

Profiles of virulence genes in enterotoxigenic *Escherichia coli* isolates from suckling piglets in Serbia¹⁾

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Summary

A total of 120 *Escherichia coli* (*E. coli*) strains from suckling piglets with diarrhoea and 30 *E. coli* strains from healthy piglets were tested for the presence of fimbrial and enterotoxin virulence genes. Out of the 120 isolates sampled from diarrheic piglets, 81 (67.5%) expressed one or more genes encoding virulence factors. Adhesin genes were detected in 52 (43.33%) out of 120 *E. coli* isolates, and the most common among them was F4 adhesin (33.33%). Genes encoding *E. coli* toxins were detected in 81 (67.5%) isolates. *E. coli* included in the study carried genes for one or more of the following toxins: STa, STb, LT and EAST1. The *astA* gene encoding EAST1 was the most prevalent and was identified in 72 (60%) *E. coli* isolates. EAST1 toxin was detected in 5 out of 30 isolates (16.7%) from healthy piglets. Among the 81 isolates expressing virulence genes, a total of 15 different combinations for fimbrial and toxin genes were found. The most common virulence pattern was F4/STb/LT/EAST1 detected in 23.45% of *E. coli* strains isolated from suckling piglets with diarrhoea. The results indicate that F4 adhesin and EAST1 toxin are the most common in *E. coli* isolates sampled from diarrheic suckling piglets in Serbia.

Keywords: *Escherichia coli*, adhesin, enterotoxin, virulence genes, diarrhoea

Diarrhoea in the neonatal period is a major health issue and a permanent challenge to the veterinary profession. At the same time, diarrhoea causes serious economic losses in pig production due to increased morbidity, mortality, treatment costs and poor growth (9, 12, 18). *Escherichia coli* (*E. coli*) live in the intestines and are part of the commensal microbiota that plays an important role in the protection of the intestinal tract of animals (21, 26). Nevertheless, there are *E. coli* strains which cause dangerous infections in suckling and/or weaned piglets. According to different virulence factors, *E. coli* strains are classified in different pathotypes or categories. Enterotoxigenic *E. coli* (ETEC) is the main diarrheagenic *E. coli* pathotype, which causes intestinal infections in newborn pigs (2, 3, 5, 27). Enterotoxigenic *E. coli* strains possess two virulence factors: adhesins and enterotoxins. Both factors are essential for the onset of the disease (13, 17).

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Enterotoxigenic *E. coli* strains responsible for neonatal diarrhoea have a set of fimbriae, such as F4 (K88), F5 (K99), F6 (987P) and F41 (6, 7).

The initial step in the pathogenesis of disease is the attachment of the ETEC strains to the microvilli of the small intestine epithelial cells (17, 18, 26). Adhesins target specific receptors on the intestinal epithelial cells, thus enabling the transfer of enterotoxins on target cells (7, 16, 21). Two enterotoxin classes are well known: the high molecular weight heat-labile toxin (LT) and low molecular weight heat-stable a and b toxins (STa and STb) (6, 12, 16). In addition, a number of ETEC strains also produce enteroaggregative heat-stable enterotoxin 1 (EAST1) (4, 26). The same toxin has previously been identified within enteroaggregative *E. coli* strains responsible for diarrhoea in humans (22). Enterotoxins stimulate the intestinal epithelium cells to secrete water and electrolytes into the intestinal lumen, thus reducing absorption, which results in diarrhoea (14, 16).

Severe diarrhoea results in dehydration and acidosis, with a possible lethal outcome (12, 21). Important factors leading to diarrhoea in piglets are the presence of enterotoxigenic *E. coli* strains in the small intestine and the absence of antibodies against *E. coli* adhesins in the colostrum of sows. *E. coli* from the environment colonize newborn piglets by the oral route, thus transferring pathogens to naive animals (14, 17). The presence of neonatal colibacillosis is quite common in Serbia. In practice, the diagnosis of this disease is based on clinical signs, the age of infected piglets, isolation of *E. coli* in pure culture and the absence of other bacteria or coccidia.

