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Age as a Factor Influencing Diversity of Commensal *E. coli* Microflora in Pigs

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Abstract

Commensal, intestinal *E. coli* microflora plays a role in maintenance of intestinal balance of the host, is responsible for defending against pathogenic *E. coli*. This study encompasses the analysis of BOX-PCR fingerprinting patterns, phylogenetic grouping and virulence genes prevalence among commensal *E. coli* isolates derived from healthy pigs. Altogether, 274 unique *E. coli* isolates were identified, 110 from weaned piglets (Piglets I and Piglets II) and 164 from adult sows (Sows I and Sows II). BOX-PCR analysis distinguished isolates from pigs in different age and indicated that during maturation the changes in *E. coli* microflora occurred. Phylogenetic grouping revealed significant differences between distribution of four phylogenetic groups among isolates derived from piglets and sows. In phylogenetic structure of isolates from the piglets group B1 prevailed significantly, while among isolates derived from the sows the majority of them were classified into phylogenetic group A. The identification of 17 virulence factors in *E. coli* isolates derived from healthy pigs was performed. Three of 13 intestinal (*escV, ehxA*, *estII*) and four extra-intestinal virulence genes (VGs) (*hlyA, fimH, papA, sfaS)* were detected in the porcine isolates. The percentage of VGs positive isolates among piglets is higher than among sows, moreover, the VGs occurring in *E. coli* isolates from piglets revealed greater diversity than that detected among isolates from sows.

Key words: BOX-PCR fingerprinting, commensal *E. coli*, healthy pigs, phylogenetic grouping, virulence genes profiles

Introduction

Piglets are born with sterile gastrointestinal tract, but during the next few hours their guts are colonized by many different microorganisms, which probably derive from skin, teats and feces of the dams (Arbuckle, 1968; Bertschinger *et al.*, 1988). Among the earliest bacteria colonizing the gastrointestinal tract of piglets are *E. coli* (Drasar and Barrow, 1985) considered to be harmless commensals of mammals (Selander *et al.*, 1987). Intestinal population of *E. coli* of pigs was described as very diverse and dynamic (Katouli *et al.*, 1995). Factors such as weaning, age or diet of pigs are reported to be important for differentiation and changes in *E. coli* population (Hinton *et al.*, 1985).

Most strains of *E. coli* are natural components of healthy gut flora (Hartl and Dykhuizen, 1984), but some strains can be a cause of either intestinal or extra-intestinal disease (Kaper *et al.*, 2004). Diarrheas in pigs are often associated with infections by pathogenic *E. coli.* Enterotoxigenic *Escherichia coli* (ETEC) is the most common cause of neonatal, pre- and postweaning diarrhea among piglets and therefore is responsible for economic losses in pig production (Straw *et al.*, 1999; Frydendahl, 2002).

Weaning is a stressful situation for young piglets, it is connected with separation from the sow, change in diet from milk to solid food and with mixing of litters resulting in environmental instability (Spencer and Howell, 1989). It is believed that a highly diverse spectrum of commensal *E. coli* in the intestinal tract is responsible for defending against pathogenic *E. coli* (Kuhn *et al.*, 1993). However, little is still known about the occurrence, individuality, phylogeny and dynamics of commensal *E. coli* isolates from food-producing animal of different age.

Pathogenic *E. coli* strains carry and express virulence genes such as adhesins, hemolysins or enterotoxins. Definite pathotypes and serotypes have a specific set of virulence genes that are crucial for infection and mechanisms of pathogenicity (Kaper *et al.*, 2004). On the other hand, commensal *E. coli* can possess some virulence genes (Chapman *et al.*, 2006; Wu *et al.*, 2007). However, the presence of one or a few virulence genes does not determine the pathogenicity status of the strain until that strain has gained the appropriate

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combination of virulence genes to cause the disease (Gilmore and Ferretti, 2003).

The goal of the present study was to determine if the age of healthy pigs has an influence on the genetic diversity of commensal *E. coli* isolates. The clonal relationships between isolates were characterized using BOX-PCR fingerprinting method, additionally phylogenetic structure was assigned. The purpose of this study was also to determine the occurrence of virulence genes among commensal *E. coli* isolates from healthy pigs.

