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(19)

(54) VACCINE AND METHOD FOR ITS PRODUCTION

(71) We, RECHERCHE ET INDUSTRIE THERAPEUTIQUES, R.I.T., of Rue de Tilleul, 13, B—1320 Genval, Belgium, a body corporate organised under the laws of Belgium, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to new colibacillosis vaccines, more particularly vaccines against piglet colibacillosis.

Neonatal colibacillosis of swine is an important cause of death in piglets and numerous attempts have been made to control this illness by actively immunizing the dam against *Escherichia coli (E. coli)* either by killed or live vaccines or by giving the pigs *E. coli* antiserum.

Active immunization of the sows has been performed by parenteral administration of *E. coli* vaccines (see for instance M.R. Wilson and J. Svendsen, Amer. J. Vet. Res. *32*, 1971, 891—898) but because the *E. coli* strain or strains used for immunization may only generate specific antibodies while there may be several and different strains associated with a given outbreak, these attempts at prophylaxis based on antibacterial immunity have given inconsistent and generally unsatisfactory results. Even if in some cases good protection was obtained, it was restricted to those serotypes of *E coli* present in the vaccine.

Administration of antiserum (see for instance O.P. Miniats, Can. Vet. J. *11*, 1970, 52—56) cannot constitute a practical prophylactic means against piglet colibacillosis. The protection afforded by administration of antiserum indeed is of short duration and requires continuous administration to the piglets.

We have now surprisingly found that when administering to pregnant sows from three to six weeks before farrowing heat labile (LT) *E. coli* enterotoxin with an adjuvant instead of administering whole cells of *E. coli*, the vaccinated sows not only develop antibodies in their colostrum and milk but also protect therethrough the piglets against the action of enterotoxins produced by *E. coli* serotypes either homologous or heterologous to the *E. coli* strain from which the heat labile (LT) enterotoxin was isolated.

In other words, the administration of the heat labile (LT) *E. coli* enterotoxin to the dam does protect the piglets not only against colibacillosis due to the heat labile (LT) *E. coli* enterotoxin but also against colibacillosis due to the heat-stable (ST) enterotoxin.

It is thus the object of the present invention to afford a method of protection of piglets against colibacillosis, said method consisting in administering to the sows and with an adjuvant a heat labile (LT) *E. coli* enterotoxin preparation isolated from a culture of an enteropathogenic *E. coli* serotype. Such an enteropathogenic *E. coli* serotype has been deposited at the American Type Culture Collection, Rockville, Maryland, U.S.A., where it received accession number 21,972.

According to this invention, an effective amount of the heat labile (LT) *E. coli* enterotoxin preparation with the adjuvant is administered to pregnant sows by intramuscular or subcutaneous route. The dosage unit is at least 5 mg. and preferably from 10 to 150 mg. of freeze-dried extract.

The adjuvant — the role of which is to assure high immune response — may be any product or composition known to the art for having an adjuvant effect on vaccines. Examples of adequate adjuvants are aluminium compounds gels such as aluminium hydroxide, aluminium phosphate and ALHYDROGEL (trademark of an aluminium hydroxide gel manufactured and sold by SUPERFOS Export Co.), Copenhagen, Denmark) and water-in-oil emulsions such as adjuvant 65

(a water-in-oil emulsion of antigen in peanut oil emulsified by the addition of mannide monooleate and stabilized by the addition of aluminium monostearate) and complete FREUND'S adjuvent (a water-in-oil emulsion of light mineral oil emulsified with mannide monooleate and supplemented with killed Mycobacterium tuberculosis).

The said heat labile (LT) *E. coli* enterotoxin preparation can be isolated from any *E. coli* serotype susceptible to provoke piglet colibacillosis and containing necessarily the heat labile (LT) *E. coli* enterotoxin. An example of such *E. coli* serotype is *E. coli* strain ATCC 21,972.

The procedure for isolating the heat labile (LT) *E. coli* enterotoxin is well known. It has been described by GYLES and BARNUM (J. Infect. Dis. *120*, 1969, 419—426) and variations of this procedure have been described e.g. by H.W. SMITH and C.L. GYLES (J. Med. Microbiol. *3*, 1970, 387—401), H.W. SMITH and S. HALLS (J. Path. Bact. *93*, 1967, 531—543) and M.R. Wilson, C. GYLES and J. SVENDSEN (Can. J. Comp. Med. *35*, 1971, 294—297).

