ISOLATION OF *ETEC* STRAINS FROM PIGLETS WITH DIARRHEA IN THE NEONATAL PERIOD AND THEIR TYPIZATION BASED ON SOMATIC AND FIMBRIAL ANTIGENS

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Among different pathogens, enterotoxic E. coli (ETEC) has been for many years an important etiological agent in the occurrence of digestive system disease of newborn animals. In counties with developed pig breeding (farming), including our country, diarrhea in the neonatal period, caused by ETEC strains is one of the most present and economically most significant diseases. The aim of this investigation wais to determine the prevalence of ETEC strains in piglets (weaning pigs), originated from 5 (five) pig farms in the Republic of Serbia, as well as their serological typization based on characteristics of somatic O antigens, presence of fimbrial antigens-adhesins and hemolytic activity.

The material for this investigation was targeted and sampled from piglets that have shown clinical signs of neonatal diarrhea or pathoanatomical changes characteristic for enteritis caused by ETEC strains. The total number of isolated ETEC strains were 148, of which 91 (61.48 %) were determined on the basis of somatic O antigen characteristics. The largest number of strains, 42 (46.15 %) belonged to serotype O149. Serological types O8 and O147 were represented, each with 15 strains (16.48 %). In 13 (14.28 %) strains the somatic antigen which belonged to serotype O138 was determined and in 6 (6.59 %) strains the antigen belonged to serotype O157. No strain agglutinated with hyperimmune O139 serotype serum.

The presence of fimbrial adhesins was determined in 47 (51.64%) strains and of that number the F4 type of fimbrial adhesins was detected in 38 (80.85 %) strains. The presence of F5 adhesins was determined in 4, and F6 in 3. In 2 strains, the paralell presence of two adhesin types, F4 and F6 was detected.

The greatest number of strains 30 (71.42 %) with adhesin F4 belonged to O149 serotype, a considerably smaller number, 4 (26.66%) to serotype O8, 2 strains to serotype O157 and to each serotype O147 and O138 1 strain. The fimbrial adhesin of F5 type was detected in 3 strains which belonged to serotype O8 and in 1 strain of serotype O149. All 3 strains with F6 adhesin, belonged to serotype O8. From 2 strains

498

Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

which had, at the same time, adhesins F4 and F6 one belonged to serotype O8 and the other to serotype O138. Hemolytic activity was present in 42 (46.15 %) strains, of which 34 strains belonged to O149 serotype, 6 strains to O157 serotype and 2 strains to O147 serotype.

Key words: diarrhea, E. coli O149, pigs

INTRODUCTION

In modern pig farming are numerous the factors that influence the presence of various infective diseases of pigs in large agglomerations. Due to their high incidence and importance, infections of the digestive system stand out as the most common pathology in pig farming. These disorders are caused by different agents out of which the most often mentioned are rota and corona viruses, TGE virus, apovirus, coccidiae, *E. coli, Clostridium* spp. etc (Katsuda *et al*., 2006; Zhang *et al*., 2008; Darong *et al*., 2010).

Amongst different pathogens the enterotoxic *E. coli* (ETEC) for quite some time represents the most significant causative agent in the development and pathogenesis of digestive tract disorders in suckling newborns (Francis, 2002; Do *et al.*, 2006; Chen *et al.*, 2008).

During the neonatal period diarrhea caused by ETEC strains is one of the most common and economically relevant diseases in intensive pig production (Zhang *et al*., 2007; Huang *et al*., 2008; White 2009; Yan *et al*., 2009).

Due to the poorly developed center for thermoregulation, newborn piglets are very sensitive to low temperatures, and are prone to hypoglycemia and infections caused by bacteria present in the environment, especially to *E. coli* (Darong *et al*., 2010).

Diarrhea in neonatal piglets can be recorded as early as 2-3 hours after birth and can affect individual piglets or the whole litter. Litters form gilts are more often affected compared to litters from sows. In the first days of life mortality can be very high, averaging in some farms up to 15% loss (Ngeleka *et al*., 2002; Mahan *et al*., 2007) if treatment is not implemented entire litters may be lost.

After infection enterotoxic *E. coli* strains adhere onto the intestinal mucosal cells, multiply and produce enterotoxins. One of the resulting effects of enterotoxins is fluid and electrolyte accumulation in the lumen of the gut, resulting in subsequent diarrhea (Blanco *et al.*, 2006; Darong *et al*., 2010). In more severe cases diarrhea leads to dehydratation and acidosis which can result in death.

