Oral immunization against enterotoxigenic colibacillosis in weaned piglets by non-pathogenic *Escherichia coli* strain with K88 (F4) colonizing factors

P. Alexa, J. Hamrik, K. Stouracova, E. Salajka

Veterinary Research Institute, Brno, Czech Republic

ABSTRACT: Experiments were focused on the prevention of diarrhoea in weaned piglets by means of enterotoxigenic strains of *Escherichia coli* (ETEC) with colonizing factors K88 (F4). The process of immunization consisted of intramuscular administration of ETEC strain bacterin one day prior to weaning and oral administration of a live culture of non-pathogenic *E. coli* strain containing colonizing factors (O149:K88; STa–, LT–) in 3 hours after weaning. The shedding of the K88 positive *E. coli* strains was monitored for 3 weeks after weaning by the culture of rectal swabs. The efficacy of such immunization protocol was tested by challenge exposure to enterotoxigenic *E. coli* O149:K88, LT+ strain on the third or the tenth day after weaning. Following the oral administration of non-pathogenic *E. coli* strain containing colonizing factors K88 to piglets, the shedding of the administered strain continued for 9 days. No or very small protection against diarrhoea following the challenge exposure to enterotoxigenic *E. coli* was found in immunized piglets.

Keywords: piglet; diarrhoea after weaning; mucosal immunity; F4 fimbriae; K88

Enterotoxigenic strains of *E. coli* bacteria (ETEC) frequently cause diarrhoeal diseases in both suckling and weaned piglets. Their pathogenicity is conditioned by two types of virulence factors – colonizing factors and enterotoxins. Colonizing factors (adhesins) are fimbrial antigens which enable adherence of ETEC to the intestinal mucosa. The most commonly found adhesins of ETEC in piglets are F4 (K88) and F4 adhesins are morphologically fimbriae 2.1 nm in diameter. Guinee and Jansen (1979) classified three antigenic variants designated K88ab, K88ac and K88ad with a common epitope "a" and type specific epitopes "b", "c" and "d". Antibodies to fimbrial antigens of ETEC are generally assumed to be protective antibodies (Rutter, 1975; Nagy et al., 1978; Acres et al., 1979).

Suckling piglets are protected against ETEC infection by antibodies ingested with colostrum and milk. This passive local immunity is limited to the period of suckling (Salajka and Mensik, 1973). The occurrence of enteral *E. coli*-infections in weaned piglets is associated with the termination of the intake of antibodies present in maternal milk. It is necessary to replace passive local immunity with active immunity to enhance defence mechanisms against diseases caused by *coli*-infections. It is a well-known fact that active mucosal immunity cannot be obtained by parenteral immunization (Porter, 1973). Holmgren et al. (1992) described the induction of mucosal immunity after repeated (up to tenfold) oral administration of antigen. Salajka et al. (1979) detected immunity against diseases in gnotobiotic piglets after infection with ETEC at the age of three weeks. The piglets were repeatedly orally administered the inactivated ETEC O149: K88 culture for ten days before challenge. The repeated oral administration to piglets before weaning at the age of 28 days that do not spontaneously and regularly take feed is quite laborious. Felder et al. (2000) examined the feasibility of oral immuni-

Supported by the Ministry of Agriculture of the Czech Republic (Grant No. MZE 0002716201).

