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1 **Cytokine mRNA expression in porcine cell lines stimulated by**
2 **enterotoxigenic *Escherichia coli***

3
4 Barbora Pavlova ^{1,2}, Jiri Volf ¹, Pavel Alexa ¹, Ivan Rychlik ¹, Jan Matiasovic ¹,
5 Martin Faldyna ^{1*}

6
7 ¹Veterinary Research Institute, Brno, Czech Republic

8 ²Institute of Experimental Biology, Faculty of Science, Masaryk University, Brno,
9 Czech Republic

10

11 *Corresponding author

12 Correspondence address:

13 Veterinary Research Institute,

14 Hudcova 70, Brno, 621 00, Czech Republic

15 Phone: +420 533 331 301

16 Fax: +420 541 211 229

17 Email: faldyna@vri.cz

18 **Abstract**

19 Enterotoxigenic *Escherichia coli* (ETEC) is an extracellular bacterium that
20 causes post-weaning diarrhoea (PWD) in piglets with different severity of clinical
21 signs. The pathogenesis of ETEC is ascribed to the effect of enterotoxins. ETEC
22 colonizes ileum and probably can penetrate the epithelium and stimulate
23 macrophages. The aim of study was to examine whether there is any difference in
24 cytokine response *in vitro* produced by two porcine cell lines, intestinal epithelial
25 cell line (IPI-2I) and macrophage cell line (3D4/31) after stimulation with
26 different serotypes of ETEC associated with different clinical course of PWD in
27 piglets. Three serotypes, O149:K88 (F4), O147:F18 and O8:K88, were used. We
28 observed that all the used serotypes were unable to induce IL-8 and TNF- α
29 mRNA expression in IPI-2I cell line as measured by the real-time RT-PCR. In
30 3D4/31 cell line, we detected differences in cytokine response among the used
31 serotypes. The highest IL-8 and TNF- α mRNA expression in 3D4/31 was detected
32 after stimulation with serotype O149:K88 frequently associated with hemorrhagic
33 gastroenteritis.

34

35 **Key words:** enterotoxigenic *Escherichia coli*, porcine cell lines, post-weaning
36 diarrhoea, IL-8, TNF- α .

37 1. Introduction

38 Enterotoxigenic *Escherichia coli* (ETEC) is one of the most important
39 causes of post-weaning diarrhoea (PWD) in piglets (Hampson et al., 1994; Khac
40 et al., 2006). ETEC that causes PWD is mostly of the O149 serotype and carries
41 the K88 (F4) adhesin that permits adhesion of the bacteria to the intestines
42 (Salajka et al., 1992; Imberechts et al., 1994). ETEC is characterized by
43 production of different combinations of enterotoxins called heat-labile
44 enterotoxins (LT) and/or heat-stable enterotoxins (STa or STb).

45 Especially serotype O149:K88 is frequently associated with hemorrhagic
46 gastroenteritis (HGE) characterized by serious inflammation with concurrent
47 hyperaemic, oedematous or hemorrhagic mucosa and strong infiltration of
48 neutrophils in lamina propria (Faubert and Drolet, 1992). Other ETEC serotypes
49 have been associated with mild or moderate enteritis. We found a large number of
50 O149:K88 serotype in the intestine practically in all cases of dead weaned piglets
51 with symptoms of HGE characterized by marked inflammatory infiltration of
52 intestine and lymph nodes. Moreover, this serotype was also isolated from
53 mesenteric lymph nodes (Alexa et al., unpublished data).

54 While much progress has been made in serotyping of *E. coli* isolated from
55 weaned pigs (Imberechts et al., 1994) and identification of virulence genes
56 (Noamani et al., 2003), no studies concerning cytokine response in relation to
57 PWD have been described prior this report. There are few *in vivo* studies which
58 investigated the cytokine production after enteropathogenic *E. coli* stimulation
59 (Girard et al., 2005; Splichal et al., 2006).

60 The first step in pathogenesis of ETEC is adhesion to the ileal epithelium
61 (Nagy et al., 1992; Girard et al., 2005) with subsequent production of enterotoxins
62 which are responsible for an increase in fluid secretion (Nagy and Fekete, 2005).
63 Then, *E. coli* can be probably engulfed by antigen-presenting cells, e.g. dendritic
64 cells or macrophages and evokes different courses of inflammation. Therefore the

65 aim of the study was to ascertain whether there is any difference in the *in vitro*
66 proinflammatory cytokine (IL-8, TNF- α) production by the continuous porcine
67 cell lines: the intestinal epithelial cell line (IPI-2I) and the macrophage cell line
68 (3D4/31) after stimulation with different ETEC serotypes.