Fimbrial adhesins and enterotoxins are considered to be the major factors of ETEC virulence (Alexa *et al*., 2001; Lee *et al*., 2008). As the factors of virulence are linked to certain serological groups *E. coli* (Ecl, 2004; Do *et al*., 2006) their identification is of major importance for the determination of pathogenicity of isolated strains. It is known that strains are classified into serologic types according to the presence of the somatic O, capsular K and flagellar H antigens. Later on were discovered different types of fimbrial (F) antigen (adhesins), and some of the previously described K antigens were determined to be identical to F antigens, thus have a parallel label such as: K88\F4, K99\F5 (Francis, 2002).

Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.   499
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

Due to their extreme importance the labels for F antigens are added to the serological strain formula, especially for *ETEC* of animal origin (Jin and Zhao, 2000; Vu-Khac *et al*., 2004; Lee *et al*., 2008). Up to now more than 30 types of fimbrial proteins have been discovered, the most common is considered to be adhesin F4 (Yan *et al*., 2009; Moon *et al*., 2010). Bacterial attachment via the F4 adhesin is specific mainly for piglets; however some individuals lack the receptor on epithelial cells thus becoming resistant to infection with ETEC strains with F4 adhesin. As this resistance type is inherited (Mendelian laws) in some countries it is used as a parameter for selection of pigs in intensive farming (Huang *et al*., 2008; Erume *et al*., 2008; Jacobsen *et al*., 2009).

As *E. coli* is a part of the gut's microbiome its isolation during diagnostic procedures does not confirm the ethiological connection with enteritis in newborn piglets, as at the same time it is necessary to establish the virulence factors of the isolated strains such is production of toxins, haemolysins, presence of adhesins etc. In laboratories, for practical purposes, the procedure for the determination of the antigenic structure of isolated strains is often used. The rationale for this is the high degree of correlation between some of the antigens, the possibility of enterotoxin production and the presence of fimbrial adhesions (Blanco *et al*., 2006; Vu-Khac *et al*., 2007). Due to the high number of antigens and the possibility for over 10.000 combinations (Kaper and Karmali, 2008), the somatic O and fimbrial F antigen are most commonly identified. There is a high correlation between the results of standard serological and biological tests and the tests used for the detection of marker genes responsible for fimbrial adhesins and enterotoxins, which are the most important virulence factors in ETEC strains originated from pigs.

E. coli strains which can cause intestinal diseases are divided according to epidemiological data, phenotype, clinical symptoms and specific virulence factors into a number of groups commonly described as pathotypes. The pathotype expressied is used in order to identify pathogenic *E. coli* types according to virulence mechanisms defined by a number of virulence genes which belong to (Ecl, 2004/B). This system identifies a number of classes of pathogenic *E. coli* strains amongst which ETEC, EDEC, AIDA, AEEC, STEC/VTEC (Ngelka, 2002; Taillon *et al*., 2008) are for piglets the most significant. Enterotoxic *E. coli* strains produce the two major classes of enterotoxins: the thermostable ST and thermolable LT (Omnia, 2009). Also *STEC* strains wich produce *SHIGA* toxin were isolated (Kim *et al*., 2010).

These strains are able to attach to specific receptors on the epithel lining of the small intestine and/or non specific onto the mucus present in the intestine by one or more fimbrial adhesins F4 (K88), F5 (K99), F6 (987P), F41 etc. (LeBouguenec, 2005; Antao *et al*., 2009; Bardian *et al*., 2010). The most often recorded ETEC strains in neonatal piglets suffering from diarrhea belonged to O8, O147, O149 and O157 strains with dominant F4 fimbrial adhesins (Vu-Khac *et al*., 2004; Yan *et al*., 2009). Eradication of diarrhea in neonatal piglets has been tried in a number of different approaches, however with variable outcomes. In pigs there is no transplacental transfer of immunoglobulins and newborns receive antibodies by means of colostrum and milk in the later stages of lactation. This fact

500   **Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.**
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

indicated that active immunisation of pregnant saws against infection with ETEC strains could protect the litter.

Good results are obtained with vaccines prepared from intact and inactivated *E. coli* stains (Pravieux *et al*., 2007; Eggen, 2009). Due to the fact that the vaccine is prepared on request with strains isolated from the localities on which there is a need to protect the newborn piglets, the vaccine can be considered to be experimental autogenous (Hera and Bures, 2004). The goal of our investigation on pathogenic *E. coli* prevalence, observed continuously on several farms in Serbia, is to prepare experimental autogenous vaccines against neonatal diarrhea caused by ETEC strains.