Any of these procedures as well as any other equivalent known by those skilled in the art may be used for the purpose of preparing the heat labile (LT) *E. coli* enterotoxin isolate which is employed in the present invention.

This invention does not require to get pure heat labile (LT) *E. coli* enterotoxin which could be eventually obtained e.g. either by electrophoresis or density gradient ultracentrifugation or adsorption/elution from hydrophilic cross-linked dextrans such as Sephadex (registered Trade Mark of a product manufactured and sold by Pharmacia Fine Chemicals AB, Uppsala 1, Sweden).

Essentially the invention only requires that the enterotoxin be liberated and separated from the cells of *E. coli*, preferably it is substantially free from lipopolysaccharides.

The following examples illustrate the present invention; they should not be construed as limiting its scope.

EXAMPLE 1.

Production medium (solid).

Water (10 liters) is added to 130 g. of BACTO-TRYPTOSE broth and 150 g. of BACTO-AGAR (BACTO-TRYPTOSE and BACTO-AGAR are trade names of products manufactured and sold by DIFCO Laboratories, Detroit 1, Michigan, U.S.A.). The mixture is heated with stirring up to 121°C for 60 minutes. Twenty g. of BACTO-DEXTROSE (trade name of a product manufactured and sold by DIFCO Laboratories) are dissolved in 20 ml. of distilled water and the solution is passed through a 0.45 micron Millipore sterilizing filter (Millipore Corporation, Bedford, Massachusetts, U.S.A.). Both preparations are then pooled and 110 ml. aliquots are distributed into 500 square centimeter Roux flasks.

Seed preparation.

E. coli strain ATCC 21972 is re-hydrated with sterile saline and incubated for 18 hours at 37°C in Petri dishes containing each 20 ml. of TRYPTOSE-AGAR solid medium prepared by mixing 26 g. of TRYPTOSE broth, 30 g. of AGAR DIFCO (registered Trade Mark) (TRYPTOSE broth and AGAR DIFCO are products manufactured and sold by DIFCO Laboratories) up to one liter with water, the mixture being heated for 45 minutes at 115°C.

A liquid culture medium (named PP_3) is then prepared as follows: Proteose peptone No. 3 (a product manufactured and sold by DIFCO Labs) 30 g., yeast extract (4 g.) and dextrose (5 g.) are dissolved in one liter of water at 60°C. After cooling, NaCl (5 g.), $NaHPO_4$ (5.05 g.) and KH_2PO_4 (1.2 g.) are added thereto. The medium, the pH of which is 6.9—7.0 is filtered on Seitz (registered Trade Mark) EKS filter and distributed into 100 ml. culture flasks.

These culture flasks are inoculated with the smooth colonies obtained on Petri dishes, using one colony per 20 ml. of PP_3 liquid medium and incubated for six hours at 37°C with shaking on rocking shelves (22 to 24 rockings per minute).

E. coli production.

Four milliliter aliquots of the obtained *E. coli* culture in PP_3 liquid medium (i.e. about 4.10^9 bacteria) are inoculated in each 500 square centimeter Roux flasks containing the production medium which are then incubated for one day at 37°C with shaking on rocking shelves (22 to 24 rockings per minute). Each cell culture is harvested in 25 ml. of distilled water and the harvests of 15 Roux flasks (one series) are pooled in one liter flasks. A sample (1 ml.) is taken from each series for purity testing.

Therefore, 0.5 ml. is seeded on TRYPTOSE-AGAR broth in Petri dishes and incubated for seven days at 34°C and the other 0.5 ml. fraction is seeded in Petri dishes containing 20 ml. of Sabouraud dextrose agar prepared by dissolving 65 g. of Sabouraud dextrose agar DIFCO (a product manufactured and sold by DIFCO Laboratories) in one liter of hot distilled water and further sterilized and the culture are incubated for seven days at 22°C.