69

70 **2. Materials and methods**

71 *2.1 Bacterial strains and growth conditions*

72 All the used serotypes of ETEC were isolated from the intestines of dead
73 weaned piglets from different herds in the Czech Republic. We used 3 serotypes:
74 O149:K88 (3 strains), O147:F18 (3 strains) and O8:K88 (3 strains). The
75 phenotypes of the used ETEC were summarized in Table 1. The overnight
76 cultures of *E. coli* were prepared by culture from a frozen stock at 37°C in Luria
77 broth (LB) (Difco, USA). Before the use in adhesion and infection experiments,
78 an overnight culture was subcultured in LB medium and incubated for 6 h at
79 37°C.

80

81 *2.2 Cell line experiments*

82 The following two porcine cell lines were used in this study: the porcine
83 ileal epithelial cell lines (IPI-2I) (Kaeffer et al., 1993) obtained from the European
84 Collection of Animal Cell Cultures and the porcine macrophage lines (3D4/31)
85 established from primary porcine alveolar macrophages obtained from Weingartl
86 et al. (2002).

87 IPI-2I cells were grown in Ham's Nutrient F 12 (Sigma, USA)
88 supplemented with 10% foetal bovine serum. 3D4/31 cells were grown in
89 Dulbecco's DMEM medium (Sigma) supplemented with 1% of non-essential
90 amino-acids and 10% foetal bovine serum. This medium was replaced with RPMI
91 1640 (Sigma) containing 1% of non-essential amino-acids and 10% porcine serum
92 (Invitrogen, USA) the day before infection. The cells were seeded into a 24-well

93 microplate and grown at 37°C under 5% CO₂. When the cultures reached
94 semiconfluent growth, ETEC were added at the multiplicity of infection equal to
95 50 for 2 h. The positive control included phorbol myristyl acetate (PMA) (0.01
96 mg/ml) (Serva, Germany) and ionomycin (0.05 mg/ml) (Sigma), which
97 maintained nonspecific stimulation of the cells. The negative control included 10
98 µl PBS instead of *E. coli*.

99 After incubation, the cells were lysed to purify total RNA using the
100 RNeasy Mini Kit according to the manufacturer's instruction (Qiagen, Germany).
101 RNA was checked spectrophotometrically as well as by agarose gel
102 electrophoresis.

103 In a parallel experiment, the numbers of adhered ETEC were determined.
104 The monolayers were washed two times with PBS 2 h after infection and lysed in
105 1% Triton X-100 (Sigma) for 20 min in order to release the bacteria. The
106 suspensions were serially diluted 5-fold and 50 µl of each dilution was plated on
107 LB agar. The percentages of attached bacteria related to infection doses were
108 calculated based on numerical CFU values. Because of variations among tests, we
109 randomly chose one strain of serotype O149:K88 as a reference, which has been
110 ascribed a value of 1 and percentage results were recalculated to this reference.

111

112 2.3 Real-time RT-PCR

113 Total RNA was reverse transcribed with M-MLV reverse transcriptase
114 (200U) (Invitrogen) using oligo-dT primers (Qiagen). cDNA was synthesized first
115 at 37°C for 50 min and the reaction was inactivated at 70°C for 15 min. cDNA
116 was purified by QIAquick PCR Purification Kit (Qiagen) and used either
117 immediately or was stored at – 20°C until use.

118 Real-time PCR was performed with the LightCycler 1.2 (Roche, USA),
119 using QuantiTect SYBR Green PCR Kit (Qiagen). Primers for IL-8, TNF- α and
120 HPRT as reference gene were purchased from Generi Biotech (Czech Republic).

121 All primers were adopted from Volf et al. (2007). Each run included a no-
122 template control to test the assay reagents for contamination. Isolation of total
123 RNA from 3D4/31 and IPI-2I was performed in three independent infections and
124 the real-time RT-PCR was repeated twice on each isolated RNA. The relative
125 expression ratio of a cytokine gene is calculated according to $2^{-\Delta\Delta C_T}$ (Livak and
126 Schmittgen, 2001).

127

128 *2.4 Statistical analysis*

129 The statistical analysis of differences of cytokine expression was
130 performed by nonparametric Kruskal-Wallis test and differences between
131 serotypes were tested with Dunns test (Prism, Graph Pad Software, USA).
132 Significant differences were defined as $p < 0.05$.