MATERIAL AND METHODS

The samples needed for this study were rectal swabs taken from piglets with clinically manifested diarrhea. Samples of the small intestines with visible pathoanatomic changes were taken from dead piglets. The piglets were raised in pens with concrete floors, with or without straw and floor heating and were born from saws not vaccinated against *E. coli* infections. Sampling was performed at the first signs of diarrhea. All samples were taken from piglets aged between 1 and 30 days.

Isolation of *E. coli* was done by standard bacteriological procedures. For the final *E. coli* strain identification commercial API 20E kits (bioMerieux) were used.

The attribution of *E.coli* strains to different serological groups which were established according to the characteristics of the somatic O antigen was carried out by rapid agglutination on slides with specific antisera (SII Diagnostica, Denmark and BD Difco™ *E. coli* O antiserum O157). For the identification of fimbrial antigens commercial specific antisera K88, K99 and 987P (Toxigenic *E. coli* pili antisera, Denka Seiken, Japan) were used.

Cultivation of strains needed for the identification of adhesins, as well as the agglutination test and the results interpretation were done according to the manufacturer's instructions.

The production of hemolizins was followed on Colombia blood agar with added 6% sheep blood (bioMerieux). Hemolytic strains were considered to be only those with a clear areal of hemolysis surrounding the developed colonies.

RESULTS AND DISCUSSION

A total of 148 ETEC strains were isolated. Out of this number 91 strains (61.48%) were defined according to the somatic O antigen. The largest number of strains (42; 46.15%) belonged to the group with the somatic antigen O149. Serological groups O8 and O147 were present with 15 (16.48%) strains. In 13 (14.28%) strains the somatic antigen O138 was present. In 6 (6.59%) strains the O157 was detected. Not a single strain agglutinated with the hyperimmune serum of the O139 serogroup. Sum of 57 strains were untypified because none of the

Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

501

used sera showed positive agglutination with these strains and is unclear to which
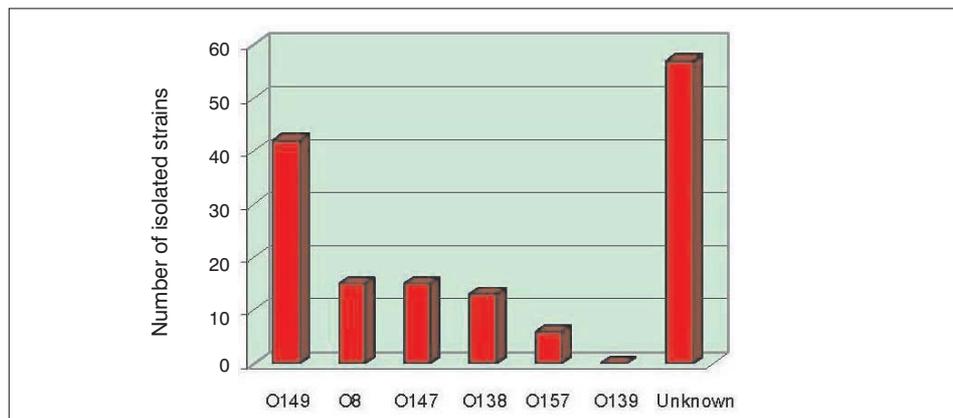type they are attributed (Figure 1).

Figure 1. Serotypes of *ETEC* strains detected in pigs in Serbia in 2010

The presence of fimbrial adhesins was established in 47 (51.64%) strains.
The F4 fimbrial antigen was the most dominant, as it was detected in 38 (80.85%)
strains. Adhesin F5 was in present 4 strains, F6 in 3, and in 2 strains adhesins F4
and F6 were present concurrently.

The largest number of strains, 30 (71.42%) with adhesin F4 belonged to
group O149. A considerably smaller incidence i.e. 4 (22.60%) was for group O8, 2
strains for group O157 and 1 strain each for O147 and O138.

Fimbrial adhesin type F5 was present in 3 strains from O8 group and in 1
strain from group O149. All 3 strains with present adhesin F6 belonged to group
O8. Out of the 2 strains with concurrent adhesins F4 and F6 one belonged to
group O8 and one to group O138 (Table 1).