zation of pigs with microencapsulated F18 *E. coli* and detached F18 fimbriae to prevent enterotoxigenic *E. coli* infections. They did not observe any significant induction of formation of serum antibodies, and *E. coli* colonization was not reduced by oral administration of various antigen-loaded microspheres. However Van den Broeck et al. (1999) demonstrated that it was possible to orally immunize F4 (K88ac) receptor-positive pigs with isolated F4 fimbriae in phosphate buffered saline. Bertschinger et al. (2000) orally vaccinated piglets with F18ac culture ten days before weaning on three consecutive days. The pigs were challenged 9 or 11 days after weaning. Piglets were protected against colonization by a strain of the homologous fimbrial variant. It was concluded that only oral vaccines containing fimbriae induced protection against the homologous fimbrial variant. Hence the oral use of a live culture of the *E. coli* strain with appropriate antigenic outfitting appears to be promising. In our previous experiments (Alexa et al., 1995) we immunized piglets by means of a combined immunization which consisted in intramuscular administration of inactivated ETEC culture with colonizing factors F18ac a day before weaning followed by oral administration of a live culture of non-toxigenic strain with identical colonizing factors on the day of weaning. The purpose of the present study is to investigate whether a similar defence is feasible by immunization to ETEC with colonizing factors K88.

MATERIAL AND METHODS

***E. coli* strains**. *E. coli* strain No. 9486 (O149: K88ac) that did not produce either heat stable enterotoxin (STa) or heat labile enterotoxin (LT) but the produced heat stable enterotoxin (STb) was used in immunization experiments. The absence of LT encoding gene was proved by PCR and both the strains were found to be negative when examined in Y1 cells. After oral administration to piglets, the strains did not produce any diarrhoeal diseases. ETEC strain No. 9437 (O149:K88, STa-, STb+, LT+) isolated from diarrhoeic piglets after weaning was used for the preparation of bacterin and for the challenge of piglets after immunization.

E. coli strains were cultured for 16 h in a medium consisting of 12.5 g acidic casein hydrolysate, 12.5 g enzymatic casein hydrolysate and 0.5 yeast extract (Imuna Sarisske Michalany, Slovakia) in 1 litre of distilled water (pH 7.4). Bacterin was prepared by the inactivation of the culture by adding 0.5% formalin. The culture for oral administration was pelleted by centrifugation and incorporated into an inert paste.

Animals. Piglets from 2 litters weaned at the age of 35 days were used in the experiments. Piglets of each litter were allocated into two groups. In the first litter, five piglets were assigned to each (control and experimental) group. In the second litter, 4 and 5 piglets were assigned to the experimental and control group, respectively. Experimental and control piglets were housed separately. Control and experimental groups were kept and fed alike.

Immunization design. The piglets from 2 litters were immunized similarly like in our previous experiments with immunization against F18ac (Alexa et al., 2002). Experimental piglets were intramuscularly administered 1 ml of bacterin of the ETEC strain with the density 2×10^9 CFU/ml, inactivated with 0.5% formalin one day before weaning. Three hours after weaning, piglets of the experimental groups were orally administered the non-pathogenic *E. coli* 9486 strain in the dose of 5×10^{10} CFU per animal.

Challenge of piglets. Both groups (control and experimental) from the first litter were challenged with ETEC strain in the dose of 5×10^{10} CFU/animal when the shedding of 9486 strain by piglets of experimental group finished. In the second litter, both groups were infected with the same strain and dose on the third day after weaning. The health status of piglets was clinically monitored. Faecal excretion of the administered strains was determined by rectal swab cultures.

Microbiological examination of rectal swabs. Rectal swabs from piglets were diluted in PBS and spread on blood agar containing 5% lamb blood, and on Mc Conkey agar, and cultured in an incubator at 37°C. Ten randomly selected isolated colonies of *E. coli* from each swab were examined and the percentages of haemolytic colonies were assessed. Haemolytic colonies were inoculated onto a nutrient broth (Imuna Sarisske Michalany, Slovakia). After the incubation at 37°C for 16 h, intravital staining of cultures was performed by adding TTC (triphenyltetrazolium-chloride) for 1 h, heated to 100°C and examined by agglutination with antisera to O149 (Salajka et al., 1992). The presence of K88 colonizing factors was detected according to agglutination (after inactivation with phenol) in the tested colonies cultured on Minca medium and broth (Salajka

et al., 1992). The percentage of the administered strain present in faeces was calculated according to the numbers of haemolytic O149:K88 type colonies in diluted culture on blood agar. The strain used for immunization was not discriminated from the ETEC strain used for challenge. The strains were morphologically and serologically identical. From the aspect of the intestinal colonization with K88 positive strain, it was not important whether the strain was enterotoxigenic or not. Both ETEC and non-ETEC colonizations should be prevented during anti-K88 immunity formation.