As the clinical picture is not specific, diarrhoea caused by ETEC strains can only be diagnosed by detecting virulence factors, that is, by determining the pathogenic potential of the *E. coli* isolate. The aim of this study was to evaluate the presence and prevalence of genes encoding the virulence factors in ETEC strains isolated from newborn piglets with diarrhoea. We believe that these results will offer information useful in the prevention and control of neonatal colibacillosis, especially because there is a lack of reliable data from Serbia.

Material and methods

Sampling. A total of 120 rectal swabs from piglets with diarrhoea and 30 swabs from healthy piglets aged 1-15 days were taken for analysis. The samples were taken during the morning round of visits by the veterinary service of the farms before treatment. The sampled piglets were housed on 3 different conventional farrow-to-finish pig farms located in three epizootiological areas in the north-west of Serbia. Piglets originated from sows not vaccinated against *E. coli* infections. Sampling was performed at the first signs of diarrhoea. One specimen from one piglet from each litter with diarrhoea was used for bacteriological investigation. On each farm, 40 rectal swabs from diarrhoeic piglets and 10 swabs from healthy piglets were sampled. These farms, with a capacity of 800-1000 sows, were located 50 to 70 km from Belgrade. After sampling, the swabs were delivered within 2 to 3 hours to the Institute of Veterinary Medicine of Serbia, Belgrade.

Bacterial isolates. Samples were inoculated on MacConkey agar (bioMerieux, France) and incubated aerobically overnight at 37°C. From each sample, 3 lactose ferment-

ing colonies from pure culture were selected for *E. coli* identification by conventional biochemical procedures (indole, methyl red, Voges-Proskauer and citrate utilization tests). BBL Crystal E/N ID kits (Becton Dickinson, USA) were used as a confirmation test. After the identification of strains, only 1 colony was stored in brain heart infusion broth (HiMedia, India) with 20% glycerol at -70°C pending further analyses.

Detection of virulence genes by standard PCR and multiplex PCR. DNA was prepared from *E. coli* strains in the following way (27). The colony of an overnight bacterial culture was suspended in 50 µl of water and boiled for 10 minutes, and then cooled on ice for 10 minutes. After brief centrifugation at 13 000 g, the supernatant was used as the template for PCR. PCR reactions for F4, F5, F6, F41, LT and EAST1 were performed in a 20 µl volume using the PCR Hotstar Master Mix Kit (Qiagen) as well as the primers and protocols provided in Table 1. Ten picomoles of each primer and 2 µl of DNA supernatant were added to the reaction and constituted the sample. The PCR reaction for STa and STb was performed with primers (20) as multiplex PCR under the following cycling conditions: 1 cycle of 5 min at 94°C; 40 cycles of 30 sec at 94°C, 60 sec at 55°C and 120 sec at 72°C, followed by final elongation at 72°C for 5 min.

A volume of 6 µl of the amplified PCR products was analyzed by standard gel electrophoresis (120 V, 30 mA, 60 min.) on 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. Primer sequences used for all PCR reactions are given in Table 1.

Results and discussion

The frequency of virulence factors in *E. coli* strains from suckling piglets with diarrhoea. A total of 120 *E. coli* strains isolated from suckling piglets with diarrhoea were tested for the presence of genes encoding virulence factors. Out of the 120 *E. coli* isolates tested, 81 (67.5%) strains from the clinical cases expressed 1 or more genes encoding virulence factors. Thirty-nine (32.5%) isolates were negative and could not be labelled as an ETEC pathotype.

Frequency of adhesin genes. Adhesin genes were detected in 52 (43.33%) *E. coli* isolates from diarrhoeic piglets. The most common among them was F4, which was detected in 40 (33.33%) *E. coli* isolates. Other adhesin genes were identified in a smaller number of isolates. The F6 gene was detected in 9 isolates

Tab. 1. PCR primers used for enterotoxin and fimbrial gene detection in *Escherichia coli* isolates from suckling piglets

