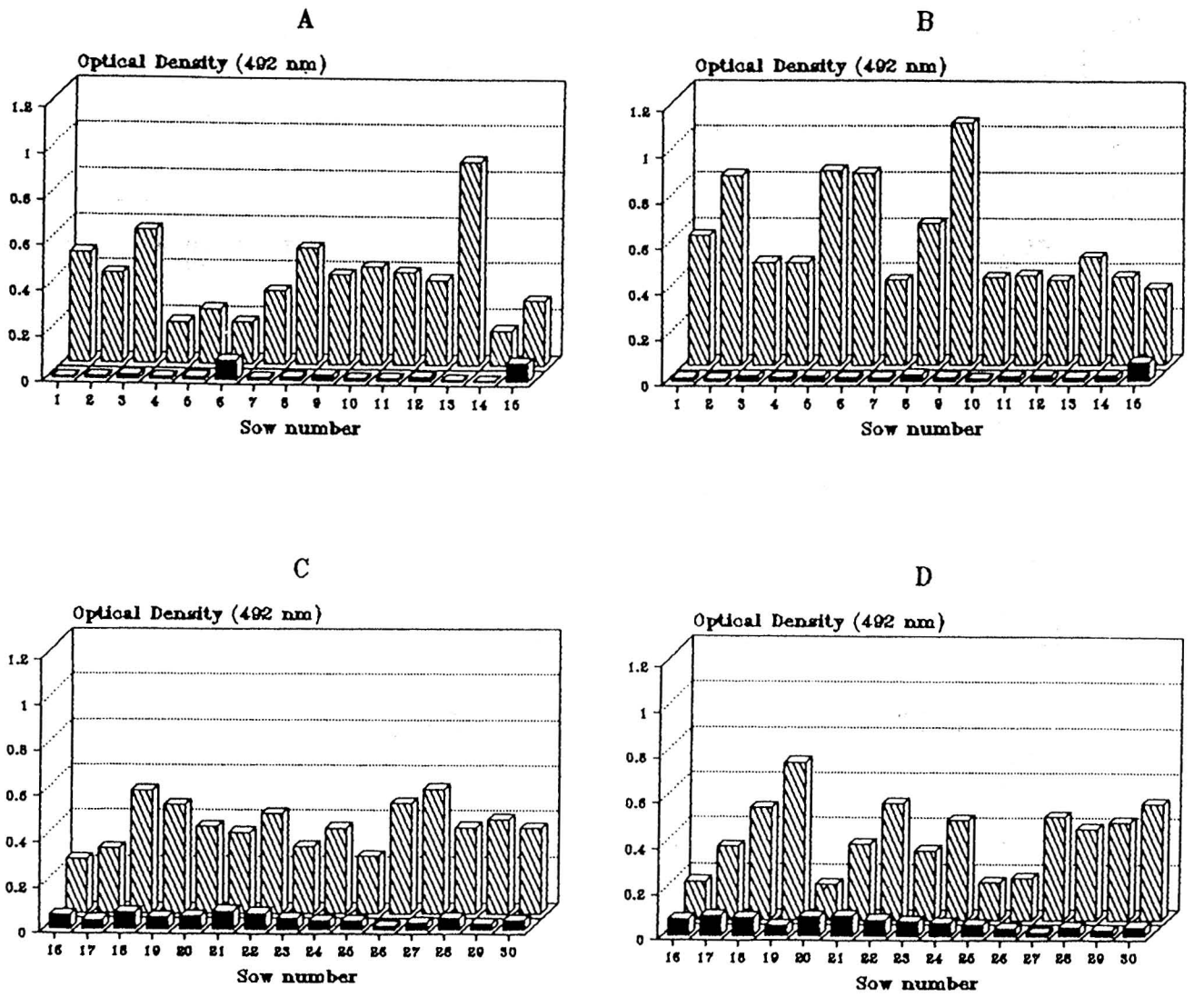

Fig.1. Antibody titers against K88ab (A,C) and K99 (B,D) antigens in serum from vaccined sows before vaccination (dark bars) and before parturition (striped bars).