RESULTS

After administration the non-toxigenic strain O149:K88 was excreted with faeces of piglets of the experimental group for 9 days (Figure 1). One day later, 10 days after weaning, the challenge of piglets with ETEC strain was carried. The challenging strain shortly prevailed in the faeces of piglets and was excreted for a period of 14 days after weaning. Two days after the challenge, severe diarrhoea occurred in one piglet; in the other piglets, only moderate transient diarrhoea was recorded. In the control group, transient, at first severe diarrhoea occurred; it spontaneously disappeared within four days. The excretion of ETEC strain continued for 18 days after weaning (Figure 2). The percentage of the administered strain detected in the faeces of two piglets exceeded 50% during 5 and 7 days, respectively.

In the next experiment, piglets were infected already on day 3 after weaning to mimic the situation under field conditions. Considering the similarity of strains used for immunization and challenge, discrimination between excreted ETEC and non-ETEC strains was not performed. The shedding of K88 positive *E. coli* strains in faeces after the administration of non-pathogenic strain and after the challenge with ETEC strain 3 days after weaning is shown in Figures 3 and 4. The shedding of the strain in the experimental and the control group continued for 10 and 13 days after weaning, respectively. In the experimental group, one piglet died within 24 h after infection with ETEC without apparent signs of diarrhoea and dehydration. O149:K88 *E. coli* shedding with faeces of the piglet that had died was not marked; it was similar in piglet No. 2 from the same group. Moderate shedding of the strain was also detected in 2 piglets of the control group. In piglets of both groups, diarrhoea of medium-severity was observed from day 2 after infection; it continued for 3 to 6 days. Recovery occurred spontaneously.

DISCUSSION

The role of fimbriae, which are ETEC colonizing factors, in the induction of protective immunity with enterotoxigenic *E. coli* in piglets was proved by Sarrazin and Bertschinger (1997). Van den Broeck et al. (1999) and Verdonck et al. (2004) demonstrated that F4 fimbriae are strong oral im-

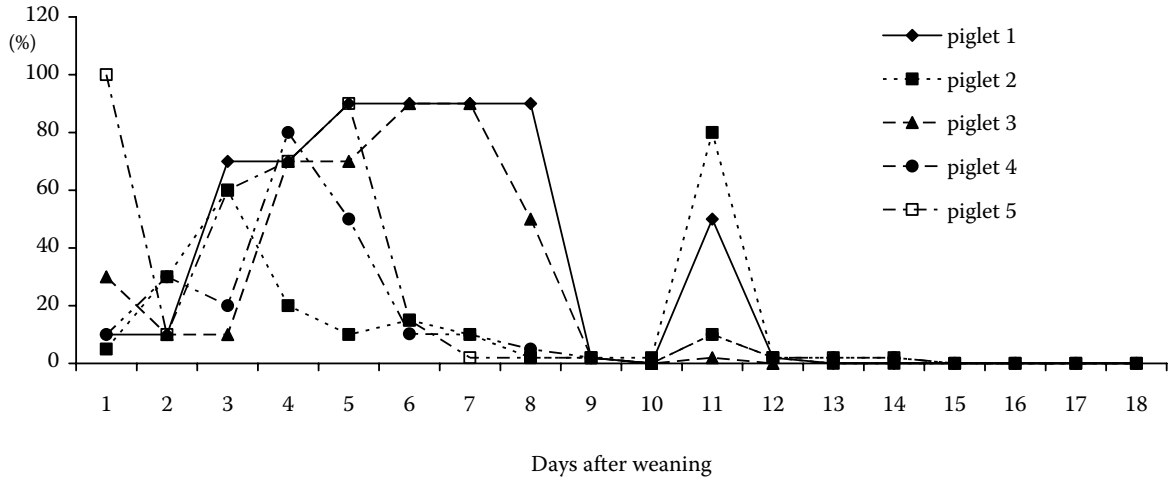

Figure 1. Percentage of the O149:K88 strain in rectal swabs from the experimental group after oral administration of non-pathogenic strain on the day of weaning and chalenge with ETEC 10 days after weaning

