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Use of vaccine and probiotic in the control of swine diarrhea caused by enterotoxigenic *Escherichia coli*

(Utilização de vacina e probiótico no controle de diarréia enterotoxigênica em suínos causada por Escherichia coli)

F.A. *Ávila*[1], R.P. *Schocken-Iturrino*[1], J.L. *Quintana*[1], A. *Basso*[2], J.A. *Ambrosim*[2]

[1]Departmento de Medicina Veterinária da Faculdade de Ciências Agrárias e Veterinárias - UNESP
Rodovia Carlos Tonani, km 5
14870-000 – Jaboticabal, SP
[2]Estudante de Veterinária da FCAV-UNESP
Bolsista do CNPq, FAPESP

ABSTRACT

A total of 42 pregnant sows were divided into eight groups and submitted to the following treatments: group I with seven unvaccinated sows whose piglets did not receive probiotic, was used as control, group II with five vaccinated sows whose piglets did not receive probiotic, groups III, IV and V with five vaccinated sows each whose piglets received probiotic for 5, 15 and 28 days, respectively, and groups VI, VII and VIII with five unvaccinated sows each whose piglets received probiotic for 5, 15 and 28 days, respectively. Each animal in the vaccinated groups received subcutaneously two doses of 5.0ml of vaccine containing pili K88, K99, 987P and F42 of Escherichia coli. *The probiotic contained* Lactobacillus acidophilus *at the dose of 2.0×10^8 live cells in 20ml of milk and was administered orally. All animals were observed clinically and bacteriologically and the titers of anti-K88, anti-K99, anti-987P and anti-F42 antibodies were determined in serum and colostrum. The results showed that the vaccine associated to the probiotic administered for 28 days was the most effective treatment for the control of diarrhea caused by enterotoxigenic* Escherichia coli.

Keywords: Swine, Escherichia coli, Lactobacillus acidophilus, *vaccine, probiotic*

RESUMO

Quarenta e duas porcas prenhes foram divididas em oito grupos e submetidas aos seguintes tratamentos: grupo I-com sete porcas não vacinadas, cujos leitões não receberam probiótico, as quais permaneceram como controle, grupo II-com cinco porcas vacinadas e seus leitões que não receberam probiótico, grupos III, IV e V-com cinco porcas cada, vacinadas, e seus leitões que receberam probiótico durante 5, 15 e 28 dias, respectivamente, e grupos VI, VII e VIII-com cinco porcas cada, não vacinadas, e seus leitões que receberam probiótico durante 5, 15 e 28 dias, respectivamente. Cada animal dos grupos vacinados recebeu duas doses de 5,0ml de vacina contendo os pili K88, K99, 987P e F42 de *Escherichia coli* por via subcutânea. O probiótico continha *Lactobacillus acidophylus* na dose de $2,0\times10^8$ células vivas em 20ml de leite e administrado por via oral. Em todos os animais observados clinica e bacteriologicamente, foram determinados os títulos de anticorpos anti-K88, anti-K99, anti-987P e anti-F42 no soro e no colostro. Os resultados mostraram que a combinação de vacina com o probiótico por 28 dias foi o mais eficiente tratamento para o controle de diarréia causada por *Escherichia coli* enterotoxigênica.

Palavras-Chave: Suíno, *Escherichia coli*, *Lactobacillus acidophilus*, vacina, probiótico

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e-mail: faavila@fcav.unesp.br

INTRODUCTION

Escherichia coli has been frequently isolated as an agent causing diarrhea in suckling piglets in several countries (Gossling & Rhoades, 1966; Gyles & Barnum, 1967; Ávila et al., 1983; Castro et al., 1984; Carvalho et al., 1991).

The development of *Escherichia coli*-induced diarrhea depends on two important factors, i.e., enterotoxin production and colonization of the small intestine (Hadad & Gyles, 1982; Smith & Halls, 1967). The enterotoxins produced cause water and electrolyte leakage into the intestinal lumen, provoking diarrhea and dehydration. Colonization of the small intestine is another important factor for the onset of diarrhea (Smith & Hallis, 1967; Smith & Huggins, 1978). The surface antigens designated as K88(F4), K99(F5) and 987P(F6) are important for the colonization of the small intestine of piglets (Jones & Rutter, 1972; Morgan et al., 1978).