The harvests are supplemented with a

sterile one percent neomycin sulfate aqueous solution (1.2 ml. of neomycin sulfate solution per 100 ml. of harvest) and the cells are disrupted by sonicating the medium for 30 minutes in a Bransone (registered Trade Mark) Europa sonicator model J 22 (Branson Europa N.V., Soest, The Netherlands), the medium being maintained in a melting ice bath.

After disruption of the cells, the suspension is centrifuged for two hours at 2,550 r.p.m. at the temperature of 5°C in order to eliminate the cell debris.

The supernatant fluid is successively passed through a clarifying filter and through a 0.45 micron Millipore sterilizing filter (Millipore is a trademark of Millipore Corporation, Bedford, Massachusetts, U.S.A.).

Ammonium sulfate previously sterilized by ethylene oxide is added to the filtrate up to reaching 50% saturation (380 g. of ammonium sulfate per liter of filtrate) and the mixture is allowed to stand for three hours at room-temperature. The obtained precipitate is centrifuged for two hours at 2,550 r.p.m. at the temperature of 5°C.

The sediment is poured into a cellophane (registered Trade Mark) bag and dialyzed against running water until the concentration in ammonium sulfate is lowered between 0.1 and 0.01%, the ammonium sulfate contents being tested with Nessler's reagent.

The exterotoxin preparation is sterilized by passage through a 0.45 micron Millipore sterilizing filter (Millipore is a trademark of Millipore Corporation) and freeze-dried.

EXAMPLE 2.

A one g. aliquot of freeze-dried enterotoxin preparation obtained at the end of example 1 is rehydrated by adding thereto 20 ml. of a sterile phosphate buffer saline (pH 7.4) consisting of NaCl (8 g.), KCl (2 g.), Na_2HPO_4 (11.5 g.), KH_2PO_4 (2 g.), $CaCl_2.2H_2O$ (1.325 g.), $MgCl_26H_2O$ (1 g.) and distilled water (10 l.).

The obtained vaccine is thoroughly mixed in sterile conditions with 20 ml. of sterile complete FREUND'S adjuvant (i.e. a water-in-oil emulsion of light mineral oil emulsified with mannide monooleate and supplemented with killed *Mycobacterium tuberculosis*) and the so-obtained adjuvant vaccine is distributed into four ml. vials which are either sealed or tightly stopped in order to obtain an (LT) enterotoxin amount corresponding to 100 mg. of freeze-dried preparation per vial.

The contents of each vial corresponds to one vaccine dosage unit. The vaccine is administered to pregnant sows by intramuscular or subcutaneous route from three to six weeks before farrowing.

Alternatively, the adjuvanted vaccine is distributed into larger vials in order to obtain integers of the dosage unit amount to constitute corresponding multi-doses vaccine preparations.

EXAMPLE 3.

The technique is that described in example 1 up to and including the incubation of *E. coli* ATCC 21, 972 for six hours at 37°C in PP_3 liquid medium.

Production of *E. coli* is then carried out in a liquid production medium as follows:

Six milliliters aliquots of the so-obtained *E. coli* (i.e. about 6.10^9 bacteria) are inoculated in production flasks containing 300 ml. of PP_3 liquid medium and the cultures are incubated for one day with shaking on rocking shelfs (22 to 24 rockings per minute).

The harvests of five production flasks (one series) are pooled and each series of harvests is centrifuged for two hours at 2,550 r.p.m., the sediment is re-suspended in 150 ml. of distilled water and a sample of the suspension is tested for purity as indicated in example 1.

The cell suspension is sonicated for 30 minutes in a Branson Europa sonicator model J 22, the medium being maintained in a melting ice bath.

After disruption of the cells, the suspension is centrifuged for two hours at 2,550 r.p.m. at 5°C in order to eliminate the cell debris. The supernatant is passed through a 0.45 micron Millipore sterilizing filter (Millipore is a trademark of Millipore Corporation) to yield 100 ml. of filtrate, the pH of which is adjusted to 6.4 with normal hydrochloric acid. Thiomersal is added in a final concentration of 0.02%.

EXAMPLE 4.

