VIRULENCE FACTORS PRESENT IN CULTURES OF *Escherichia coli* ISOLATED FROM PIGS IN THE REGION OF CONCÓRDIA, SANTA CATARINA, BRAZIL[1]

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SINOPSE.- Castro A.F.P., Serafim M.B., Brito J.R.F., Barcellos D.S.E.N. & Colli I.A.G. 1984. **[Fatores de virulencia em amostras de Escherichia coli isoladas de porcos na região de Concórdia, SC, Brasil.]** Virulence factors present in cultures of Escherichia coli isolated from pigs in the region of Concórdia, Santa Catarina, Brazil. *Pesquisa Veterinária Brasileira 4(3): 109-114.* Depto Microbiol. Imunol., Unicamp, Campinas, SP 13100, Brazil.

Quatrocentos e setenta e sete amostras de *Escherichia coli,* isoladas na região de Concórdia, SC, Brazil, foram examinadas quanto a presença de fatores de virulência a saber: enterotoxina termolábil (LT), enterotoxinas termoestáveis (STa e STb) e os fatores de colonização K88, K99 e 987P. Amostras pareadas de soro de alguns leitões doentes foram também coletadas para a pesquisa de anticorpos anti-LT. Oitenta e seis (18,02%) amostras de *E. coli* produziram a enterotoxina STa enquanto a enterotoxina LT foi detectada em apenas 8 (1.67%). Entre as 381 amostras STa^-, 49 (12.53%) foram STb^+. Entre 28 amostras $K88^+$, uma produziu LT, 9 STa e as restantes eram não enterotoxigenicas. Nenhuma das amostras LT^+ codificou para o antígeno K99, porém, este foi encontrado em 5 amostras STa^+ e em 17 não enterotoxigênicas. Quatro entre os 10 soros examinados apresentaram conversão sorológica nos níveis de anticorpos anti-LT quando os soros foram examinados pelo teste da imunohemólise passiva (PIH). Os resultados dos exames sorológicos revelaram que todas as amostras LT^+ pertenciam ao sorogrupo 0149. Entre 86 amostras STa^+, 1 foi classificada como pertencendo ao sorogrupo 09, 2 ao 010; 4 ao 035; 2 ao 064; 3 ao 0108; 2 ao 0138; 1 ao 0149; 1 ao 0157 e 70 amostras não foram classificadas. Entre as 49 amostras STb^+ 1 pertencia ao sorogrupo 09; 4 ao 010; 1 ao 035; 1 ao 0139; 2 ao 0149; 1 ao 0157 e 39 não foram classificadas. Estes resultados sugerem que na colibacilose suína da região de Concórdia as enterotoxinas termoestáveis (STa e STb) possam desempenhar um importante papel, existindo a possibilidade de que nelas possam ocorrer outros fatores de colonização que não K88, K99 e 987P. As amostras estudadas também, em sua maioria, não se enquadraram entre aqueles sorogrupos geralmente aceitos como enteropatogênicos para suínos.

TERMOS DE INDEXAÇÃO: Fatores de virulência, colibacilose suína, Concórdia, Brazil.

ASTRACT.- Four hundred and seventy-seven cultures of *Escherichia coli* isolated in the region of Concórdia, Santa Catarina, Brazil, were examined for the presence of "virulence factors", such as: thermolabile (LT) enterotoxin; thermostable (STa and STb) enterotoxins and the colonization factors K88, K99 and 987P. Paired samples of sera from some sick piglets were also collected for the search of anti-LT antibodies. Eighty-six (18,02%) *E. coli* cultures produced STa enterotoxin whereas LT enterotoxin was detected in 8 (1,67%) only. Among 391 STa^- cultures 49 (12,53%) were STb^+. Among 28 $K88^+$ cultures, 1 produced LT; 9 STa and the remaining were non-enterotoxigenic. None of the LT^+ cultures codified for K99 antigen, which, on the other hand, was found in 5 STa^+ and in 17 non-enterotoxigenic cultures. 987P antigen was found in 3 non-enterotoxigenic cultures. Four out of 10 samples of the examined paired sera showed serological conversion in relation to titres found in the passive immunehemolysis (PIH) test. The results of serogrouping revealed that all LT^+ cultures belonged to serogroup 0149. Among 86 STa^+ cultures, 1 was grouped as serogroup 09, 2 as 010, 4 as 035; 2 as 064; 3 as 0108; 2 as 0138; 1 as 0149 and 1 as 0157. Seventy STa^+ cultures were not classified. Among 49 STb^+ cultures 1 belonged to serogroup 09; 4 to 010; 1 to 035; 1 to 0139; 2 to 0149; 1 to 0157 and 39 cultures could not be serogrouped. These results suggest that in porcine colibacillosis of the region of Concórdia, SC, Brasil the ther-