Yano et al. (1986) described the adhesive antigen F42 in strains of *Escherichia coli* that are enterotoxigenic for swine, an antigen that differed from those previously described. This antigen has an agglutinating action on human, horse, guinea pig, sheep and chicken red blood cells. F42 is encoded by a plasmid and produces the Sta enterotoxin.

The ingestion of colostrum from sows immunized against *Escherichia coli* carrying pili K88, K99 and 987P has been reported to be effective in protecting piglets against colibacillosis (Avila et al., 1986). The introduction of antigen F42 into this experimental vaccine may possibly broaden its protective spectrum. On the other hand, the major factor for the good physiological performance of the intestinal tract results from the equilibrium of the bacterial flora. This equilibrium may be impaired by aggressive phenomena that modify intestinal secretion and peristalsis. In this case, pathogenic microorganisms such as enterotoxigenic *Escherichia coli* may proliferate (Ávila et al., 1987).

Probiotics are viable bacterial cultures administered orally to the animals, which basically consist of lactic acid producing-bacteria (Golding & Gorbach, 1984), i.e., lactobacilli or enterococci. *Lactobacillus acidophilus* is considered to be a good probiotic (Ávila et al., 1995; Tournut et al., 1988).

The role of probiotics as microbial bioregulators is to maintain the equilibrium of the intestinal flora, the major property being the prevention of the multiplication of pathogenic bacteria such as enterotoxigenic *Escherichia coli* (Ávila et al., 1987).

The objective of the present study was to compare the efficacy of a probiotic, a vaccine and their combination in the control of enterotoxigenic *Escherichia coli*-induced diarrhea in piglets.

MATERIAL AND METHODS

The probiotic used was a strain of *Lactobacillus acidophilus* (LBV-1) isolated from the intestinal tract of an adult cow as recommended by Wolf et al. (1975), with the following characteristics: apathogenic, lactic acid producing and resistance to pH 4.0 to 3.0.

The inoculum contained 2.0×10^8 live cells of *Lactobacillus acidophilus* (Tournut et al., 1988) in 10g powdered skim milk (lyophilized form).

The probiotic, resuspended in 20ml water, was administered individually to the piglets by the oral route starting on the first day of life.

There were 42 pregnant sows belonging to the Paineiras Farm, municipality of Jaboticabal, SP, Brazil. The animals were divided into eight groups and submitted to the following treatments: group I (N=7) consisted of unvaccinated sows whose piglets did not receive the probiotic, and was used as control, group II (N=5) consisted of vaccinated sows whose piglets did not receive the probiotic, groups III, IV and V (N=5 each) consisted of vaccinated sows whose piglets received the probiotic for 5, 15 and 28 days, respectively, and groups VI, VII and VIII (N=5 each) consisted of unvaccinated sows whose piglets received the probiotic for 5, 15 and 28 days, respectively.

The experimental vaccine was prepared with four strains of *Escherichia coli* that are producers of pili K88, K99, 987P and F42. The cultures were inactivated with commercial 40% formalin and adsorbed to aluminum hydroxide. The vaccinal dose was 5.0ml and contained 5.0×10^9 bacteria.

In the vaccinated groups, each pregnant sow received two vaccine doses of 5.0ml by the subcutaneous route. The first dose was administered during the 5th or 6th week before parturition and the second three weeks after the first. The animals that were not vaccinated received a 5.0ml dose of placebo (sterile saline) also during the 5th or 6th week before parturition, followed by other dose three weeks later.

Blood samples were collected from each sow before vaccination or placebo administration and on the day of parturition. Colostrum was also obtained for the determination of anti-K88, anti-K99, anti-987P and anti-F42 antibodies. Antibody titers were determined by the tube agglutination technique according to the method of Acres et al. (1979). The titer is expressed as the reciprocal of the highest serum dilution that presented agglutination.