Experimental

Materials and Methods

Sample collection, isolation and identification of *Escherichia coli* strains. Fresh fecal samples were collected from healthy pigs (two groups of weaning piglets and two groups of sows) living in one farm. The samples were inoculated on agar m-FC and incubated at 44°C for 24 h. The blue colonies were placed on the MacConkey's agar and incubated at 37°C for 24 h. Lactose-positive colonies were identified as a *Escherichia coli* by biochemical IMVC (indol, methyl-red-voges-proskauer, citrate) tests. Bacterial isolates were stored at –80°C and non-passaged bacteria were used in all investigations.

DNA extraction. All isolates were prepared by inoculating of a single colony to 2 ml of LB-medium and incubation at 37°C overnight. DNA was extracted using cell lysis method, 150 µl overnight cultures were centrifuged, pellets were suspended in 25 µl of sterile water and heated at 99°C for 10 min. and transferred into ice for 5 min. Cell debris was pelleted by centrifugation at 18 000 × g for 4 min. The supernatants were the source of DNA used in PCR reactions.

BOX-PCR fingrerprinting. The genotypic analyses of *E. coli* strains were carried out by BOX-A1R primer as described earlier (Versalovic *et al*., 1994). Amplification products were analyzed electrophoretically in 1.5% agarose gel stained with ethidium bromide and then washed in water. Gels were documented as TIFF files and analyzed with Fingerprintig II informatix software (BioRad). The similarity matrix was calculated on the basis of Jaccard similarity coefficient and dendrogram was generated by the unweighted pair group method with arithmetic mean analysis (UPGMA). The similarity relations were expressed as percentage. The similarity of BOX-PCR patterns of the orders of 80% was established as a cut-off value for determination of unique strains.

Phylogenetic grouping. The four major *E. coli* phylogenetic groups (A, B1, B2 and D) were determined as described previously (Clermont *et al*., 2000), by triplex PCR of the *chuA* and *yjaA* genes and the DNA fragment TspE4.C2.

Identification of unique isolates. Among isolates derived from the same probe and revealed 80% similarity of genomic patterns and simultaneously belonged to the same phylogenetic group only one was chosen as a representative.

Virulence genes determination. All isolates were screened for presence of 17 virulence genes: encoding toxins (*stx1*, *stx2*, *eltA*, *estI*, *estII*), hemolysins *(hlyA*, *ehxA)*, nonfimbrial adhesin (*escV*) and fimbrial adhesins (*fimH, papA, sfaS, faeG, fanC, fasA, fedA, f41, bfpB*) associated with different *E. coli* pathotypes were identified by PCR. The primers and multiplex PCR conditions used were previously described (Chapman *et al.*, 2006; Müller *et al.*, 2006). Briefly PCR was performed on MJ Research® PTC-200 thermal cycler with a reaction volume of 25 µl. Reaction mixture contained: buffer solution (Finnzymes); 2,5 mM $MgCl_2$ (Promega); 0,5 mM of each dNTP (Promega); 0,2 µM of each primer; 1 U of DyNAzyme II polymerase (Finnzymes) and 3 µl of DNA template. PCR reactions with template DNA of strain O157:H7 were used as a positive control. PCR mixtures without template DNA were used as a negative control.

DNA sequencing. PCR products of virulence factors were purified using PCR Isolate Kit (Bioline) and sequenced (Genomed) for gene verification. The nucleotide sequences of subject genes were compared with the target sequences in genetic sequence database – GenBank (NCBI).

Statistical analysis. The distributions of the phylogenetic groups of *E. coli* among the isolates and the distributions of the virulence genes were compared by the Chi-squared test (Sneath and Sokal, 1973).

Results

Similarity analysis of isolates. Based on the similarity of BOX-PCR genomic patterns with the use of UPGMA grouping method and the Jaccard similarity coefficient 274 unique *E. coli* isolates were identified (Table I).

The obtained dendrogram grouped 274 strains into 20 clusters (Table II) of a mutual similarity lower than 50%. The particular clusters comprised different numbers of strains.