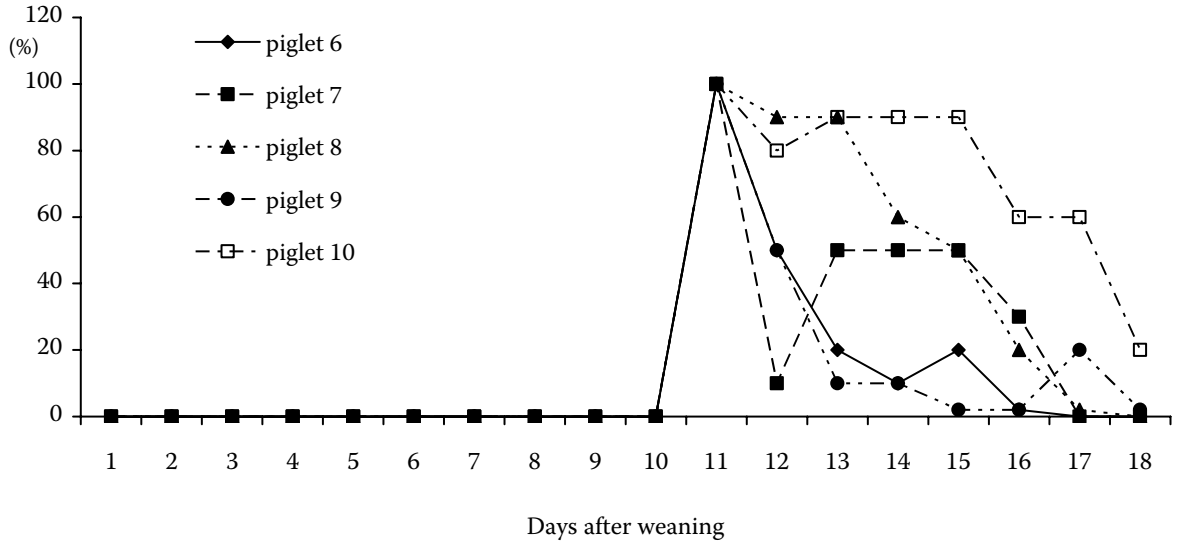

Figure 2. Percentage ot the ETEC O149:K88 strain in rectal swabs from the control group after chalenge 10 days after weaning

munogens. The oral administration of purified F4 induced antigen-specific antibody-secreting cells in the gut-associated lymphoid tissue, indicating stimulation of the mucosal immune system.

In our previous experiments with combined immunization to ETEC with colonizing factors F18ac in piglets at weaning (Alexa et al., 1995), the immunity against ETEC infection was induced with homologous colonizing factors in a relatively short time after weaning. The method consisted in intramuscular administration of bacterin on the day before weaning and a single oral administration of the non-pathogenic *E. coli* culture with colonizing factors F18ac 3 h after weaning.

It was confirmed that a certain degree of immunity was achieved by the challenge ten days after immunization. It was demonstrated that the immunity occurred later than after the administration of a strain with colonizing factors F18ac in our previous experiments where piglets were protected against challenge with F18 positive ETEC on day 7 after combined immunization. It was proved in the second experiment when the infection with ETEC strain was performed on the third day after wean-