Table 1. Pathotypes of *ETEC* strains according to detected somatic O antigen and
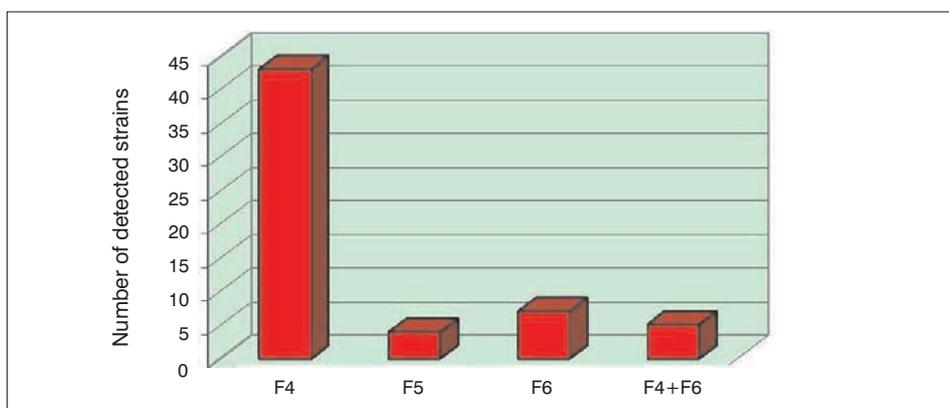fimbrial adhesins isolated from pigs in Serbia in 2010.

ETEC pathotype	O149/ F4	O8/ F4	O157/ F4	O147/ F4	O138/ F4	O139	O8/ F5	O149/ F5	O8/ F6	O8/ F4+F6	O138/ F4+F6
Number of strains	30	4	2	1	1	0	3	1	3	1	2
Heamolysins	0	0	6	2	0	0	0	34	0	0	0

Besides, out of the 57 (38.51%) serologically non standardized strains for
the presence of somatic antigens in 12 (21.05%) fimbrial antigens were detected
(Table 2).

502   **Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.**
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

Table 2. Uknown pathotypes of *ETEC* strains with detected fimbrial adhesins isolated from pigs in Serbia in 2010.

ETEC pathotype	Unknown	Unknown	Unknown
Fimbrial adhesins	F4	F6	F4+F6
Number of strains	5	4	3
Heamolysins	3	0	0

Antigen F4 was detected in 5 strains, F6 in 4 strains and in 3 strains there was a combination of adhesins F4 and F6. Thus, out of the total of 148 tested strains fimbrial antigens were present in 59 (39.86%) strains (Figure 2).

Figure 2. Types of fimbrial adhesins detected in *ETEC* strains isolated from pigs in Serbia in 2010

Haemolytic activity was proven in 42 (46.15%) strains, out of which 34 belonged to group O149, 6 to group O157 and 2 to serogroup O147. In the group of 57 non standardized strains the same activity was present in only 3 strains. Out of the 12 strains with identified fimbrial antigen in 3 with F4 fimbria type the production of haemolysin was proven.

The relatively high percentage (38.51%) of non typified strains can be the result of a number of factors. One of the most significant of which is the limited number of used antisera, the presence of bacterial and viral infections, use of drugs, presence of other factors of virulence, etc. In studies carried out by other authors the percentage of non typified isolates was lower, between 15% (Do *et al*., 2006) and 23% (Chen *et al*., 2008), this being probably the consequence of the larger number of antisera used and/or the application of diagnostic molecular procedures. According to literature data, serogroup O149 is the predominant among the ETEC strains present in Europe and USA, and as much as 40% of all ETEC strains isolated from piglets with diarrhea belongs to this serogroup

Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.
503
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

(Francis, 2002; Docic *et al*., 2003; Vu-Khac *et al*., 2007; Taillon *et al*., 2008). According to the research carried out by Katsude *et al*. in Japan serogroup O149 was detected in 36% of piglets suffering from diarrhea with confirmed ETEC strains. In our study ETEC strains which belong to serogroup O139 were not confirmed, which is in agreement with literature data stating that this serogroup is seldom found in *E coli* strains originating from piglets (Katsuda *et al*., 2006). Similarly, fimbrial adhesins of the F4 (K88) type were predominant in *ETEC* strains isolated from pigs and most often were in *E. coli* O149 (Alexa, 2009; Vidoto, 2009). In Serbia F4 adhesin was most often (in 71.42% of cases) detected in ETEC strains of the O149 serogroup. However, in our study the percentage of detected F5 and F6 adhesins in ETEC strains was extremely low while in European and world regions F5 and F6, as well as F18 and F41 are detected more often. Based upon the findings of Vidotto *et al*. (2009) in Brasil F5 and F6 were recoreded in 30% and 26% *E. coli* strains originating from piglets. The same authors documented the presence of F18 and F41 in 38% and 32% ETEC strain samples, respectively.