The created dendrogram of BOX-PCR patterns revealed the presence of 6 clusters formed only or in prevailed number by strains derived from piglets. Clusters VII, X, XII and XIV were formed only by strains derived from piglets. Moreover, cluster VII grouped strains from Piglets I and cluster X only from Piglets II. In clusters VIII and IX strains from piglets pre-

Table I
Number of animals and identified unique *E. coli* isolates in subject groups

Groups of animals/age	Piglets I/ 6 weeks	Piglets II/ 8 weeks	Sows I/ 5 months	Sows II/ 7 months	Total
Number of animals	25	24	25	25	99
Number of *E. coli* isolates	59	51	78	86	274

Table II
Distribution of commensal *E. coli* isolates derived from pigs in different age among the similarity clusters of BOX-PCR dendrogram

Similarity clusters UPGMA	Number of isolates derived from:				
	Piglets I (n = 59)	Piglets II (n = 51)	Sows I (n = 78)	Sows II (n = 86)	Total number of isolates (n = 274)
I	1	1	5	5	12
II	3	–	4	9	16
III	–	–	17	11	28
IV	1	–	10	11	22
V	1	–	2	2	5
VI	1	–	5	2	8
VII	8	–	–	–	8
VIII	14	20	1	1	36
IX	12	11	2	1	26
X	–	7	–	–	7
XI	–	–	3	3	6
XII	1	4	–	–	5
XIII	–	–	2	2	4
XIV	3	1	–	–	4
XV	7	2	2	7	18
XVI	1	–	14	10	25
XVII	–	–	4	2	6
XVIII	–	–	2	9	11
XIX	1	1	3	–	5
XX	1	1	1	2	5
Unclassified isolates	4	3	1	9	17

vailed significantly. 8 clusters gathered only or mainly the strains from sows. Clusters III, XI, XIII, XVII and XVIII grouped strains derived only from sows. Clusters I, IV and XVI encompassed strains from sows in great majority. There are also 6 clusters: II, V, VI, XV, XIX and XX formed by strains derived from both piglets and sows, which indicated the similarity of some isolates of the whole tested set.

Phylogenetic structure determination. A total number of 274 isolates were classified into one of the four main phylogenetic groups A, B1, B2 and D. The analysis of the genetic structure of *E. coli* isolates showed significant differences between strains derived from piglets and sows (Fig. 1). The four phylogenetic groups of *E. coli* were not uniformly distributed ($p < 0.001$) among piglets and sows. Particularly the distribution of groups A and B1 in the groups of piglets and sows was statistically different ($p < 0.001$). Among strains derived from piglets phylogenetic group B1 prevailed significantly (48,2%), groups A and D were represented less frequently (26.3% and 18.2% respectively), whereas strains belonging to group B2 were not numerous (7,3%). The majority of strains derived from sows were classified into phylogenetic group A (54,3%), isolates representing group B1 and D were less numerous (22% and 20, % respectively), while group B2 were the smallest (3%).

The analysis of the phylogenetic structure of *E. coli* strains among individual animals revealed significant difference between piglets and sows groups (Table III). There was no complete phylogenetic structure (A, B1, B2 and D) among *E. coli* isolates in individual piglets. The majority of individuals have strains classified into one phylogenetic group (46.9%), usually B1 or two groups (44.9%), most frequently in combination A+B1. Only 8.2% of individuals indicated the presence of isolates from three phylogenetic groups. However, among sows there was only one individual (2%) with strains revealing complete phylogenetic structure. There were the comparable, numerous representation of sows with strains classified into two (48%), usually in combination A+B1, and three (48%) phylogenetic groups, most frequently in combination A+B1+D. Considerably smaller percentage of adult individuals (2%) revealed the presence of isolates which represented only one phylogenetic group (A) (Fig. 2).

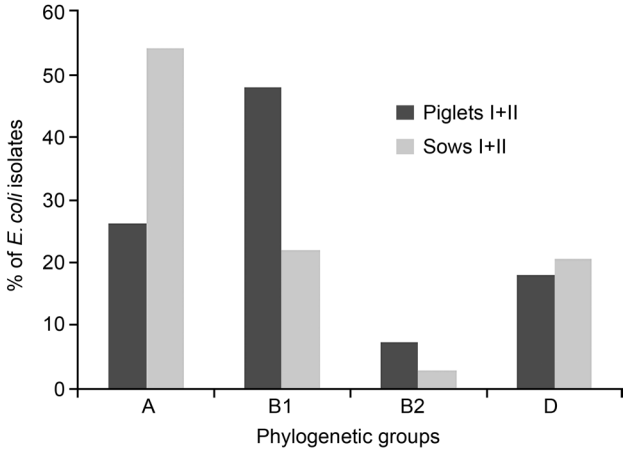

Fig. 1. The phylogenetic structure of *E. coli* isolates derived from piglets and sows

Table III
Phylogenetic structure of *E. coli* isolates with reference to individual animals