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mostable enterotoxins (STa and STb) may play an important role. It could be possible that among these cultures other colonization factors, different from K88, K99 and 987P may occur. Also, most cultures examined could not be grouped among the serogroups generally accepted as enteropathogenic for swine.

INDEX TERMS: Virulence factors, porcine colibacillosis, Concórdia, Brazil.

INTRODUCTION

It is accepted that porcine enteropathogenic *Escherichia coli* (PEPEC) can produce two different types of enterotoxin: a heat labile (LT), immunogenic, non-dyalisable toxin which is antigenically related to cholera toxin (Gyles 1974) and a heat-stable (STa), poor immunogenic dyalisable toxin (Gyles & Barnum 1967, Smith & Gyles 1970a, Smith & Gyles 1970b, Smith & Halls 1967). Both enterotoxins cause fluid accumulation when introduced into the ileal loop of rabbits and young pigs (Gyles & Barnum 1967, Gyles 1971). LT enterotoxin is usually detected by tissue culture assays (Donta et al. 1974, Guerrant et al. 1974) and several serological tests (Evans & Evans 1977, Greenberg et al. 1977, Serafim et al. 1979, Castro et al. 1980, Serafim et al. 1979, Serafim et al. 1981, Yano et al. 1982) using either cholera antitoxin or LT antiserum prepared with purified LT. STa enterotoxin is normally detected by the infant mouse test (IMT) (Dean et al. 1973).

More recently, several reports have shown that PEPEC can produce another type of heat-stable enterotoxin (STb) which is not active in the IMT but can be assayed in 5-7-weeks-old pigs by the ileal loop technique (ILT) (Burgess et al. 1978, Gyles 1979, Moon et al 1980, Whipp et al 1981). Usually PEPEC belong to a limited number of serogroups (Sojka 1965, Gyles 1979) and besides enterotoxigenicity, they must be provided with special pili, known as colonization factors which are responsible for the adherence of the bacteria to the intestinal cells (Orskov et al. 1964, Jones & Rutter 1972, Jones & Rutter 1974, Orskov et al. 1975, Guinée et al. 1977, Moon et al. 1977, Isaacson 1978, Guinée & Jansen 1979, Isaacson & Richter 1981). After adhesion the bacterial cells multiply and the production of enterotoxins reach in the gut levels capable of causing diarrhoea.

The most important colonization factors for PEPEC are K88, K99 and 987P (Gaastra & De Graaf 1982). With the exception of 987P, the other 2 are able to hemagglutinate red blood cells (RBC) in the presence of 0,5% of D-mannose. K88 agglutinates guinea-pig RBC (Jones & Rutter 1974), K99 horse RBC (Guinée et al. 1977, Morris et al. 1977) and 987P is not an hemagglutinating antigen (Gaastra De Graaf 1982). For all colonization factors specific antisera must be used for a definitive confirmation of their identities.

As far as swine breeding is concerned, the region of Concórdia in the State of Santa Catarina, Brazil, is probably one of the most important in this country. Since clinical porcine colibaciilosis has been frequently reported in this area, we decided to investigate which "virulence factors" were present among cultures of PEPEC isolated from sick piglets in Concórdia, SC, Brazil, during 1982-83.

MATERIALS AND METHODS

Cultures

Four hundred and seventy-seven cultures of *Escherichia coli* isolated from sick piglets from the region of Concórdia, SC, Brazil, were examined. The isolated cultures were stored in Brain Heart Infusion (BHI) plus 15% of glycerol.

Preparation of enterotoxins

For the detection of enterotoxins cultures were grown in Casamino Acids-Yeast Extract (CAYE) medium (Evans et al. 1973) distributed in 125 ml Erlenmeyer flasks incubated on a rotary-shaker (150 rpm) at 37°C for 18 h. Afterwards, a 1.0 ml amount of polymyxin containing 2.2 mg/ml in 0.40M phosphate buffered saline was then added to each flask. Shaking was continued for an additional 15 min. Supernatants recovered after centrifugation of the polymyxin-treated cultures were considered as the extract preparations.