characteristic facilitated the purification of the recombinant antigens. According to the results obtained in the antibody test (ELISA), the group of vaccinated sows developed a high immune response both against rec-K88ab and rec-K99 antigens (figs. 1,2). Piglets from the vaccinated group were found to have serum antibodies against both antigens, during the first week after birth. Antibodies were transferred by their dams' colostrum and after two reimmunization shots, a low incidence of diarrhea was observed (figs. 3,4)

The cumulative probability per day of healthy piglets in both groups is shown in figure 5. The differences are statistically significant ($p < 0.05$). On day 15 of birth, cumulative probability was

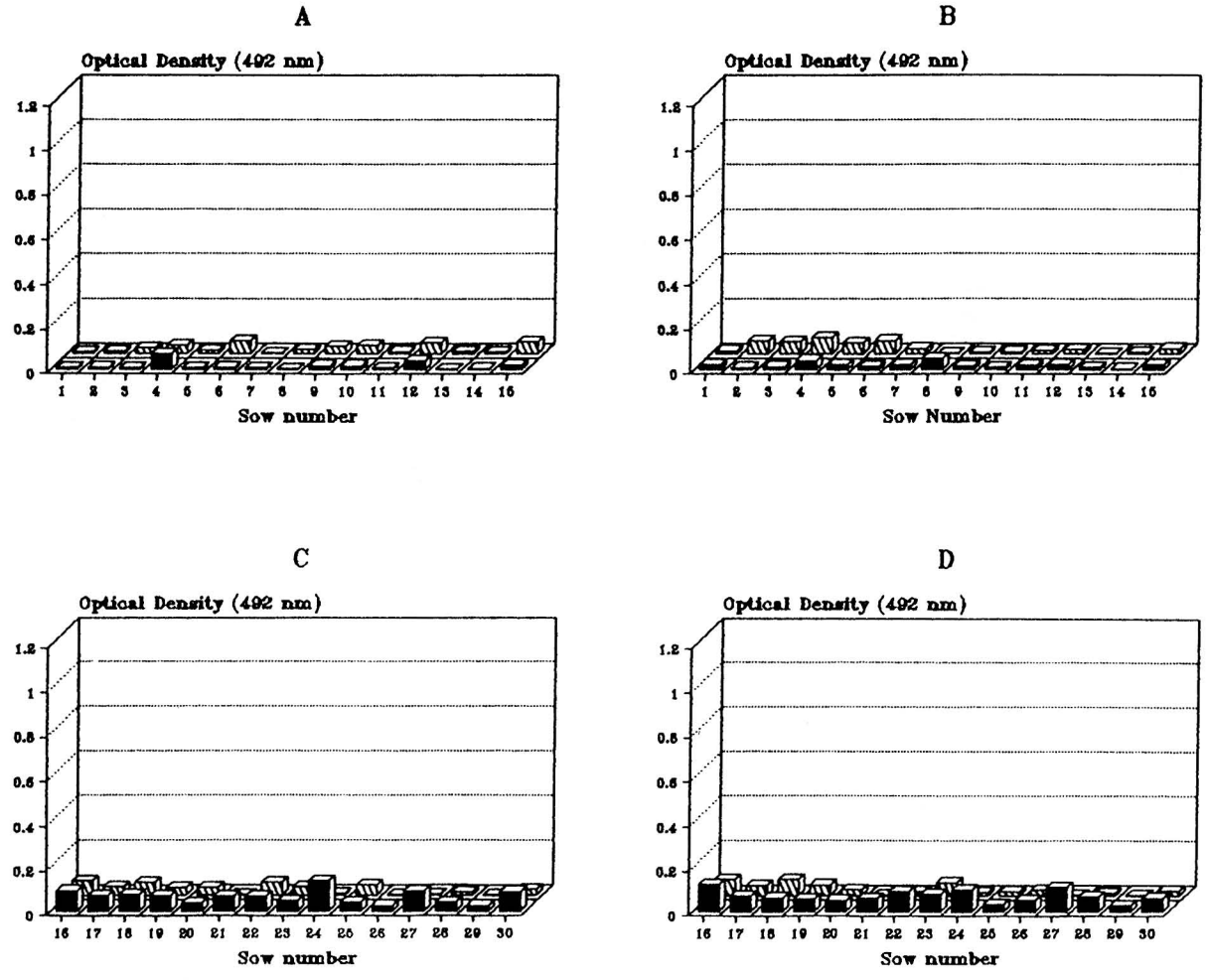

Fig. 2. Antibody titers against K88ab (A,C) and K99 (B,D) antigens in serum from control sows before vaccination (dark bars) and before parturition (striped bars).

near zero for the control group. However, in the vaccinated group, it remained near 0.8, with values of 0.5 until day 42 of lactation.

A higher percent of sick piglets in the control group appeared between days ten and twenty after birth, when the greatest number of diarrheas appeared (fig. 6).