Phylogenetic structure	Piglets I					Piglets II				
	N*	Number of isolates n = 59				N*	Number of isolates n = 51			
		A	B1	B2	D		A	B1	B2	D
A	4	5	–	–	–	1	2	–	–	–
B1	1	–	2	–	–	10	–	16	–	–
B2	1	–	–	2	–	1	–	–	1	–
D	3	–	–	–	6	2	–	–	–	2
A+B1	8	12	10	–	–	7	7	10	–	–
A+D	1	1	–	–	1	0	–	–	–	–
B1+D	4	–	5	–	5	2	–	6	–	3
A+B1+D	1	1	1	–	1	0	–	–	–	–
A+B2+D	0	–	–	–	–	0	–	–	–	–
A+B1+B2	1	1	1	2	–	0	–	–	–	–
B1+B2+D	1	–	1	1	1	1	–	1	2	1
A+B1+B2+D	0	–	–	–	–	0	–	–	–	–
Total	25	20	20	5	14	24	9	33	3	6
Phylogenetic structure	Sows I					Sows II				
	N*	Number of isolates n = 78				N*	Number of isolates n = 86			
		A	B1	B2	D		A	B1	B2	D
A	0	–	–	–	–	2	2	–	–	–
B1	0	–	–	–	–	0	–	–	–	–
B2	0	–	–	–	–	0	–	–	–	–
D	0	–	–	–	–	0	–	–	–	–
A+B1	7	9	8	–	–	7	12	9	–	–
A+D	7	10	–	–	10	3	4	–	–	3
B1+D	0	–	–	–	–	0	–	–	–	–
A+B1+D	10	12	12	–	13	10	18	11	–	10
A+B2+D	1	2	–	1	1	3	6	–	3	4
A+B1+B2	0	–	–	–	–	0	–	–	–	–
B1+B2+D	0	–	–	–	–	0	–	–	–	–
A+B1+B2+D	0	–	–	–	–	1	1	1	1	1
Total	25	33	20	1	24	25	43	21	4	18

N*– number of individuals

Distribution of virulence genes. The identification of 17 virulence genes (VGs) in *E. coli* isolates derived from healthy pigs was performed. All *E. coli* isolates were analyzed for the presence of VGs characteristic for intestinal pathotypes (EHEC: *stx1*, *stx2*, *escV*, *ehxA*; EPEC: *bfpB*, *escV*, *ehxA*; ETEC: *estI*, *estII*, *eltA*, *faeG*, *fanC*, *fasA*, *fedA*, *f41*) and that connected with extra-intestinal pathotypes (ExPEC: *hlyA*, *fimH*, *papA*, *sfaS*). Three of 13 intestinal (*escV*, *ehxA*, *estII*) and four extra-intestinal VGs (*hlyA*, *fimH*, *papA*, *sfaS*) were detected in the porcine isolates. Among the VGs positive isolates, seven different VGs profiles were identified. There were one to three VGs in the single *E. coli* strain (Table IV). The analyzes also revealed the common occurrence of *fimH* gene in isolates derived from healthy pigs (not presented in table), as reported previously (Chapman *et al*., 2006; Wu *et al*., 2007).

There were significant differences between VGs profiles characteristic for isolates from piglets and sows. Only two profiles: *escV* and *papA* occurred simultaneously in isolates from piglets and sows groups. The profiles: *sfaS*, *escV+ ehxA, hlyA+ papA* were assigned only to isolates from piglets and *estII, escV+estII+papA* were detected only among isolates from sows. The number of VGs positive isolates was statistically higher in the piglets then in the sows ($p < 0.001$) and it amounts frequency 23.6% for piglets and 7.3% for sows. The diversity of virulence genes in the set of isolates from

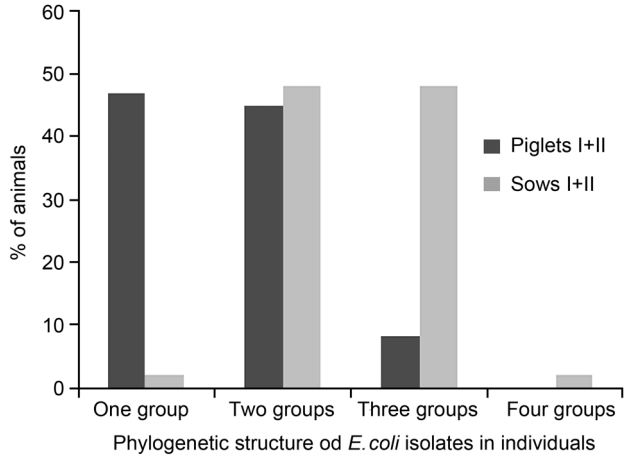

Fig. 2. The phylogenetic structure of *E. coli* isolates derived from individual animals with reference to number of phylogenetic groups