133

134 **3. Results**

135 *3.1 Adhesion*

136 The adhesion of different serotypes of ETEC was tested by infecting IPI-
137 2I and 3D4/31 cell lines (Fig.1). Serotypes O149:K88 and O8:K88 were able of
138 higher adhesion to IPI-2I than O147:F18. In the case of 3D4/31, the highest
139 adhesion was found for serotype O149:K88. Serotype O147:F18 adhesion to
140 3D4/31 was the lowest of all the used serotypes as well as in the case of adhesion
141 to IPI-2I.

142

143 *3.2 Bacterial infection of IPI-2I cell line*

144 No significant differences between all the used serotypes of ETEC were
145 noted for the tested cytokines in IPI-2I (Fig.2). The relatively weak induction of
146 cytokine expression in IPI-2I cell line was not due to the inability of this cell line
147 to express cytokines since PMA induced about 9-fold expression of IL-8 and in
148 the case of TNF- α , it was about 36-fold compared to negative control.

149 *3.3 Bacterial infection of 3D4/31 cell line*

150 IL-8 mRNA expression was significantly higher after infection of 3D4/31
151 with O149:K88 compared to O147:F18 ($p < 0.001$). IL-8 mRNA expression was
152 higher, although not significantly, after infection of 3D4/31 with O8:K88
153 compared to O147:F18 (Fig.3). PMA induced IL-8 mRNA expression in 3D4/31
154 was about 128-fold.

155 TNF- α mRNA expression was significantly higher after infection of
156 3D4/31 with O149:K88 compared to O147:F18 ($p < 0.05$). TNF- α mRNA
157 expression was higher, although not significantly, after infection of 3D4/31 with
158 O8:K88 compared with O147:F18 (Fig.3). PMA induced TNF- α mRNA
159 expression in 3D4/31 was about 866-fold.

160

161 **4. Discussion**

162 In this study all the used serotypes of ETEC were unable to induce higher
163 expression of IL-8 and TNF- α in IPI-2I cell line compared to negative control.
164 Bacterial entry is required for the IL-8 mRNA expression; neither bacterial
165 adhesion nor the presence of bacterial LPS caused an increase in IL-8 synthesis by
166 the epithelial cell lines (Eckmann et al., 1993). The principal idea of apical
167 membrane of the intestinal epithelial cells, which represent a mechanical barrier
168 against pathogens, was confirmed in our study.

169 The serotypes O149:K88 and O8:K88 were able of a higher adhesion to
170 IPI-2I than O147:F18. Different ability of the serotypes of adhesion is based on
171 the findings of Nagy et al. (1996) and Nagy and Fekete (2005) that the receptors
172 for K88 seemed to differ from the receptors for F18. Although there were found
173 differences in the adhesion ability of various serotypes to IPI-2I, there were no
174 significant differences in the cytokine response.

175 We found that 3D4/31 cell line can respond to O149:K88 stimulation by a
176 higher mRNA expression of IL-8 and TNF- α than to O147:F18 (in this case

177 significantly) and O8:K88. Moreover, the serotype O149:K88 was isolated from
178 mesenteric lymph nodes, it suggests that O149:K88 strains penetrating the
179 epithelium. Macrophages are capable of phagocytosis in contrast to epithelial
180 cells; therefore macrophages may be responsible for higher cytokine production
181 and contribute to the severity of inflammation in the affected intestine during
182 PWD. These findings are in accordance with the clinical course of PWD, when
183 serotype O149:K88 is frequently associated with HGE (Faubert and Drolet, 1992).

184 In the case of 3D4/31, serotype O149:K88 was able of the highest
185 adhesion and cytokine response. The serotype O8:K88 adherence to the cell
186 surface of 3D4/31 was higher than that of O147:F18 and also induced a higher
187 cytokine response. However, the adhesion ability of serotypes O149:K88 and
188 O8:K88 was almost at the same level; accordingly, some virulence factor
189 responsible for the highest cytokine response in the case of O149:K88 seems to
190 play a role there.

191 The pathogenesis of ETEC is ascribed to the effect of enterotoxins. The
192 enterotoxins induce a secretory diarrhoea, generally without inducing histological
193 lesions (Rose et al., 1987). The *in vivo* study on gnotobiotic piglets revealed that
194 LT is the major virulence factor of K88 ETEC, because it enhanced bacterial
195 colonization of the intestine (Berberov et al., 2004; Zhang et al., 2006). The
196 differences in PWD severity from mild, self-limited disease to more severe cases
197 described as HGE are not readily accounted for by our current knowledge of
198 ETEC pathogenesis. Although we detected significant differences in the cytokine
199 response among the used different serotypes of ETEC; we repeatedly failed to
200 induce *in vivo* infection with characteristic hemorrhagic mucosa of weaned piglets
201 challenged with O149:K88. The complex situation occurs *in vivo*, therefore we
202 assume that some additional factors (together with contribution of some virulence
203 factor) exist which is contributing to the progress of the clinical course of HGE.