Table 1 shows the mean of piglets per day during lactation (274 from vaccinated sows and 223 from unvaccinated sows).The mean of sick piglets was only 6 in the former, and 48 in the latter. Morbility due to diarrhea in the control group reached up to 21.5% and only 2% in the vaccinated group, both with an average age of 14 days. Of all samples of diarrhea analyzed, 98% corresponded to enterotoxigenic K88ab *E. coli* and only 2% to K99 *E. coli*. Among piglets from the control swine, 60 deaths were caused by colibacillosis, and 33 by other causes. Mortality by *E. coli* in this group was up to 21%, having a daily morbilethality of 2.98%. Piglets from vaccinated sows were less propense to other diseases, with less number of deaths by other causes and presenting no deaths by colibacillosis (table 2).

At weaning, the mean weight of piglets which had diarrheas during their first 20 days of life was 6.39 kg and in the clinically healthy animals it was 8.99 kg. This 2.6 kg difference in the mean weights of weaned pigs was statistically significant ($p < 0.05$) (table 3).

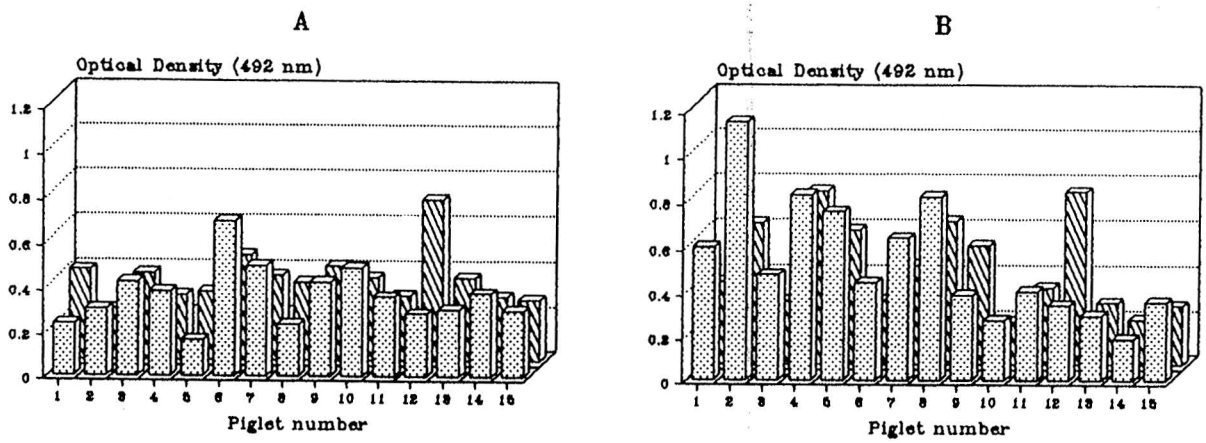

Fig. 3. Antibody titers against K88ab (A) and K99 (B) in serum of piglets from vaccined sows 5-7 days after birth (pointed bars) and 40-45 days after birth (striped bars).