piglets was higher (5 different genes: *escV, ehxA, hlyA, papA, sfaS*) than among isolates from sows (3 genes: *escV, estII, papA*). In both groups of animals the isolates with extra-intestinal virulence genes were more frequent (Table V), but the number of extra-intestinal VGs positive isolates was significantly higher in the piglets than in the sows ($p < 0.02$). Among all piglets there were 11 isolates with *papA*, 2 with *sfaS* and 2 with combination *hlyA+papA* genes, summing up, this gives 15 (13.6%). In the sows there were 7 isolates with *papA* and 1 with combination *papA+escV+estII* genes, which gives 8 (4.9%) altogether. The isolates that possess intestinal VGs were less frequent in both groups of animals, while the number of intestinal VGs positive strains was significantly higher in the piglets than in the sows ($p < 0,02$). Among piglets 8 isolates with *escV* gene and 3 with *escV+ ehxA* genes were identified, it gives 11 (10%) altogether. 1 isolate with *escV*, 3 with *estII* and 1 combination of *escV+estII+papA* genes were detected in the sows group, summing up, this gives 5 (3%). Moreover, VGs were carried by isolates of phylogenetic groups A, B1, B2 and D among piglets, while VGs positive isolates derived from sows were classified into groups A, B1 and D.

Discussion

The genetic diversity of clinical *E. coli* isolates derived from neonatal or postweaning diarrhea cases has beeen widely characterized in the literature (Wang *et al*., 2011; Bruant *et al*., 2009; Blanco *et al*., 2006), whereas data concerning the diversity of commensal, porcine *E. coli* are rather rare (Dixit *et al*., 2004; Schierack *et al*., 2006).

Table IV
The prevalence of VGs profiles among isolates from subject groups of pigs

VGs profiles	Number and phylogenetic group of isolates derived from					
	Piglets			Sows		
	I n = 59	II n = 51	Total no. of VGs-positive isolates n = 110	I n = 78	II n = 86	Total no. of VGs-positive isolates n = 164
escV	1A, 4B1, 3D	–	8	–	1A	1
estII	–	–	–	1A	1A, 1D	3
papA	2B1	6B1, 2B2, 1D	11	1B1, 1D	3B1, 2D	7
sfaS	2B2	–	2	–	–	–
escV, ehxA	1B2	2D	3	–	–	–
hlyA, papA	–	1B1, 1B2	2	–	–	–
escV, estII, papA	–	–	–	–	1D	1
Total no. of VGs-positive isolates (%)	13 (22)	13 (25,5)	26 (23,6)	3 (3,8)	9 (10,5)	12 (7,3)

Table V
The prevalence of intestinal and extra-intestinal VGs among isolates from subject groups of pigs

Virulence genes	Number of isolates with virulence genes (%)					
	Piglets			Sows		
	I n = 59	II n = 51	Total n = 110	I n = 78	II n = 86	Total n = 164
Intestinal (escV, ehxA, estII)	9 (15,3)	2 (3,9)	11 (10)	1 (1,3)	4 (4,7)	5 (3)
Extra-intestinal (*hlyA*, *papA*, *sfaS*)	4 (6,8)	11 (21,6)	15 (13,6)	2 (2,6)	6 (7)	8 (4,9)
Total (%)	13 (22)	13 (25,5)	26 (23,6)	3 (3,8)	9 (10,5)	12 (7,3)

It seems very important to gain more detailed knowledge about genetic structure and possession of VGs among commensal isolates derived from pigs in different age, this may help understand why young piglets are often susceptible to diarrhea caused by pathogenic *E. coli*. This study encompassed the complex analysis of genetic diversity of commensal *E. coli* isolates derived from healthy, weaned (Piglets I and Piglets II) and finished grow pigs (Sows I and Sows II). The diversity of porcine *E. coli* isolates was expressed by polymorphism of BOX-PCR patterns, phylogenetic grouping, phylogenetic structure of isolates among individual animals and identification of virulence profiles.