204 In summary, IPI-2I represents a mechanical barrier, which essentially does
205 not respond to ETEC infection. We detected significant differences in the
206 cytokine response in 3D4/31 among the used different serotypes of ETEC. The
207 highest IL-8 and TNF- α mRNA expression in 3D4/31 was detected after
208 stimulation with serotype O149:K88. Together with frequent isolation of this
209 serotype from mesenteric lymph nodes, the higher cytokine response can, at least
210 partly, explain different course of infection. On the other hand, we failed to induce
211 *in vivo* infection with characteristic hemorrhagic mucosa of weaned piglets
212 challenged with O149:K88, therefore further *in vivo* investigations are required
213 for better understanding of different clinical courses of ETEC infections.

214

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219

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- 287

288 **Figure legends**

289 Fig.1. Adhesion of different serotypes of ETEC (O149:K88, O147:F18 or
290 O8:K88) to intestinal porcine cell line **IPI-2I** (empty columns) and macrophage
291 porcine cell line **3D4/31** (grey columns) after 2 h of *in vitro* stimulation. The
292 percentages of attached bacteria related to infection doses were calculated. The
293 rate of adhesion represents percentage results which were recalculated to one
294 randomly chose strain of serotype O149:K88, which was ascribed a value of 1.
295 The results represent the means of three independent infections conducted in
296 triplicate.

297

298 Fig.2. IL-8 and TNF- α mRNA expression by intestinal porcine cell line
299 **IPI-2I** after 2 h of *in vitro* stimulation with different serotypes of ETEC:
300 O149:K88 – shaded columns, O147:F18 – grey columns or O8:K88 – empty
301 columns. No significant differences between all the used serotypes of ETEC were
302 noted. The results represent the means of three independent infections conducted
303 in duplicate.

304

305 Fig.3. IL-8 and TNF- α mRNA expression by macrophage porcine cell line
306 **3D4/31** after 2 h of *in vitro* stimulation with different serotypes of ETEC:
307 O149:K88 – shaded columns, O147:F18 – grey columns or O8:K88 – empty
308 columns. Significant differences between serotypes O149:K88 and O147:F18
309 were noted ($p < 0.001$ indicated by **, $p < 0.05$ indicated by *). The results
310 represent the means of three independent infections conducted in duplicate.

Table 1
 Characterization of the used enterotoxigenic *Escherichia coli* strains

O- serogroup	hemolysin	adhesin	STa	STb	LT	Stx
O149	-	F4	-	NT	+	-
O149	+	F4	-	NT	+	-
O149	+	F4	-	NT	+	-
O147	+	F18ac	+	NT	-	-
O147	+	F18ac	-	NT	+	-
O147	+	F18ac	-	+	+	-
O8	+	F4	-	NT	+	-
O8	+	F4ac	-	-	+	-
O8	-	F4ac	-	+	+	-

ST = heat-stable enterotoxin, LT = heat-labile enterotoxins, Stx = Shiga toxin (verotoxin), NT = not tested.