Forty milliliters of a 2% aqueous solution of ALHYDROGEL (a product manufactured and sold by SUPERFOS EXPORT Company) is thoroughly mixed with 80 ml. of the filtrate obtained at the end of example 3, the adsorption of the enterotoxin on the ALHYDROGEL being checked by precipitation test with trichloroacetic acid.

Twenty milliliters of the supernatant fluid is discarded and the remaining mixture is distributed into ten (10 ml.) vials which are stoppered.

The contents of each vial (9 ml.) corresponds to one vaccine dosage unit of 100 mg. (dry weight basis) of heat labile (LT) *E. coli* enterotoxin.

The vaccine is administered to pregnant sows by intramuscular or subcutaneous route from three to six weeks before farrowing.

Alternatively, the adjuvated vaccine is distributed into larger vials in order to ob-

tain integers of the dosage unit amount to constitute corresponding multi-doses vaccine preparations.

EXAMPLE 5.

The technique is that of example 3 but the mixture is distributed into 100 glass vials which are stoppered.

The contents of each vial (0.9 ml.) corresponds to one vaccine dosage unit of 10 mg. (dry weight basis) of heat labile (LT) *E. coli* enterotoxin extract.

The vaccine is administered to pregnant sows by intramuscular or subcutaneous route from three to six weeks before farrowing.

EXAMPLE 6.

Prevention of piglet colibacillosis by subcutaneous administration of heat labile (LT) *E. coli* enterotoxin preparations according to the invention has been demonstrated by the following trials I, II and III.

In each of these trials, the sows were vaccinated from three to six weeks before farrowing and one sow was used as control. Twenty-four hours after birth, the piglets were challenged with 400 mg. per kg. of weight of LT *E. coli* enterotoxin prepared according to example 1, using a stomach tube for the administration.

For three litters — as indicated in the following Tables — some piglets were also challenged with heat-stable (ST) *E. coli* enterotoxin obtained from strain ATCC 21,972, using therefore 660 mg. of ST enterotoxin.

Clinical evolution of the piglets was regularly followed regarding appearance of typical symptoms of colibacillosis.

In the following Tables:

—Vaccine A represents the vaccine of example 2 at the 100 mg. (dry weight basis) dosage unit.

—Vaccine B represents the vaccine of example 4 at the 100 mg. (dry weight basis) dosage unit.

—Vaccine C represents the vaccine of example 5 at the 10 mg. (dry weight basis) dosage unit.

TRIAL No. 1.

SOW		PIGLET					
				Diarrhoea			
No.	Vaccinated with	No.	Challenged with	Number of hrs between challenge and onset	Duration (hrs)	Mortality	Weight 24 hrs after challenge
2	A	2—10	LT	—	—	—	no change
		2—11	LT	—	—	—	+3.5%
		2—12	LT	—	—	—	+9.5%
		2—13	LT	—	—	—	no change
		2—14	ST	—	—	—	+3.6%
		2—15	ST	—	—	—	no change
		2—16	ST	—	—	—	+2.8%
3	A	3—17	LT	—	—	—	+10.6%
		3—18	LT	—	—	—	+11.8%
		3—19	LT	—	—	—	+6.3%
		3—20	LT	—	—	—	+5.8%
		3—21	ST	—	—	—	+10%
		3—22	ST	—	—	—	+11.9%
		3—23	ST	—	—	—	+10.4%
		3—24	ST	—	—	—	+7.5%
10 (control)	—	10—01	LT	6	24	—	−10%
		10—02	LT	6—12	24	—	−10.9%
		10—03	LT	6	12	—	−5.3%
		10—04	LT	6	96	+	−14.7%
		10—05	LT	6—12	10	—	−7.6%
		10—06	ST	—	—	—	no change
		10—07	ST	1	3	—	no change
		10—08	ST	—	—	—	no change
		10—09	ST	6	12	—	no change

TRIAL No. 2.