Target gene	Primer sequence	Amplified products (bp)	Reference
LT	TTA CCG CGT TAC TAT CCT CT GGT CTC GGT CAG ATA TGT GA	275	Alexa et al. (1997) (1)
STa (1,2)	TCT TTC CCC TCT TTA GTC AG ACA GGC CGG ATT ACA ACA AAG	166	Osek (2001) (20)
STb (1,2)	GCC TAT GCA TCT ACA CAA TC TGA GAA ATG GAC AAT GTC CG	278	Osek (2001) (20)
EAST1	CCA TCA ACA CAG TAT ATC CGA GGT CGC GAG TGA CGG CTT TGT	111	Yamamoto and Nakazawa (1997) (26)
F4	ATC GGT GGT AGT ATC ACT GC AAC CTG CGA CGT CAA CAA GA	601	Ojeniyi et al. (1994) (19)
F5	TGC GAC TAC CAA TGC TTC TG TAT CCA CCA TTA GAC GGA GC	450	Ojeniyi et al. (1994) (19)
F6	TCT GCT CTT AAA GCT ACT GG AAC TCC ACC GTT TGT ATC AG	333	Ojeniyi et al. (1994) (19)
F41	GCA TCA GCG GCA GTA TCT GTC CCT AGC TCA GTA TTA TCA CCT	380	Franck et al. (1998) (8)

(7.5%), 4 of which had the F4/F6 gene combination. Five isolates (4.16%) were positive for F41, and one of them also had the F5 gene. A total of 3 (2.5%) F5-positive strains were identified. Fimbrial adhesins F4, F5, F6 and F41 were not detected in twenty-nine (24.16%) *E. coli* isolates from diarrhoeic piglets.

The frequency of toxin genes. Genes encoding *E. coli* toxins were detected in 81 (67.5%) out of 120 isolates from diarrhoeic piglets. Strains carried genes for 1 or more of the following toxins: STa, STb, LT and EAST1. The *astA* gene encoding EAST1 was the most prevalent gene identified in 72 (60%) strains, followed by genes encoding LT in 42 (35%), and STb in 36 (30%), while genes encoding STa were detected in 10 (8.3%) *E. coli* isolates. All strains with LT toxins (42) had genes for EAST1, and 36 strains with STb toxins concurrently showed the EAST1 gene. Both genes encoding STb and LT toxins were detected in 19 F4-positive strains, 2 F4/F6-positive strains and 3 isolates negative for fimbriae. Among the 10 strains carrying the gene for STa toxin, 8 had only the STa gene and 2 showed the STa/STb/EAST1 pattern. Among the 72 EAST1-positive strains, 16 carried the *astA* gene as the only virulence gene, while the remaining 56 strains contained additional toxin and/or fimbrial genes. The combinations of adhesin and toxin genes in the *E. coli* isolates are shown in Table 2.

Out of 120 *E. coli* isolates, 52 (43.33%) possessed genes for fimbriae and toxins. All isolates carrying the F4 fimbrial gene possessed one or more enterotoxin genes. Thirty-eight out of 40 F4-positive isolates possessed genes for EAST1, 33 carried genes for LT, and 26 for STb. The F6 adhesin was present in 7 strains, including 2 F4/F6-positive strains out of 10 STa-positive isolates. In addition, 2 isolates showing F5 adhesin genes encoding STa toxin were identified, as well as 1 isolate with F5/F41 adhesins. Four isolates with F41 adhesins carried genes for individual toxins: 3 isolates for EAST1, and 1 isolate for STb. In general, fimbrial isolates with 1 toxin gene were present in 12 isolates, and 40 isolates contained a number of different toxin genes. Among the 81 isolates with virulence genes, 15 different combinations of genes for fimbriae and toxins were found. The most common virulence pattern was F4/STb/LT/EAST1, present in 19 isolates. In 12 isolates, the combination observed was F4/LT/EAST1. A less common virulence pattern, present in 5 isolates, was F4/STb/EAST1, while F4/F6/STb/LT/

Tab. 2. Combinations of adhesin and toxin genes observed in 81 *E. coli* isolates from piglets with diarrhoea

Toxin gene profile	No. of isolates carrying genes for fimbriae							Total
	F4	F5	F6	F41	F4/F6	F5/F41	None ^a	
STb/LT/EAST1	19							19
LT/EAST1	12							12
STb/EAST1	5							5
STb/LT/EAST1					2			2
STa					2			2
STa		2						2
STa						1		1
STa			3					3
STa/STb/EAST1			2					2
EAST1				3				3
STb				1				1
STb/EAST1							4	4
LT/EAST1							6	6
STb/LT/EAST1							3	3
EAST1							16	16
Total	36	2	5	4	4	1	29	81