Assay of LT enterotoxin

The passive immunohemolysis (PIH) test (Evans & Evans 1977, Serafim et al. 1979) was used for this purpose. The performance of the PIH test was similar to that previously described (Serafim et al. 1979) except that veronal buffered saline plus Ca^{++} and Mg^{++} (VBS) was used instead of phosphate buffered saline, in order to improve the sensitivity of the test (Castro et al. 1980).

Briefly, fresh sheep red blood cells (SRBC) stored at 4°C in Alsever solution, were washed twice with 8 volumes of 0.04M phosphate buffered saline and finally resuspended in VBS and standardized so that, with part of the suspension, after lysis at 1:20 in distilled water, the resulting hemoglobin solution gave a reading of 1.3 at 420 nm in a Coleman Jr. spectrophotometer, which corresponded to 2.10^9 SRBC/ml. Duplicate 10 x 100 tubes received 50 μl of enterotoxin extract and 100 μl of the standardized SRBC suspension. Next, 100 μl of cholera antitoxin diluted 1:80 was added and the tubes were incubated for another 30 min at 37°C to allow antibody (cholera antitoxin) to react with the LT-SRBC complexes. Afterwards, 100 μl of guinea-pig complement (diluted 1:10 in VBS) was added and the tubes were returned at 37°C for 60 min. Hemolysis was thereafter quantitated as follows: the reaction mixtures (0.4ml) were diluted 1:10 by the addition of 3.6 ml of VBS and the tubes were centrifuged at 2000 x g for 10 min to sediment the unhemolyzed SRBC. Hemoglobin concentration in the supernatant fluids was determined spectrophotometrically at 420 nm. Any reaction giving $A_{420} = 0.17$ or more was considered positive (Castro et al. 1980). In addition to known LT^+ and LT^- polymyxin extracts which were included as controls of the PIH test, tubes containing the following reagents were also examined to correct for non-immune hemolysis: 1) SRBC, extract under test, and VBS; 2) SRBC, extract, VBS and complement; 3) SRBC, VBS, complement and cholera antitoxin.

Assay of STa enterotoxin

Newborn swiss albino mice (2–4 days old) were separated from their mothers immediately before use and randomly divided into groups of 4 animals. Procedures for assaying STa by the infant mouse test (IMT) were similar to those reported previously (Dean et al. 1972, Gianella 1976, Gomes et al. 1979), that is, aliquots of 0,1 ml from each enterotoxin preparation to which 0.1% of blue Evans dye (2%) solution had been added, were inoculated in each mice by the intragastric route. Mice were kept at 28°C for 4 h and then euthanized with ether. After necropsy, gut and remaining carcass for each lot

were weighed. Any gut/carcass weight ratio equal or above 0.085 was considered positive for STa production.

Assay of STb enterotoxin

For that purpose the 6-week old pig ILT (Gyles 1979) with slight modifications was performed in Landrace pigs. Only STa$^-$ negative strains were examined. Animals were starved 24 h prior to surgery and water was given "ad libitum". A laparotomy was carried out under anesthesia and ligated 10 cm-segments were prepared in the anterior small intestines. The number of loops in each pig varied from 15 to 17. Afterwards, each loop was incolulated with 2 ml of supernatants from cultures under test. Sixteen hours after the injection animals were electrocuted with 220 volts and the loops examined for the presence of dilatation. Next, volume of fluid accumulation in the loops over the entire length was calculated. Considering the mean results obtained with positive and negative controls, values equal to 0.7 or more were considered positive. For each supernatant under test three different animals were used.

Detection of colonization factors

For the detection of K88 antigen, *E. coli* cultures were grown on glucose phosphate agar (Jones & Rutter 1974). Cultures to be examined for K99 antigen were inoculated on Minca Medium (Guiné et al. 1977) and those to be tested for 987P colonization factor were grown on blood agar plates (Nagy et al. 1977). All media were incubated at 37°C for 24 h. Afterwards, from each plate 10 individual colonies were examined against specific antisera by a slide agglutination technique, using antisera prepared as previously reported (Simões et al. 1981).