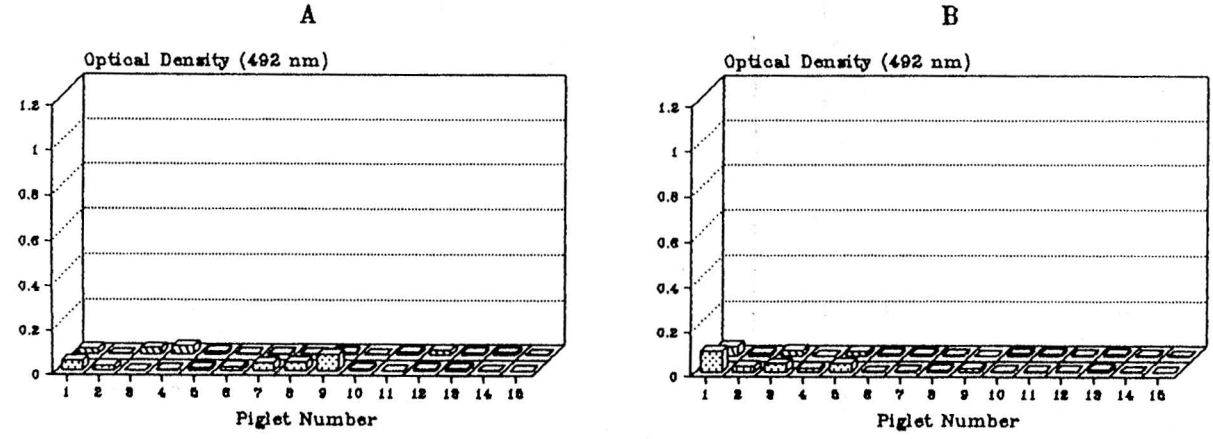

Fig.4. Antibody titers against K88ab (A) and K99 (B) in serum of piglets from control sows 5-7days after birth (pointed bars) and 40-45 days after birth (striped bars).

The results 15 days after weaning are recorded in table 4. The morbility and morbilethality in the vaccinated group were significantly lower than that seen in the control group ($p < 0.01$). The mortality due to *E. coli* infection was 0.37% in the vaccinated group and 21.7% in the control group. VACOLI causes no side effects or adverse reactions and offered a total protection of 93% in suckling piglets and 98% in weaned piglets.

CONCLUSIONS

The possibility of developing recombinant vaccines against *E. coli* infections in pigs permits the application of "home-produced" safe and efficient vaccines allowing the inclusion of the prevalent antigens, thus reducing the cost of the vaccine preparations. The administration of the vaccine VACOLI together with an adequate hygiene and proper handling of animals can reduce