Explanation: a – *E. coli* isolates negative for F4, F5, F6 and F41

EAST1 and F4/F6/STa were found in 2 isolates each. Adhesin F5 was found in 2 F5/STa isolates and 1 F5/F41/STa isolate. In total, adhesin F6 was discovered in 5 isolates, 3 of which showed the F6/STa pattern and 2 showed the F6/STa/STb/EAST1 pattern. In addition, 3 isolates had the F41/EAST1 combination, and 1 isolate had the F41/STb combination.

The strains from all three farms showed no significant differences in their profiles of virulence genes. The farms are situated in three bordering epizootiological locations in the north-western part of Serbia. The distance between the farms does not exceed 50 km. That distance could have a significant effect on pathogen transmission between them, since shorter distances result in greater transmission risk.

The frequency of virulence factors in *E. coli* isolates from healthy piglets. A total of 30 *E. coli* isolates from clinically healthy piglets were examined, that is, 10 isolates from each of the 3 farms. *E. coli* strains positive for the *astA* gene were detected on all 3 farms. Three EAST1-positive isolates were found on farms A and C, whereas 2 positive isolates were identified on farm B. None of the fimbrial adhesin genes and toxic genes STa, STb, LT were found in *E. coli* isolates from healthy piglets.

Diarrhoea in suckling piglets caused by *E. coli* strains is a problem which causes significant health and economic losses worldwide. For this reason we decided to publish the results of our research on the prevalence of some virulence factors in *E. coli* isolates from diarrhoeic and non-diarrhoeic piglets at 15 days of age. Among the 120 *E. coli* isolates from diarrhoeic piglets, 81 (67.5%) carried one or more virulence genes.

E. coli isolates with one or more enterotoxigenic genes are classified as ETEC. Previous results indicate that the most common among virulence genes were *astA* genes, which encode EAST1 enterotoxin in ETEC isolates from suckling piglets with diarrhoea. Thus, in Slovakia the presence of the EAST1 toxin gene was established in 144 out of 220 isolates (65%) from diarrhoeic piglets aged up to 14 days (25). In the Czech Republic, EAST1 was present in 277 out of 800 strains (34.6%) (27). In Korea, 48 out of 116 isolates (41.4%) were positive for EAST1 (3), and in China the percentage of EAST1 strains was slightly lower (31.5%) (13). In our studies, we also detected a high prevalence of the *astA* gene, which was found in 72 out of 120 (60%) *E. coli* isolates from diarrhoeic piglets. Similar to results published by Yamamoto and Nakazawa (26), we detected *astA* genes in F4-positive strains which concurrently carried genes for LT enterotoxin. We found that all 42 LT-positive strains from diarrhoeic piglets contained the *astA* gene. Similar results of previous investigations were also reported in the Czech Republic, where 174 out of 180 LT-positive strains (96.7%) possessed the *astA* gene (27). During our investigation we detected EAST1 toxin in 5 out of 30 isolates (16.7%) from healthy pigs. The prevalence of EAST1 in *E. coli* isolates from non-diarrhoeic piglets was reported as 10.8% in Canada (18) and 9% in Mexico (24). In Slovakia, 27% of isolates from healthy piglets were EAST1-positive (25). As a possible explanation for such a high prevalence of EAST1 in healthy piglets, the authors state asymptomatic infections (25), as well as the possibility that, without the presence of other enterotoxins, EAST1 alone may not be able to cause diarrhoea in piglets (4, 18, 24). Some authors note a varying connection between the *astA* gene and F4 adhesin. For example, in Slovakia 100% of *astA* gene-positive strains were also positive for F4 adhesin (25), in Japan 88.2% (26), in the Czech Republic 64.6% (27), and in Uganda 3 out of 4 F4-positive strains carried the EAST1 gene (9). Our results are in agreement with the above, as 38 out of 43 EAST1-positive strains (88.4%) carried genes for F4, which indicates a close relationship between EAST1 and F4 genes in isolates from suckling piglets with diarrhoea in Serbia. On the other hand, in Mexico EAST1 was most often found along with genes encoding F41 (24). Studies on the incidence of STb and LT enterotoxins reveal significant differences between countries. In Switzerland, the prevalence of STb toxin was 90% (23), in Vietnam 83% (5), in Slovakia 50% (25), in Uganda 39% (9) and in China 20% (13). Our results show a prevalence of 35% and 30% for LT and STb toxins, respectively. Similar results on the prevalence of LT toxin were obtained in the Czech Republic, where 62.8% of 277 EAST1-positive isolates carried the gene for LT (27). In Korea, LT toxin was the most common (23%) in 2010 (11), but 3 years later LT and STb toxins were found to be present with an equal incidence (31%) (3). Contrary to the above findings,