Detection of LT antibodies by the PIH test

Paired sera of 10 animals from which no LT$^+$ *E. coli* had been isolated were examined by the PIH test (Castro et al. 1980). Before that, all sera were exhaustively absorbed with SRBC. The performance of the test was equal to that described for LT enterotoxin with appropriate changes aiming the detection of LT antibodies; extracts used were known to contain LT enterotoxin (*E. coli* 40 T-human origin provided by L.R. Trabulsi, Escola Paulista de Medicina and strain 0149/8-porcine origin– Maria S.V. Gatti, Department of Microbiology and Immunology, University of Campinas, SP, Brazil) and sera under test were added to the reactions in place of cholera antitoxin. The end-point titre of each serum was considered as the reciprocal of the highest dilution of it which still gave A_{420} values $\geqslant 0.17$ against the standard extracts of LT$^+$ cultures.

To ascertain whether a determined animal showed a serological conversion for LT antibodies within a specific period of time (25 days), after an outbreak of diarrhea, the ratio of the reciprocal end-point titres between the second and the first samples of paired sera collected from this animal was calculated.

When this ratio showed an increase of 4-fold or more it was considered that serological conversion has occurred. Briefly, for example, if the second sample of serum of an animal gave A_{420} values $\geqslant 0.17$ when diluted up to 1/64, whereas the first sample of serum collected 25 days before gave A_{420} values $\geqslant 0.17$ up to 1/8 dilution, the ratio will be 64/8 i.e.8. In other words, serological conversion for LT antibodies has occurred with this animal and it is highly probable that it was infected with LT$^+$ *E. coli* by the time of the first bleeding.

Serogrouping

Serogrouping using specific OK antisera of all PEPEC was performed as recommended by Sojka (1965). All test were carried out in the Instituto de Pesquisas Veterinárias Desidério Finamor, Porto Alegre, Brazil.

RESULTS

Table 1 shows the distribution of "virulence factors" among the PEPEC isolated from piglets with diarrhoea in the region of Concórdia, SC, Brazil. Only 8 (1.67%) out of 477 cultures produce LT whereas 86 (18.02%) and 49 (12.53%) produce STa and STb enterotoxins respectively. Figure 1 shows positive and negative results in the pig ileal loop technique for the detection of STb enterotoxin. Table 1 shows also that in most enterotoxigenic cultures K88, K99 and 987P colonization factors were not detected. Among the LT$^+$ cultures only one had K88 antigen which was present in 9 STa$^+$ and 10 STb$^+$ cultures. K99 antigen was found in 5 STa cultures whereas all 987P cultures were non-enterotoxigenic. Among the non-enterotoxigenic cultures K88 and K99 antigens were found in 8 and 31 cultures respectively. The results of the examination of paired sera from 10 piglets with diarrhoea from which no enterotoxigenic *E. coli* was isolated is shown in Table 2. Four out of 10 paired sera showed a positive serological conversion, that is, a 4-fold or more increase in the PIH test titres between samples collected in the beginning of the disease and after 25 days, during recovery. Five antisera (3527, 3728, 3731, 3734 and 3736) showed in the PIH test stronger reactions with the first samples of sera than with the second ones and one sera (3397) gave a very weak positive reaction with pig LT enterotoxin but did not show serological conversion. Among the positive reactions in the PIH test carried out either with the first samples or the second

Table 1. *Virulence factors present in PEPEC[a] isolated from piglets with acute diarrhoea in the region of Concórdia, SC, Brazil, 1983*

Enterotoxigenic properties	Nº of positive/ total examined (%)	Colonization factors			
		K88$^+$	K99$^+$	987P$^+$	K89$^-$, K99$^-$
LT	8/477 (1.67)	1	0	0	7
STa	86/477 (18.02)	9	5	0	72
STb	49/391 (12.53)[b]	10	0	0	39
Non-ETEC	334/477 (70.02)	8	21	3	302
Total	477	28	26	3	420

[a] Porcine enteropathogenic *Escherichia coli*.

[b] Only STa$^-$ negative strains were examined by the 6 week-old pig ileal loop test.