SOW		PIGLET					
				Diarrhoea			
No.	Vaccinated with	No.	Challenged with	Number of hrs between challenge and onset	Duration (hrs)	Mortality	Weight 24 hrs after challenge
47	A	47—12	LT	—	—	—	no change
		47—13	LT	—	—	—	+9.2%
		47—14	LT	—	—	—	no change
		47—15	LT	—	—	—	+6.4%
		47—16	LT	—	—	—	+6.6%
48	B	48—08	LT	—	—	—	+5.2%
		48—09	LT	—	—	—	+6.7%
		48—10	LT	—	—	—	+3.7%
		48—11	LT	—	—	—	+10.5%
50 (control)	—	50—01	LT	5	21	+	–19.8%
		50—02	LT	—	—	—	no change
		50—03	LT	—	—	—	+2.6%
		50—04	LT	6	14—21	+	–22.1%
		50—05	LT	14	48	+	–18.8%
		50—06	LT	21	10	—	–3.2%
		50—07	LT	6	21	+	–28.5%

TRIAL No. 3.

SOW		PIGLET					
				Diarrhoea			
No.	Vaccinated with	No.	Challenged with	Number of hrs between challenge and onset	Duration (hrs)	Mortality	Weight 24 hrs after challenge
13	B	13—119	LT	—	—	—	+8.4%
		13—120	LT	—	—	—	+11.4%
		13—121	LT	—	—	—	+2%
		13—122	LT	—	—	—	no change
		13—123	LT	—	—	—	+7.1%
		13—124	LT	4	2	—	+8.1%
		13—125	LT	—	—	—	+8%
		13—126	LT	—	—	—	+5.8%
		13—127	LT	8	40	+	−21%
		13—128	LT	—	—	—	+8.4%
21 x	C	21—110	LT	3	48	+	−15%
		21—111	LT	3	6—11	—	−6.9%
		21—112	LT	4	48	+	−14.3%
		21—114	LT	—	—	—	−5.9%
		21—115	LT	6	6	—	−6.3%
		21—116	LT	—	—	—	−3.6%
		21—117	LT	—	—	—	−2%
		21—118	LT	6	5	—	−12.5%
15	C	15—129	LT	—	—	—	+7.4%
		15—130	LT	—	—	—	+4.9%
		15—131	LT	—	—	—	+13.2%
		15—132	LT	—	—	—	+16%
		15—133	LT	—	—	—	no change

SOW		PIGLET						
				Diarrhoea				
No.	Vaccinated with	No.	Challenged with	Number of hrs between challenge and onset	Duration (hrs)	Mortality	Weight 24 hrs after challenge	
20 (control)	—	20—101	LT	5	6	—	–3.6%	
		20—102	LT	6	6	—	–16.5%	
		20—103	LT	25	4	—	–4.5%	xx
		20—104	LT	19	6	—	–5.3%	
		20—105	LT	6	6	—	–4.7%	
		20—106	LT	3	4—9	—	no change	
		20—107	LT	—	—	—	–3.2%	
		20—108	LT	6	6	—	–5.7%	
		20—109	LT	6	6	—	–4.2%	

(x) Mastitis appeared 48 hours after farrowing;

(xx) Loss of weight 4 hours after appearance of diarrhoea.

The result of the trials show that six out of the seven litters from vaccinated sows were protected against oral challenge with *E. coli* enterotoxins. The afforded protection was 90% for one litter and 100% for the five other ones. No litter of the control sows showed resistance to the same challenge with exterotoxins while the mortality and morbidity among the control piglets ranged from 71.4 to 88.8%.

In five out of seven litters from vaccinated sows, all piglets were completely protected; in one litter, eight out of ten piglets were completely protected. In the piglets challenged with LT enterotoxin and derived from control sows, the figures were as follows: in one litter, five out of five animals showed typical symptoms; in another litter five out of seven and in the third litter eight out of nine.

EXAMPLE 7.

A 12 g. aliquot of freeze-dried enterotoxin preparation obtained according to the process described in example 1 is suspended in one liter of pyrogen free distilled water and the suspension is mixed with a solution of 20 g. of ALHYDROGEL (a product manufactured and sold by SUPERFOS EXPORT Company) in one liter of pyrogen free distilled water. The pH is adjusted to 6 with N hydrochloric acid and the mixture is allowed to stand in the dark at room temperature for 40 hrs. with intermittent stirring in order to allow adsorption or the enterotoxin on ALHYDROGEL. The final pH is 6.3.