All passively immunized, probiotic-treated and control piglets were examined clinically during the experiment and the cases with diarrhea were recorded. Fecal swabs were obtained from piglets with diarrhea and submitted to bacteriologic examination. The sick animals were then treated.

The fecal samples were plated into McConkey agar and MRS agar and incubated at 37°C for 24 hours. Typical *Escherichia coli* colonies were identified using the following tests: lactose fermentation, indole production, methyl red and Voges & Proskauer reactions, utilization of citrate, urease formation, and H_2S production. For identification *Lactobacillus acidophylus* slids were made and stained using Gram methods. The suspect colonies were identified by bioquimical and serological tests.

Serologic identification of the *Escherichia coli* strains was carried out with OK antiserum produced in rabbits against the following strains: E68I (O141:K85ab:K88ab), E145 (O141:K85ab), P155 (O149:K91:K88ac), E68II (O141:K85ab:K88ac), P307 (O8:K87:K88ab), P104 (O139:K82), E65 (O45:K"65"), V17 (O157:"V17":K88ac), E57 (O138:K81), G491 (O138:K81:K88ac) G1253 (O147:K89:K88ac), P16 (O9:K103), Moon 637 (O64:K?), Troyer (O9:K35), Moon 431 (O101:K30:K99), MT5821 (O149:K91:987P). The strains were identified by the slide agglutination tests by the method of Carvalho et al. (1991).

Anti-K88, andi-K99, anti-987P and anti-F42 antisera were prepared by immunizing rabbits using semi-purified antigens. The *Escherichia coli* strains used for the detection of colonization factors were examined by the slide agglutination test using the technique recommended by Carvalho et al. (1991).

The *Escherichia coli* strains were cultured in brain-heart infusion (BHI) broth in a waterbath with shaking at 150-200 rpm, at 37°C for 18 hours and then centrifuged. Evans Blue (2%) was added to the supernatant of each strain and 0.1ml of the mixture was inoculated intragastrically into three mice aged three to four days. Another mouse of the same age was similarly inoculated with 0.1ml BHI broth and Evans Blue and used as control according to the technique recommended by Dean et al. (1972).

The strains were cultured in BHI broth in a waterbath with shaking at 150 to 200 rpm for 18 hours and then centrifuged for the determination of STb enterotoxin. The method employed was that of the ligated loop of piglets aged five-seven weeks (Gyles, 1979). Laparotomy was performed to expose the small intestine, the intestinal lumen was washed with physiological saline, the strains were inoculated, and the intestinal loops were ligated. Tem milliliters of the culture supernatant were inoculated into each loop, for a total of 15 to 20 loops measuring 10cm each. The animals were sacrificed 18 hours later, the loops examined for the presence of dilatation and the liquid volume/loop length ratio was calculated for each loop. Values of 1.0 or more were considered to be positive.

Cholera toxin (SIGMA C-8052 Lot 81 H 40161) was used for the production of anti-cholera toxin antiserum.

Two young male rabbits were used for the production of anti-cholera toxin antiserum. The

animals were injected intramuscularly with cholera toxin (CT) at the following schedule: day 1, 100µg CT emulsified in complete Freund adjuvant, day 25, 50µg CT emulsified in complete Freund adjuvant, day 50, 50µg CT emulsified in incomplete Freund adjuvant, day 60, test bleeding.

For extraction of LT enterotoxin, each strain was cultured in casamino acid yeast extract medium (Evans et al., 1972) divided into two 125ml Erlenmeyer flasks and incubated with shaking (150 rpm) at 37°C for 18 hours. Polymixin B (1.0ml) was then added at the concentration of 2.2mg/ml in 0.40M buffered saline (PBS) to one flask, with shaking, for 15 minutes, The extracts of the two flasks were obtained after culture centrifugation.

In order to standardize the radial immunohemolysis flask, different concentrations of cholera toxin (0.1 to 0.0125µg) were also tested to determine sensitivity.