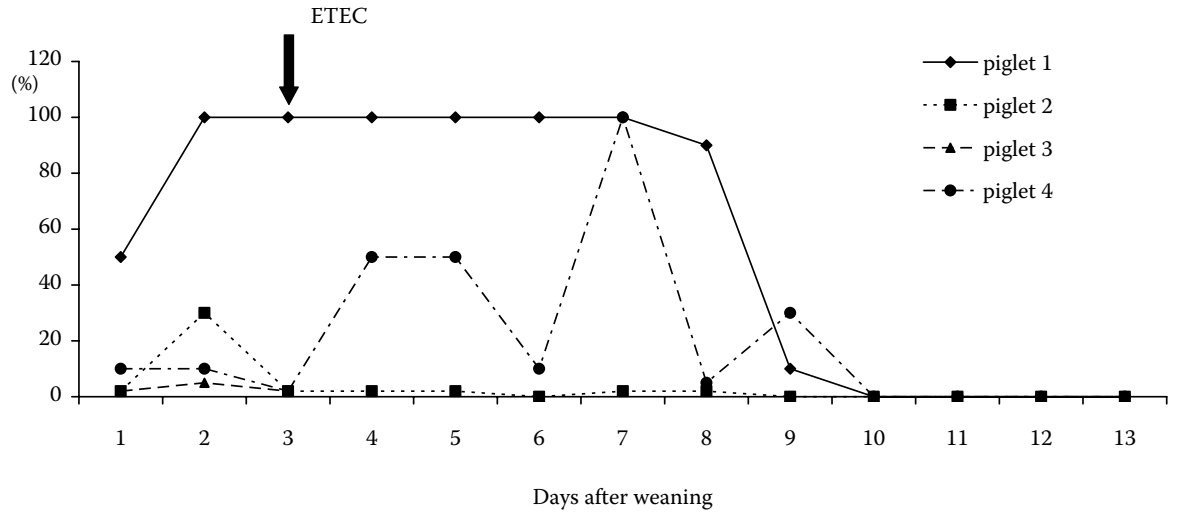

Figure 3. Percentage of the *E. coli* O149:K88 in rectal swabs from the experimental group after administration of the non-pathogenic strain on the day of weaning and after chalenge by ETEC three days after weaning

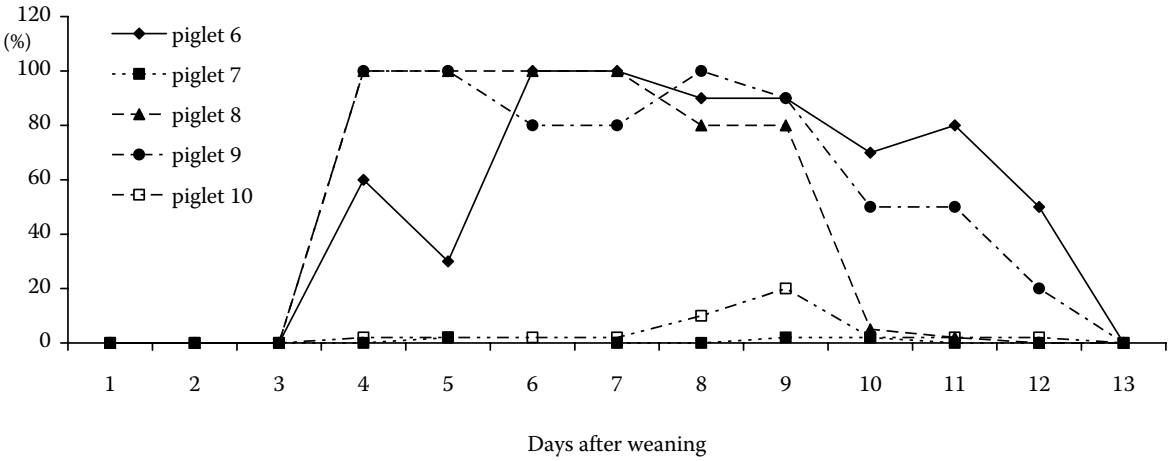

Figure 4. Percentage of *E. coli* O149:K88 strain in rectal swabs from the control group after chalenge by ETEC three days after weaning

ing. In the study of Verdonck et al. (2002) the F18ab strain was less immunogenic than the F4ac ETEC strain. Different results might have been caused by different immunogenicity of F18ab strain used by the quoted authors and of F18ac fimbriae of the strain used in our study. The time of immunity development is also affected by the presence of thermolabile enterotoxins during ETEC infection. It is likely that LT has adjuvant properties similarly like cholera toxin (Clements et al., 1988; Cheng et al., 1999; Freytag and Clements, 2005). The strain used in the present study did not produce LT.