After centrifugation, the supernatant is discarded and the sediment is re-hydrated by addition of 2.2 l. of a 50/50 mixture of physiological serum and buffer (22 g. Na_2HPO_4 (M/10) and 78 g. KH_2PO_4 (M/10) with 10 mg. of thiomersal for 100 ml.; pH 6.3).

The mixture is stirred at 4°C for two hrs. and divided into two 1100 ml. batches, respectively named A and B. Batch A (which contains 5.4 mg. of enterotoxin preparation and 9 mg. of ALHYDROGEL per ml.) is divided into 5 ml. aliquots (thus containing 27 mg. of exterotoxin preparation) which are distributed into glass vials

which are then sealed. Batch B is diluted by addition of 303 ml. of a 50/50 mixture of physiological serum and buffer (22 g. Na_2HPO_4 (M/10) and 78 g. KH_2PO_4(M/10) with 10 mg. of thiomersal for 100 ml.; pH 6.3) supplemented with 2.7 g. of ALHYDROGEL. Batch B (which contains 4.3 mg. of enterotoxin preparation and 9 mg. ALHYDROGEL per ml.) is divided into 2.5 ml. aliquots (thus containing 10.75 mg. of enterotoxin preparation) which are distributed into glass vials which are then sealed.

The vaccine preparation of batches A and B have been tested as follows on sows in breeding centres contaminated by colibacillary diarrhoea.

Therefore the sows were subcutaneously inoculated between 3 and 6 weeks before farrowing with one single dose of vaccine: 59 sows receiving one dose of batch A vaccine and 28 sows receiving one dose of batch B vaccine. Fifty sows were used as control receiving the same volumes of the same ALHYDROGEL/buffer mixture without enterotoxin extract.

All piglets were observed for the appearance of diarrhoea.

On and after the second day of clinical symptom of illness, the piglets were treated by daily administration of chloramphenicol, ampicillin or sulfadoxin/trimethoprim.

The results are summarized in the following Table I taking into account feces consistance duration of diarrhoea, number of antibiotic administrations and mortality by dehydration.

TABLE I.

Vaccine dose	Total number of litters	Number of litters showing severe diarrhoea (x)	Percentage of litters showing severe diarrhoea (x)	Number of litters with mortality	Percentage of litters with mortality
5 ml. of batch A	59	15	25.4	2	3.3
2.5 ml. of batch B	28	10	35.7	2	7.1
placebo (xx)	50	24	48	8	16

(x) i.e. liquid feces for more than two days, several antibiotic administrations, with and without mortality.
(xx) a group of 25 sows receiving 2.5 ml. and the other group of 25 sows receiving 5 ml.

The results of Table I indicate that the administration of vaccines according to the invention considerably reduces the percentage of illness and mortality, better protection being obtained after administration of the 27 mg. enterotoxin dosage unit to the sows.

WHAT WE CLAIM IS:—

1. A piglet colibacillosis vaccine for intramuscular or subcutaneous administration to pregnant sows comprising an effective amount of cell-free heat labile (LT) *E. coli* enterotoxin and an adjuvant.

2. A piglet colibacillosis vaccine according to claim 1, wherein the heat labile (LT) *E. coli* enterotoxin is a cell-free extract of *E. coli* substantially free from lipopolysaccharides.

3. A piglet colibacillosis vaccine according to claim 2, wherein the enterotoxin dosage unit is from 10 to 150 mg. of freeze-dried extract.

4. A piglet colibacillosis vaccine according to any of claims 1 to 3, wherein the adjuvant is an oily adjuvant or an aluminium compound gel adjuvant.

5. A method for preventing piglet colibacillosis consisting in administering intramuscularly or subcutaneously to pregnant sows from three to six weeks before farrowing cell-free heat labile (LT) *E. coli* enterotoxin with an adjuvant.

6. A method according to claim 5, wherein said cell-free *E. coli* heat labile (LT) enterotoxin is a cell-free extract of *E. coli* cells substantially free from lipopolysaccharides.