The concurrent presence of two types of adhesins in one ETEC strain is not a rare finding and it was documented also in Slovakia in *E.coli* strains isolated from diseased piglets. These strains exibited concomitantly F5 and F41 adhesins (Vu-Khac *et al*., 2004).

Haemolytic activity was shown in 46.15% isolated strains. All *E. coli* haemolitic strains belonged to one of the tested serogroups with adhesins present, thus indicating that the presence of haemolisin in *E. coli* strains can be interpreted as a sign of pathogenicity. It must be emphasized that as the most common etiological agents which cause diarrhea in newborn piglets are Rota and Corona viruses which in over 70% cases are joined to *Cryptosporidia spp* (Katsuda *et al*., 2006). ETEC strains are the primary ethiological agents in less than 20% cases of diarrhea in piglets.

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504   **Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.**
                                              Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
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Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.   505
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IZOLACIJA SOJEVA ETEC OD PRASADI SA DIJAREJOM U NEONATALNOM PERIODU I NJIHOVA TIPIZACIJA NA OSNOVU SOMATSKOG I FIMBRIJALNIH ANTIGENA

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SADRŽAJ

Među različitim patogenima, enterotoksična *E.coli* (ETEC) je već dugo značajan etiološki agens u nastanku oboljenja digestivnog sistema novorođenih životinja. U zemljama sa razvijenom svinjarskom proizvodnjom, uključujući i našu zemlju, dijareja u neonatalnom periodu, uzrokovana sojevima ETEC je jedna od najprisutnijih i ekonomski najznačajnijih bolesti. Cilj istraživanja je bio da se utvrdi prevalencija sojeva ETEC kod prasadi, poreklom sa 5 farmi svinja u Srbiji, kao i nji-

506

Acta Veterinaria (Beograd), Vol. 60, No. 5-6, 497-506, 2010.
Žutić Jadranka *et al*.: Isolation of *ETEC* strains from piglets with diarrhea in
the neonatal period and their typization based on somatic and fimbrial antigens

hova serološka tipizacija na osnovu karakteristika somatskog O antigena, prisu-
stva fimbrijalnih antigena – adhezina i hemolitičke aktivnosti.

Materijal za istraživanja ciljano je uzorkovan od prasadi koja su pokazivala
kliničke znake neonatalne dijareje ili patoanatomske promene karakteristične za
enteritise izazvane sojevima ETEC. Ukupno je izolovano 148 sojeva ETEC od
čega je 91 soj (61,48%) bio tipiziran na osnovu karakteristika somatskog O anti-
gena. Najveći broj sojeva, 42 (46,15%) pripadao je serotipu O149. Serološke
grupe O8 i O147 su bile zastupljene sa po 15 (16,48%) sojeva. Kod 13 (14,28 %)
sojeva, utvrđen je somatski antigen serogrupe O138, a kod 6 (6,59%) sojeva anti-
gen serogrupe O157. Nijedan soj nije aglutinirao sa hiperimunim serumom sero-
grupe O139.

Prisustvo fimbrijalnih adhezina ustanovljeno je kod 47 (51,64 %) sojeva, a
od tog broja F4 tip fimbrijalnih adhezina detektovan je kod 38 (80,85 %) sojeva.
Prisustvo F5 adhezina ustanovljeno je kod 4, F6 kod 3, a kod 2 soja detektovano
je istovremeno prisustvo dva tipa adhezina, F4 i F6.

Najveći broj sojeva, 30 (71,42 %) sa F4 adhezinom pripadao je serogrupi
O149, znatno manji broj, 4 (26,66 %) serogrupi O8, 2 soja serogrupi O157 i po 1
soj serogrupama O147 i O138. Fimbrijalni adhezin tipa F5 otkriven je kod 3 soja
serogrupe O8 i kod 1 soja iz serogrupe O149. Sva 3 soja sa F6 adhezinom pripa-
dala su serogrupi O8. Od 2 soja sa istovremenim prisustvom adhezina F4 i F6, je-
dan je pripadao serogrupi O8, a drugi serogrupi O138. Hemolitička aktivnost je
bila prisutna kod 42 (46,15%) soja, od kojih su 34 soja pripadala serotipu O149, 6
sojeva serotipu O157 i 2 soja serotipu O147.