For the radial immunohemolysis test, agarose was prepared in triethanolamine buffer at 1% final concentration. The gel was dissolved by heating and placed in test tubes (2.7ml per tube) which were kept at 45°C in a waterbath. The sheep red blood cells used for the test were washed three times in triethanolamine buffer and diluted to 10% concentration in the same buffer. For sensitization, 1ml of the culture supernatant in casamino acid yeast extract containing polymixin B was mixed with an equal volume of red blood cells. The mixture was incubated at 37°C for 30 minutes and centrifuged. Sensitized red cell pellets were resuspended in the same buffer at 5% concentration. Volumes of 0.3ml of sensitized red cells were added to the tubes containing 2.7ml agarose and mixed. The contents of the tubes were placed on slides and cooled to room temperature. Two orifices of 4mm each separated by a 2.5cm distance were punched into the red cell-agarose preparation. A 20µl aliquot of anti-cholera toxin at 1:80 dilution was added to each orifice. The slides were stored at 4°C for 24 hours in a humid chamber. After this period, 2ml of guinea pig serum diluted 1:10 in triethanolamine buffer (complement source) was pipetted onto the surface of the gel and incubated at 37°C in a humid chamber for six hours. Excess complement was removed and the diameter of the hemolytic haloes was measured.

The effects of treatment were evaluated by the chi-square test for the qualitative characteristic (diarrhea) according (Anderson et al., 1980).

RESULTS AND DISCUSSION

Table 1 shows the number of animals (sows and piglets) from each group, with the respective numbers of enterotoxigenic *Escherichia coli* isolates. On the basis of the results of the slide agglutination test against the four adhesive antigens and of the tests for the detection of enterotoxins, it can be seem that 51 of the *Escherichia coli* strains isolated from piglets with diarrhea (15.4%) proved to be enterotoxigenic (Table 1).

Table 2 lists the titers of the agglutinating antibodies against antigens K88, K99, 987P and F42 present in the serum and colostrum of the sows from each group.

Table 1. Number of piglets born and enterotoxigenic *Escherichia coli* strains isolated from the feces of piglets with diarrhea in each group.

Group	Number of sows	Number of piglets	Number of strains						
			K88$^+$	K99$^+$	987P$^+$	F42$^+$	Sta$^+$	Stb$^+$	LT$^+$
I	7	58	26	6	-	-	12	8	6
II	5	40	2	1	-	-	2	-	1
III	5	38	2	-	-	-	-	2	-
IV	5	41	2	-	-	-	1	1	-
V	5	37	-	1	-	-	1	-	-
VI	5	40	3	1	-	-	2	1	1
VII	5	41	2	2	-	-	1	1	2
VIII	5	36	2	-	1	-	2	-	1

Table 2. Reciprocal values of the titers of agglutinating antibodies against antigens K88, K99, 987P and F42 present in serum before immunization, at the time of parturition and in the colostrum of sows from each group.

Group	Number of sows	Period	Reciprocal value of the titer			
			K88	K99	987P	F42
I	7	Before immunization	0-2	0-2	0*	0
		Parturition	0-4	0-2	0	0
		Colostrum	0-4	0-4	0	0
II	5	Before immunization	0-2	0-2	0	0
		Parturition	80-160	40-160	20-80	80-160
		Colostrum	80-320	80-320	80-160	160-640
III	5	Before immunization	0-2	0	0	0
		Parturition	40-160	80-160	40-160	80-160
		Colostrum	80-640	160-320	40-320	160-640
IV	5	Before immunization	0	0-2	0	0
		Parturition	40-160	80-160	40-160	80-160
		Colostrum	80-320	160-640	80-640	80-1280
V	5	Before immunization	0	0	0	0
		Parturition	40-160	80-160	40-160	80-160
		Colostrum	80-320	80-320	80-320	160-640
VI	5	Before immunization	0	0-2	0	0
		Parturition	0	0-4	0	0
		Colostrum	0	0-4	0	0
VII	5	Before immunization	0	0	0	0
		Parturition	0	0	0	0
		Colostrum	0	0	0	0
VIII	05	Before immunization	0-2	0-2	0	0
		Parturition	0-4	0-4	0	0
		Colostrum	0-4	0-4	0	0