The duration of diarrhoea was shorter in the experimental group; however, the differences were not significant. In both groups, the excretion of the administered *E. coli* strains was marked in some animals. Differences in the excretion intensity did not seem to be caused by the time of local immunity establishment, because weak excretion of O149:K88 strain through piglet faeces persisted about as long as marked excretion in the other piglets. In our experiment, we did not discriminate between ETEC and non-ETEC strains excreted.

Francis and Willgohs (1991) orally immunized piglets weaned at the age of 10 days. Two doses of the live non-pathogenic *E. coli* strain with colonizing factors K88 were administered to the animals at the age of 15 days and repeatedly 5 days later. The authors recorded a protective effect of challenge with the ETEC strain 14 days after the first immunization, which is consistent with our results. A similar trend in the development of local immunity was described by Van den Broeck et al. (1999). They observed a marked increase in the antibody secreting cell levels in the intestinal *lamina propria* on the eleventh day after oral immunization. However, the induction of immunity later than 5 days after weaning does not protect piglets at the age when the highest losses are recorded due to diarrhoea caused by *E. coli*-infections, i.e. at about 4 to 7 days after weaning. It is necessary to investigate other possibilities how to accelerate the induction of mucosal immunity. Van der Stede et al. (2003) reduced the shedding of F4 positive *E. coli* after intramuscular immunization of suckling piglets by F4 fimbriae and by addition of 1α,25-dihydroxyvitamin D_3 or CpG-oligonucleotides. Using this kind of adjuvants could have improved the results.

The demonstration of a long-lasting excretion of the strains with K88 colonizing factors also confirmed that immunity was established approximately ten days after the administration of *E. coli* strain. As the results obtained in our two experiments did not suggest that protective immunity might be induced in piglets by the end of the first week after weaning, the experiments were finished.

REFERENCES

Acres S.D., Isaacson R.E., Babiuk L.A., Kapitany R.A. (1979): Immunization of calves against enterotoxigenic colibacillosis by vaccinating dams with purified K99 antigen and whole cell bacterins. Infection and Immunity, 25, 121–126.

Alexa P., Salajka E., Salajkova Z., Machova A. (1995): Combined parenteral and oral immunization against diarrhoea in weaned piglets caused by enterotoxigenic

strains of *Escherichia coli*. Veterinarni Medicina, 40, 365–370.

Alexa P., Stouracova K., Hamrik J., Salajka E. (2002): Gene typing of the colonisation factors F18 of *Escherichia coli* isolated from piglets suffering from post-weaning oedema disease. Veterinarni Medicina, 47, 132–136.

Bertschinger H.U., Nief V., Tschape H. (2000): Active oral immunization of suckling piglets to prevent colonization after weaning by enterotoxigenic *Escherichia coli* with fimbriae F18. Veterinary Microbiology, 71, 255–267.

Cheng E., Cardenas-Freytag L., Clements J.D. (1999): The role of cAMP in mucosal adjuvanticity of *Escherichia coli* heat-labile enterotoxin (LT). Vaccine, 18, 38–49.

Clements J.D., Hartzog N.M., Lyon F.L. (1988): Adjuvant activity of *Escherichia-coli* heat-labile entero-toxin and effect on the induction of oral tolerance in mice to unrelated protein antigens. Vaccine, 6, 269–277.