Table 2. *Results of PIH[(a)] test with paired sera collected from piglets with diarrhoea, Concórdia, SC, Brazil, 1983*

Serum identification	LT-toxin used			Serological conversion
	sample	*E. coli* 40T (human)	*E. coli* 0149 (pig)	
3382	1st (b)	0.20(c)	0.27	+ (8) (d)
	2nd	0.16	1.30	
3395	1st	0.11	0.18	+ (4)
	2nd	0.34	0.70	
3396	1st	0.10	0.11	+ (16)
	2nd	1.10	1.50	
3397	1st	0.10	0.26	–
	2nd	0.10	0.18	
3727	1 st	0.39	1.4	–
	2nd	0.27	0.85	
3728	1st	0.15	0.54	–
	2nd	0.13	0.45	
3731	1st	0.69	1.10	–
	2nd	0.24	0.37	
3734	1st	0.83	1.50	–
	2nd	0.31	0.72	
3735	1st	0.20	0.40	–
	2nd	0.87	1.50	
3736	1st	0.85	1.30	–
	2nd	0.23	0.46	

(a) Passive immune hemolysis test.

(b) Interval of time between the collection of each sample of serum was 25 days.

(c) Any A_{420} value equal to above 0.17 ($\geqslant$30 μg of hemoglobin release) was considered as positive.

(d) Number between parenthesis represent the increase in the PIH titre between each sample of serum from the same animal.

Table 3. *Relationship between enterotoxigenicity and serogroups of PEPEC[(a)] strains isolated from piglets with acute diarrhea in the region of Concórdia, SC, Brazil 1983*

PEPEC serogroups founds	Enterotoxigenic properties		
	LT	STa	STb
09	0	1	1
010	0	2	4
035	0	4	1
064	0	2	0
0108	0	3	0
0138	0	2	0
0139	0	0	1
0149	8	1	2
0157	0	1	1
Not classified among PEPEC	0	70	39
TOTAL	8	86	49

(a) Porcine enteropathogenic *Escherichia coli*

ones, A_{420} values were higher for those reactions made with pig LT enterotoxin than those performed with human LT enterotoxin.

Serogrouping of all enterotoxigenic cultures demonstrated that most of them could not be classified among the PEPEC serogroups (Sojka 1965). All LT^+ cultures belonged to serogroup 0149. Among 86 STa^+ 1 was grouped as serogroup 09; 2 as 010, 4 as 035; 2 as 064; 3 as 0108; 2 as 0138; 1 as 0149 and 1 as 0157. Seventy STa^+ cultures were not classified. Among 49 STb^+ cultures 1 belonged to serogroup 09; 4 to 010; 1 to 035; 1 to 0139; 2 to 0 149; 1 to 0157 and 39 cultures could not be serogrouped (Table 3).

DISCUSSION

Although all specimens of stools were collected from piglets with clinical symptoms of neonatal diarrhoea, the percentage of LT^+ culture found (1.65%) can be considered fair low as compared to findings by other authors (Guinée & Jansen 1979, Söderlind & Molby 1979, Simões et al. 1982) who reported a higher incidence of LT^+ strains in cases of porcine colibacillosis. On the other hand, the high prevalence of STa^+ cultures (18.02%) in relation to LT^+ ones was very clear-cut and suggestive of an important role of STa enterotoxin in the pathogenesis of porcine colibacillosis in the region of Concórdia, Santa Catarina, Brazil.

The low number of LT^+ cultures, however, cannot be taken as an indication that LT^+ strains of *E. coli* are not involved in the cases of clinical colibacillosis selected for study. In other words, the results of the PIH test carried out with paired sera from 10 piglets, which recovered from an outbreak of neonatal diarrhoea, demonstrated that at least in four of them, from which LT^+ cultures of *E. coli* could not be isolated, a serological conversion of 4-fold or more had occurred within a range of 25 days. This increase of titres in the PIH test carried out with paired sera suggests that these animals had been surely infected with PEPEC strains which produce LT. Therefore, the results of the PIH test with paired sera showed that this test may be a very useful tool for the retrospective diagnosis of colibacillosis caused by LT^+ strains of *E. coli*. Whenever coproculture is not carried out or fails to detect this sort of bacteria, enterotoxins prepared from strains of different sources were included in the PIH test, because common and different antigens have been reported in human and porcine LT enterotoxins (Honda et al. 1981). These antigenic differences may explain why titres in the PIH test were always higher with porcine LT than with human LT (Table 2). In our case, we have not any explanation why the bacteriological examination of stools made to detect LT^+ colibacilli have not been successful. However, it should be important to point out that some strains of *E. coli* may loose the ent plasmid very soon when stored "in vitro" (Evans et al. 1977). Another possibility is that recovering of piglets took place earlier than expected, decreasing therefore the number of LT^+ bacterial cells present in the specimens of stools collected from them for examination. Since only cultures derived from single colonies were testd, some LT^+ strains might have been overlooked in our studies.