BOX-PCR fingerprinting analysis has shown closer relationship between strains grouped in common clusters and simultaneously the differences between isolates derived from piglets and sows which grouped mainly in separate clusters. In general, analysis of polymorphism of genomic patterns distinguished strains from pigs in different age and indicated that during growing up the changes in *E. coli* microflora occurred.

Phylogenetic grouping revealed significant differences between distribution of four phylogenetic groups among isolates derived from piglets and sows. These results confirm earlier reports concerning the phylogenetic structure of isolates from healthy piglets with predominance of group B1 (Bibbal *et al*., 2009). It was also previously described (Cortés *et al*., 2010; Carlos *et al*., 2010) that the most commensal strains in sows belonged to group A. In present studies differences between phylogenetic structure of isolates from the particular individual animals were analyzed. Among isolates from individual piglets the simple phylogenetic structure consisted of one or two predominating groups. A more complex phylogenetic structure, encompassing two or three groups, was characteristic for the individual sows.

BOX-PCR fingerprinting as well as phylogenetic grouping analysis indicated on significant genomic diversity between isolates derived from piglets and sows, but on the another level. Genomic patterns and phylogenetic structure diversities do not correspond to each other but increase genomic characterization of tested isolates. Both these analyses confirmed that the age of the host has a considerable influence on the genomic diversity of isolates. In the present studies the analysis of phylogenetic structure of isolates in particular individuals revealed lower diversity in piglets than in sows. It was stated previously by another method (biochemical fingerprinting) that among young animals *E. coli* flora is not diverse (Katouli *et al*., 1995).

This study encompasses the analysis of occurrence of 17 VGs among commensal, porcine isolates. Among piglets, isolates carrying genes associated with the EPEC/EHEC pathotypes (*escV* and *escV+ehxA*) as well as these characteristic for ExPEC (*papA, sfaS* and *hlyA+papA)* were identified. The occurrence of various combinations of these genes and the same virulence profiles among strains belonging to different phylogenetic groups is consistent with horizontal transfer of genetic elements harboring these VGs. In sows isolates, which carry VGs associated with profile characteristic for ETEC pathotypes (*estII gene*), and for ExPEC (*papA gene*), were identified. Moreover, among sows the isolate possesses the profile *escV+estII+papA* which is the combination of genes characteristic for the EPEC/EHEC, ETEC and ExPEC pathotypes were also detected. The appearance of such a combination and occurrence of these genes among isolates belonged to separate phylogenetic groups confirmed that prevalence of these genes is connected with horizontal transfer. Two profiles: *escV* and *papA* occurred simultaneously in isolates from piglets and sows groups and some isolates carrying these genes belong to the same phylogenetic groups, which indicated the possibility of transmission of these isolates from sows to their litters.

It was reported (Johnson and Stell, 2000) that strains belonging to phylogenetic groups B2 and D and carry *hlyA* and *papA* genes cause extra-intestinal disease. One of the identified VGs positive isolates, has a combination of *hlyA+papA* and belongs to B2 group.

Significant differences between isolates derived from piglets and sows were showed. The percentage of VGs positive isolates among piglets is higher than among sows, moreover, also the VGs occurring in *E. coli* isolates from piglets revealed greater diversity than those detected among isolates from sows. These results indicated that with the evolving of the host organisms there appeared some changes in the genetic structure of commensal *E. coli*. Piglets during growing up lose strains which constitute a reservoir of the virulence genes, therefore among sows VGs positive isolates were detected less frequently but still maintained.

In this study comparative analysis of fingerprinting genomic patterns, phylogenetic grouping and virulence profiles of commensal porcine isolates, in the aspect of the age of pigs, were carried out. This complex analysis confirmed, as was previously partially reported (Katouli *et al*., 1995), that age is an important factor influencing the diversity of commensal microflora among pigs. It was stated that simple phylogenetic structure of commensal *E. coli* derived from piglets occurred in pair with more complex genetic diversity among separate phylogenetic groups.

There is also some evidence that weaning is the key moment in the piglets life, it is connected with stress during separation from the dam and with changes in diet from milk to solid food, which cause environmental instability (Spencer and Howell, 1989). It is obvious that environmental stabilization and physiological

development of the intestinal tract influenced the increase of the diversity of the natural microflora (Pielou, 1975), as the piglets aged. The great diversity of *E. coli* strains colonizing the gut epithelium guarantee a competitive barrier against enteric pathotypes (Dixit *et al*., 2004; Wu *et al*., 2007).

Acknowledgments

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