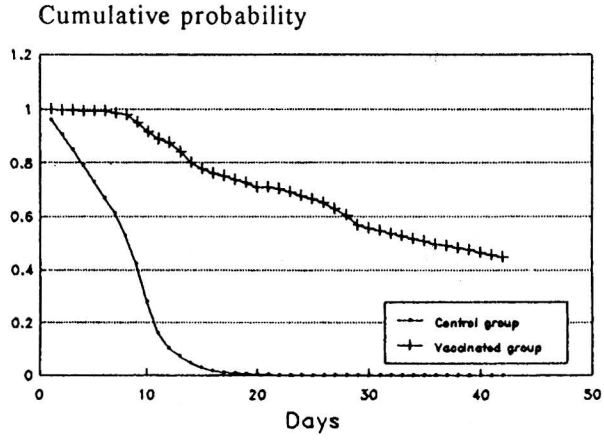

Fig.5. Cumulative probability of healthy piglets

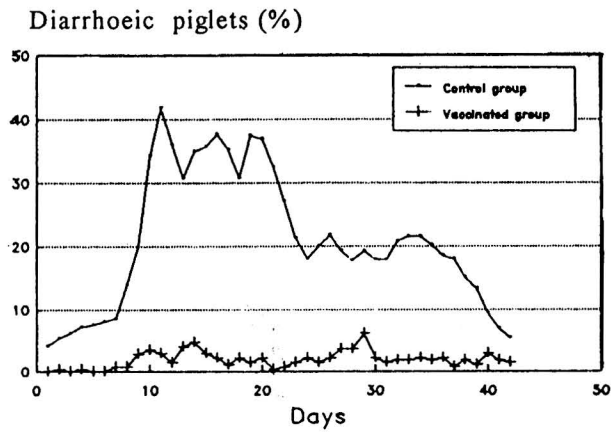

Fig. 6. Daily percentage of diarrhoeic piglets

Table 1
Performance of suckling piglets during lactation.

	Group Vaccinated	Group Control
Piglets[a]	274	223
Piglets per family[b]	7	8
Sick piglets[c]	6	48
Morbility (%)[d]	2	21.5

(a) Daily mean number of piglets.

(b) Calculated as the ratio between the total number of piglets and the number of families.

(c) Calculated as the ratio between the sum of daily sick piglets and the number of days of lactation.

(d) Calculated as the ratio between the number of sick piglets per day and the mean number of piglets per day.

Table 2.
Morbility of suckling piglets during lactation

	Group Vaccinated	Group Control
Deaths due to *E.coli*	0	60
Mortality by *E. coli* (%)[a]	0	21
Deaths by other causes[b]	19	33
Mortality by other causes (%)[c]	7	12
Morbilethality by *E.coli* (%)[d]	0	2.98

(a) The mortality caused by *E. coli* infection (%) was calculated as the ratio between the total number of deaths due to *E. coli* and the total number of births multiplied by 100.

(b) Represents the difference between the total number of deaths and the number of deaths caused by colibacillosis.

(c) The mortality (%) by other causes was calculated as the ratio between (b) and the total number of births, multiplied by 100.

(d) Represents daily morbilethality percentage caused by *E.coli* and was calculated as the ratio between the number of deaths per day caused by *E. coli* and the daily mean of diarrhoeic piglets, multiplied by 100.

Table 3
Weight of suckling piglets during lactation.

	Group Vaccinated	Group Control
Mean weight at birth (kg)[a]	0.9	0.9
Mean weight at weanig (kg)[b]	8.99	6.39
Overall weight increase (kg)[c]	7.31	5.49

(c) Represents the overall weight increase during lactation, and was determined as the difference (b)-(a).

Table 4.
Morbility and mortality 15 days after weaning

	Group Vaccinated	Group Control
Piglets[a]	271	180
Morbility(%)[b]	1.73	29
Morbilethality(%)[c]	1.43	5
Mortality (%)[d]	0.37	21.7

(a) Represents the mean number of piglets daily and was calculated as the ratio between the daily count of animals and the number of days evaluated after weaning.

(b) Represents the daily morbility and was calculated as the ratio between the mean of sick piglets each day and the mean of animals arriving daily at the post weaning period, multiplied by 100.

(c) Represents the daily morbilethality and was calculated as the ratio of the mean number of deaths per day and the daily mean number of sick animals.

(d) The mortality caused by *E. coli* (%) was calculated as the ratio between the number of deaths caused by *E. coli* infection and the daily mean number of animals, multiplied by 100.

mortality, improve feed conversion and decrease other second degree infections, caused by entero-toxigenic *E. coli*.

This vaccine is now in use in all the swine units in the country and a long-term evaluation of the effects of the vaccine in production is in progress.

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