a number of studies report low incidence (4.3%, 4.7%, and 5.6%) for LT toxin (5, 12, 24). The results of Ikwap et al. (9) are interesting, as they show absence of both LT and STa toxins in 18 isolates from suckling piglets with diarrhoea. Among enterotoxins, the least common in the present study was STa toxin, whose genes were determined in 10 isolates, that is 8.3%, which was somewhat more than 3.4% reported from China (13) for *E. coli* isolates from suckling piglets with diarrhoea. On the other hand, there are reports of a high prevalence of STa toxin, which amounted to 92.1% in Vietnam, 80.7% in Japan, and 37.5% in Mexico (5, 10, 24). Previous studies report that fimbrial adhesins are widespread in *E. coli* strains isolated from piglets with diarrhoea. In this study, 33.33% of all *E. coli* isolates from diseased piglets were positive for the F4 gene, which suggests that adhesin F4 is the most prevalent among the *E. coli* strains tested in Serbia. These findings are in agreement with other studies from different countries in which ETEC F4-positive strains were isolated from piglets with diarrhoea. Thus, 84.1% of isolates were F4-positive in the Czech Republic (2), 77% in Switzerland (23), 46% in Japan (10), 38% in Slovakia (25), and 31.9% in Korea (3).

Compared to our previous results, a smaller incidence of F4 adhesin can be seen: F4 was present in 33.33% of isolates compared to 80.85% previously found in piglets up to 30 days of age (28). In accordance with a report by Luppi (14), we ascribe this fact to the less frequent occurrence of F4 strains in younger piglets. However, some reports are contradictory, as they state that F4-positive isolates from diarrhoeic piglets are very rare. F4 adhesin was found in 3% of isolates in Mexico (24), and in 3.9% in China (13). Our results show a markedly lower presence of F5, F6 and F41 adhesins. In some countries, some of them were reported to be dominant, for example, F41 in Mexico (24). In this study, the variability of adherence factors was highest for STa toxin in combination with F5, F6, F4/F6 and F5/F41. Similar combinations of genes encoding adhesins and STa toxin were discovered in other countries – for example F5/STa, F6/STa and F4/F6/STa in Korea and Belgium (12, 15), as well as F5/F41/STa and F6/STa/STb/EAST1 in Slovakia (25). It is interesting that not a single strain with STa toxin was associated with F4 or F41 alone, except when in combination with other adhesins. Three of the 5 F41-positive isolates also possessed the *astA* gene, and 1 isolate harboured the genes for F41 and STb respectively. Findings on the presence of F5, F6, F18 and F41 reported from Uganda are very interesting, as these adhesins were not detected in any of 18 isolates examined there (9).

The present study shows different profiles of virulence factors in *E. coli* isolates from suckling piglets with diarrhoea in Serbia. The results indicate that the most common genes in ETEC isolates from suckling piglets with diarrhoea were those coding for EAST1

enterotoxin and, to a lesser degree, for LT and STb enterotoxins. Among the colonization factors tested, the most commonly detected were genes for F4 fimbrial adhesin. Our results confirm the presence of virulence factors in 15 different combinations of fimbriae and toxin genes. The most common virulence pattern was F4/STb/LT/EAST1. The research presented here is interesting from the epidemiological point of view and will be useful in prevention programmes, such as the vaccination of sows to protect piglets from ETEC strains in the suckling period.

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