If these considerations can be accepted to explain the low prevalence of LT^+ cultures in this investigation, on the other hand, they reinforce the significance of the high prevalence of STa^+ and STb^+ cultures which apparently was not affected either by the storage of the cultures "in vitro" or by the examination of cultures from single colonies. The high prevalence of STa^+ cultures may be considered unexpected but that of STb^+ cultures is surprising. Other reports in the literature have described the production of STb enterotoxin by strains from serogroups 08, 09, 064, 0138, 0139, 0141, 0147, 0149 and 0157 (Gyles 1979, Moon et al. 1980) but the number of strains studied by those authors, in each serogroup, was low. In the present study the production of STb enterotoxin is reported in 49 out of 391 (12.53%) Sta^- cultures of *E. coli*. Among these STb^+ *E. coli* only 10 cultures could be serogrouped, using specific antisera against the known PEPEC (Sojka 1965). Production of STb enterotoxin was also detected in serogroups 010 and 035 which, as far as we know, had not been previously reported as producing this enterotoxin. Also, with regard to STa^+ *E. coli*, 70 out of 86 cultures could not be grouped, using the same antisera. Since Sta and STb enterotoxins are recognizably involved in porcine colibacillosis (Gyles 1979, Whipp et al. 1981) the relationship between enterotoxigenic strains isolated and the respective serogrouping studies suggest that serogroups other than those accepted as belonging do PEPEC (Sojka 1965, Gyles 1979) may be involved in porcine colibacillosis, in the region of Concórdia, SC, Brazil.

It was puzzling that the prevalence of K88 and K99 colonization factors was very low among the enterotoxigenic *E. coli* studied. With regard to K88 antigen, since it usually comes together with LT production (Smith 1976), our data cannot be taken as discrepant because of the low number of LT^+ cultures found. However, the association between Sta and K88 (Table 1) which was found among 9 PEPEC cannot be neglected and should be better investigated. The coproductions of K99 and STa found in 5 cultures is not uncommon being reported mainly among strains from serogroup 0101, which though originally enteropathogenic for calves, has frequently been found in cases of diarrheal illness in piglets (Moon et al. 1977, Moon et al. 1980, Simões et al. 1981).

Because 987P antigen is found only among Sta^+ strains of PEPEC (Gaastra & De Graaf 1982), a high prevalence of this colonization factor was expected among our 86 STa^+ cultures. Thus, it was amazing that none of these cultures were $987P^+$. However, because the cultivation "in vitro" may favour the overgrowth on non-piliated forms ($987P^-$) (Nagy et al. 1977) and we have not used the "in vitro" experimental infection of piglets to stimulate the growth of piliated forms ($987P^+$), the presence of this adhesin among our STa^+ cannot be ruled out completely.

Because STa^+ and STb^+ *E. coli* isolated from diarrheic piglets totallized 135 out of 477 stool specimens examined, from which only 24 cultures codified for either K88 or K99 antigens, we feel that other different colonization factors may occur in these cultures which should be further investigated concerning the presence of adhesins.

Finally, we would like to recall the attention to the fact that the high number of *E. coli* cultures producing thermostable enterotoxins (STa and STb) constitutes a very interesting finding which deserves additional studies directed towards the prophylaxis of the disease in that region of Brazil. Since both enterotoxins are non-immunogenic (Gyles 1971, Burgess et al. 1978, Whipp et al. 1981) the prophylaxis against *E. coli* strains producing these enterotoxins should be based on bacterins prepared with local strains, which, as we know, in their majority did not belong to the known PEPEC serogroups, Alternatively, vaccines could be prepared with colonization factors harboured by these strains. However, since, in most cultures according to our findings, they are still unknown, one may conclude that the first alternative – bacterins prepared with local strains – remains as an advisable initial step for controlling porcine colibacillosis in Concórdia, Santa Catarina, Brazil.

Acknowledgements. - This work was supported by grants awarded by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Financiadora de Estudos e Projetos (Finep) and Empresa Brasileira de Pesquisa Agropecuária (Embrapa).

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