Felder C.B., Vorlaender N., Gander B., Merkle H.P., Bertschinger H.U. (2000): Microencapsulated enterotoxigenic *Escherichia coli* and detached fimbriae for peroral vaccination of pigs. Vaccine, 19, 706–715.

Francis D.H., Willgohs J.A. (1991): Evaluation of a live avirulent *Escherichia-coli* vaccine for K-88+ Lt+ enterotoxigenic colibacillosis in weaned pigs. American Journal of Veterinary Research, 52, 1051–1055.

Freytag L.C., Clements J.D. (2005): Mucosal adjuvants. Vaccine, 23, 1804–1813.

Guinee P.A.M., Jansen W.H. (1979): Behaviour of *Escherichia coli* K antigens K88ab, K88ac and K88ad in immunoelectrophoresis, double diffusion and haemagglutination. Infection and Immunity, 13, 1369–1377.

Holmgren J., Czerkinsky C., Lycke N., Svennerholm A.M. (1992): Mucosal immunity – implications for vaccine development. Immunobiology, 184, 157–179.

Nagy B., Moon H.W., Isaacson R.E., Brinton C.C. (1978): Immunization of suckling pigs against enteric enterotoxigenic *Escherichia coli* infection by vaccinating dams with purified pili. Infection and Immunity, 21, 269–274.

Porter P. (1973): Intestinal defence in the young pig – A review of the secretory antibody system and their possible role in oral immunization. Veterinary Record, 92, 658–664.

Rutter J.M. (1975): *Escherichia coli* infections in piglets: Pathogenesis, virulence and vaccination. Veterinary Record, 96, 171–175.

Salajka E., Mensik J. (1973): Implication of total and local immunity in protection against current infections of piglets in early postnatal period (in Czech). Veterinarství, 23, 163–164.

Salajka E., Salajkova Z., Alexa P., Hornich M. (1979): Prevention of intestinal *Escherichia coli* infection in suckling and weaned piglets. In: XXIst World Vet. Cong. Moscow, Summaries No. 6, 32, 64.

Salajka E., Salajkova Z., Alexa P., Hornich M. (1992): Colonization factor different from K88, K99, F41 and 987P in enterotoxigenic *Escherichia-coli* strains isolated from postweaning diarrhoea in pigs. Veterinary Microbiology, 32, 163–175.

Sarrazin E., Bertschinger H.U. (1997): Role of fimbriae F18 for actively acquired immunity against porcine enterotoxigenic *Escherichia coli*. Veterinary Microbiology, 54, 133–144.

Van den Broeck W., Cox E., Goddeeris B.M. (1999): Induction of immune responses in pigs following oral administration of purified F4 fimbriae. Vaccine, 17, 2020–2029.

Van der Stede Y., Cox E., Verdonck F., Vancaeneghem S., Goddeeris B.M. (2003): Reduced faecal excretion of F4(+)-*E. coli* by the intramuscular immunisation of suckling piglets by the addition of 1 alpha,25-dihydroxyvitamin D-3 or CpG-oligodeoxynucleotides. Vaccine, 21, 1023–1032.

Verdonck F., Cox E., Van der Stede Y., Goddeeris B.M. (2004): Oral immunization of piglets with recombinant F4 fimbrial adhesin FaeG monomers induces a mucosal and systemic F4-specific immune response. Vaccine, 22, 4291–4299.

Verdonck F., Cox, E., van Gog, K., Van der Stede, Y., Duchateau, L., Deprez, P., Goddeeris, B.M. (2002): Different kinetic of antibody responses following infection of newly weaned pigs with an F4 enterotoxigenic *Escherichia coli* strain or an F18 verotoxigenic *Escherichia coli* strain. Vaccine, 20, 2995–3004.

Received: 05–04–05
Accepted after corrections: 05–05–18

Corresponding Author

Pavel Alexa, Veterinary Research Institute, Hudcova 70, 621 32 Brno, Czech Republic
Tel.: +420 533 331 201, fax +420 541 211 229, e- mail: alexa@vri.cz