7. A process for preparing a piglet colibacillosis vaccine for intramuscular or subcutaneous administration to pregnant sows according to any of claims 1 to 4 which comprises isolating heat labile (LT) *E. coli* enterotoxin from cells of an heat labile enterotoxin producing *E. coli* strain and mixing the enterotoxin with an adjuvant for vaccine.

which are then sealed. Batch B is diluted by addition of 303 ml. of a 50/50 mixture of physiological serum and buffer (22 g. Na_2HPO_4 (M/10) and 78 g. KH_2PO_4(M/10) with 10 mg. of thiomersal for 100 ml.; pH 6.3) supplemented with 2.7 g. of ALHYDROGEL. Batch B (which contains 4.3 mg. of enterotoxin preparation and 9 mg. ALHYDROGEL per ml.) is divided into 2.5 ml. aliquots (thus containing 10.75 mg. of enterotoxin preparation) which are distributed into glass vials which are then sealed.

The vaccine preparation of batches A and B have been tested as follows on sows in breeding centres contaminated by colibacillary diarrhoea.

Therefore the sows were subcutaneously inoculated between 3 and 6 weeks before farrowing with one single dose of vaccine: 59 sows receiving one dose of batch A vaccine and 28 sows receiving one dose of batch B vaccine. Fifty sows were used as control receiving the same volumes of the same ALHYDROGEL/buffer mixture without enterotoxin extract.

All piglets were observed for the appearance of diarrhoea.

On and after the second day of clinical symptom of illness, the piglets were treated by daily administration of chloramphenicol, ampicillin or sulfadoxin/trimethoprim.

The results are summarized in the following Table I taking into account feces consistance duration of diarrhoea, number of antibiotic administrations and mortality by dehydration.

TABLE I.

Vaccine dose	Total number of litters	Number of litters showing severe diarrhoea (x)	Percentage of litters showing severe diarrhoea (x)	Number of litters with mortality	Percentage of litters with mortality
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2.5 ml. of batch B	28	10	35.7	2	7.1
placebo (xx)	50	24	48	8	16

(x) i.e. liquid feces for more than two days, several antibiotic administrations, with and without mortality.
(xx) a group of 25 sows receiving 2.5 ml. and the other group of 25 sows receiving 5 ml.

The results of Table I indicate that the administration of vaccines according to the invention considerably reduces the percentage of illness and mortality, better protection being obtained after administration of the 27 mg. enterotoxin dosage unit to the sows.

WHAT WE CLAIM IS:—

1. A piglet colibacillosis vaccine for intramuscular or subcutaneous administration to pregnant sows comprising an effective amount of cell-free heat labile (LT) *E. coli* enterotoxin and an adjuvant.

2. A piglet colibacillosis vaccine according to claim 1, wherein the heat labile (LT) *E. coli* enterotoxin is a cell-free extract of *E. coli* substantially free from lipopolysaccharides.

3. A piglet colibacillosis vaccine according to claim 2, wherein the enterotoxin dosage unit is from 10 to 150 mg. of freeze-dried extract.

4. A piglet colibacillosis vaccine according to any of claims 1 to 3, wherein the adjuvant is an oily adjuvant or an aluminium compound gel adjuvant.

5. A method for preventing piglet colibacillosis consisting in administering intramuscularly or subcutaneously to pregnant sows from three to six weeks before farrowing cell-free heat labile (LT) *E. coli* enterotoxin with an adjuvant.

6. A method according to claim 5, wherein said cell-free *E. coli* heat labile (LT) enterotoxin is a cell-free extract of *E. coli* cells substantially free from lipopolysaccharides.

7. A process for preparing a piglet colibacillosis vaccine for intramuscular or subcutaneous administration to pregnant sows according to any of claims 1 to 4 which comprises isolating heat labile (LT) *E. coli* enterotoxin from cells of an heat labile enterotoxin producing *E. coli* strain and mixing the enterotoxin with an adjuvant for vaccine.

* C L M S *

8. A process according to claim 7 as hereinbefore described in Examples 1 and 2, Examples 3 and 4 or Example 5.

9. A process according to claim 7 as hereinbefore described in Examples 1 and 7.

R.A.A. HURST
Agent for the Applicants.

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