Figure 1

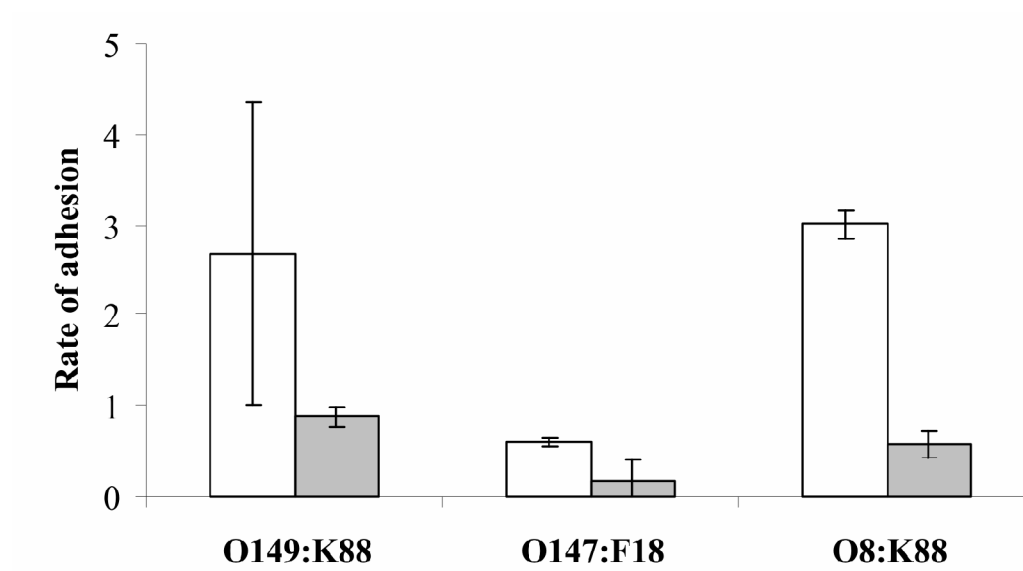
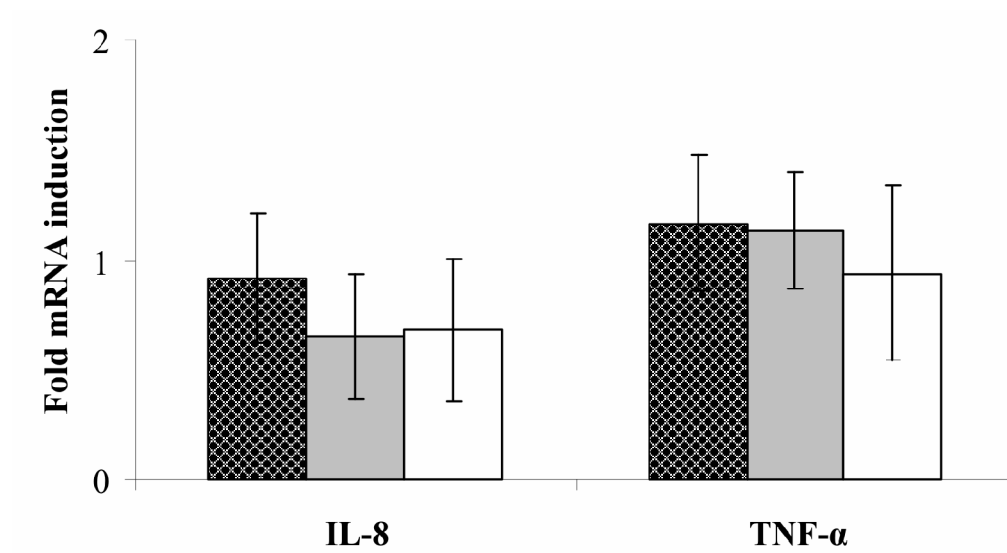
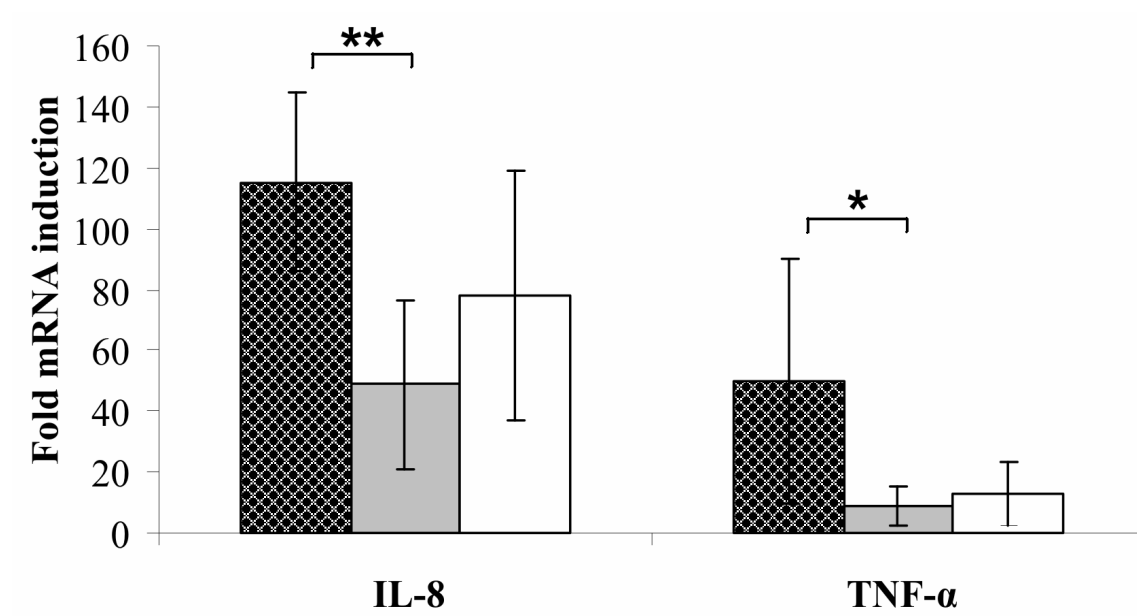


Figure 2



Accepted Man

Figure 3



Accepted M.