*No titer

Agglutinating titers ranging from 2 to 4 for pili K88 and K99 were detected in the serum of 12 sows before immunization or application of placebo. The antibody titers present in the sera from control sows and from unvaccinated sows whose piglets received the probiotic presented incipient elevation in colostrum. No animal in groups V and VII presented antibodies against the four antigens before immunization. The animals in the other groups presented antibodies against one or two adhesive antigens (K88 and K99) before vaccination. No animal in group VII presented an agglutinating titer in serum or colostrum against the adhesive antigens before application of the first dose of placebo or at the time of parturition. Significant increases in serum titers were observed in the sows after vaccination. These increases were also observed in the antibody titers of the colostrum of vaccinated sows, especially against antigen F42. These results agree with those reported by Ávila et al. (1986) with respect to passive post-vaccination antibody transfer from cow serum to colostrum and from colostrum to calves.

The number and percentage of piglets with dirrhea in each group are presented in Table 3. The number of piglets with diarrhea was 32 in group I, in which the sows were not vaccinated and their piglets did not receive the probiotic. Statistical analysis by the chi-square test demonstrated significant differences ($P<0.01$) between groups ($\chi^2=87.14$) and between the control group and the treated groups ($\chi^2=85.81$) (Table 3).

Table 3. Percentage and number of piglets that presented diarrhea induced by *Escherichia coli* in each group.

Group	Number of piglets born	Piglets with diarrhea	
		Number	%
I	58	32	55,2
II	40	3	7,5
III	38	2	5,3
IV	41	2	5,3
V	37	1	2,7
VI	40	4	10,0
VI	41	4	9,8
VIII	36	3	8,3

The percentage, number and serotypes of *E. coli* isolated from the feces of piglets with diarrhea in each group are listet in Table 4. Only one serotype carrying the K99 pilus was isolated and found to belong to serogroup O101. Similarly, only one serotype carrying the 987P pilus was isolated and found to belong to somatic group O149 (Table 4). The serotypes carrying pilus K88 were the most frequent and belonged to somatic groups O8, O138, O141 and O157. Among the animals that received the probiotic and presented diarrhea, *Escherichia coli* predominated in the feces, whereas *Lactobacillus acidophylus* was difficult to isolate. However, in the animals with no diarrhea, *Lactobacillus acidophylus* seamed to predominate in the feces, so that it could be easily isolated. This observation has also been reported by Savage (1980) and Tournut et al. (1988). These facts seem to confirm the function of the probiotic reported by Ávila et al. (1995) e Tournut et al. (1988), i.e., the prevention of intestinal colonization by enterotoxigenic *Escherichia coli*. The results show that the vaccine in combination with the probiotic administered for 28 days was the most effective treatment for the control of diarrhea caused by enterotoxigenic *Escherichia coli*.

Table 4. Percentage, number and serotypes of *E. coli* isolated from the feces of piglets with diarrhea in each group

Group	N° of piglets born	% Isolations	N° of serotypes	Isolated serotypes
I	58	55,2	6	O101:K30:K99
			8	O8:K87:K88ab
			6	O138:K81:K88ac
			12	O141:K85ab:K88ac
II	40	7,5	2	O149:K91:K88ac
			1	O101:K30:K99
III	38	5,3	2	O8:K87:K88ab
IV	41	5,3	2	O138:K81:K88ac
V	37	2,7	1	O101:K30:K99
VI	40	10,0	2	O138:K81:K88ac
			1	O101:K30:K99
			1	O157:"V17":K88ac
VII	41	9,8	2	O8:K81:K88ac
			2	O101:K30:K99
VIII	36	8,3	1	O149:K91:987P
			2	O141:K85ab:K88ac

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