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CONJUGATIVE IncF AND IncI1 PLASMIDS WITH *tet*(A) AND CLASS 1 INTEGRON CONFERRING MULTIDRUG RESISTANCE IN F18$^+$ PORCINE ENTEROTOXIGENIC *E. COLI*

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Enterotoxigenic *E. coli* (ETEC) bacteria frequently cause watery diarrhoea in newborn and weaned pigs. Plasmids carrying genes of different enterotoxins and fimbrial adhesins, as well as plasmids conferring antimicrobial resistance are of prime importance in the epidemiology and pathogenesis of ETEC. Recent studies have revealed the significance of the porcine ETEC plasmid pTC, carrying tetracycline resistance gene *tet*(B) with enterotoxin genes. In contrast, the role of *tet*(A) plasmids in transferring resistance of porcine ETEC is less understood. The objective of the present study was to provide a comparative analysis of antimicrobial resistance and virulence gene profiles of porcine post-weaning ETEC strains representing pork-producing areas in Central Europe and in the USA, with special attention to plasmids carrying the *tet*(A) gene. Antimicrobial resistance phenotypes and genotypes of 87 porcine ETEC strains isolated from cases of post-weaning diarrhoea in Austria, the Czech Republic, Hungary and the Midwest USA was determined by disk diffusion and by PCR. Central European strains carrying *tet*(A) or *tet*(B) were further subjected to molecular characterisation of their *tet* plasmids. Results indicated that > 90% of the ETEC strains shared a common multidrug resistant (MDR) pattern of sulphamethoxazole (91%), tetracycline (84%) and streptomycin (80%) resistance. Tetracycline resistance was most frequently determined by the *tet*(B) gene (38%), while *tet*(A) was identified in 26% of all isolates with wide ranges for both *tet* gene types between some countries and with class 1 integrons and resistance genes co-transferred by conjugation. The virulence gene profiles included enterotoxin genes *(lt*, *sta* and/or *stb)*, as well as adhesin genes *(k88/f4, f18)*. Characterisation of two representative *tet*(A) plasmids of porcine F18$^+$ ETEC from Central Europe revealed that the IncF plasmid (pES11732) of the Czech strain (~120 kb) carried *tet*(A) in association with *catA1* for chloramphenicol resistance. The IncI1 plasmid (pES2172) of the Hungarian

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strain (~138 kb) carried *tet*(A) gene and a class 1 integron with an unusual variable region of 2,735 bp composed by two gene cassettes: *estX-aadA1* encoding for streptothricin-spectinomycin/streptomycin resistance exemplifying simultaneous recruitment, assembly and transfer of multidrug resistance genes by the *tet*(A) plasmid of porcine ETEC. By this we provide the first description of IncF and IncI1 type plasmids of $F18^+$ porcine enterotoxigenic *E. coli* responsible for co-transfer of the *tet*(A) gene with multidrug resistance. Additionally, the unusual determinant *estX,* encoding for streptothricin resistance, is first reported here in porcine enterotoxigenic *E. coli*.

Key words: Enterotoxigenic *Escherichia coli*, tetA plasmid, multiresistance, antimicrobial resistance, virulence

Severe watery diarrhoea of newborn and weaned pigs as well as of newborn calves is often caused by enterotoxigenic *Escherichia coli* (ETEC) strains (Nagy and Fekete, 2005). ETEC infection often leads to traveller's diarrhoea among humans and may cause significant morbidity and mortality of children in the developing countries (Qadri et al., 2005). Due to the pathogenic similarities of ETEC infections in animals and humans, ETEC has been the subject of intensive studies in human and veterinary medicine over the past three decades.

In weaned pigs two main types of virulence factors are essential for the development of diarrhoea caused by ETEC: bacterial attachment to the mucosal surface of the small intestine mediated by fimbrial adhesins (i.e. K88/F4 and/or F18ac), and enterotoxins – heat-labile toxin (LT) and/or heat-stable toxins (STa, STb) – changing the absorptive function of the small intestine to a secretory function leading to diarrhoea and loss of water and electrolytes.

Genes encoding the above virulence factors are located on ETEC virulence plasmids (Gyles, 1994; Fekete et al., 2012); however, several other mobile virulence elements such as pathogenicity islands (PAIs), bacteriophages and transposons may also contribute to the transfer of virulence genes between *E. coli* strains (Hacker et al., 1997). As an example, heat-stable enterotoxin genes *sta* and *stb* are part of transposons: *sta* is carried by Tn*1681* (So and McCarthy, 1980), and *stb* was described as a part of Tn*4521* (Hu and Lee, 1988). The spread of the heat-labile toxin gene *lt* is accomplished by IS-mediated transfer mechanisms (Schlör et al., 2000).

In our previous studies on the representative porcine post-weaning $F18^+$ ETEC strain 2173 we described and sequenced the large conjugative plasmid pTC (sta^+, stb^+, $tetB^+$) as being responsible for the enterotoxigenicity and tetracycline resistance [*tet*(B)] of the host strain (Nagy et al., 1990; Fekete et al., 2012). Among *Enterobacteriaceae*, tetracycline resistance is encoded mainly by tetracycline efflux proteins that remove tetracycline from the bacterial cytoplasm by a process most often encoded by *tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), and *tet*(G) genes. Tetracycline resistance genes are often located on mobile elements

such as plasmids, transposons and/or conjugative transposons, which can be transferred between bacterial species (Chopra and Roberts, 2001; Michalova et al., 2004). Studies on tetracycline resistance mechanisms are justified by data about tetracyclines being the far most frequently used antimicrobials in the EU (Schwarz and Chaslus-Dancla, 2001; Moulin et al., 2008; EMA, 2014).

In addition to our above studies on the pTC plasmid of F18$^+$ ETEC carrying *tet*(B), we intended to study the prevalence and genetic associations of *tet*(B) and *tet*(A) with emphasis on characterisation and transfer of the less well explored *tet*(A) plasmids of porcine ETEC. Therefore, in this study we aimed to provide a comparative description of antimicrobial resistance and virulence profiles of porcine post-weaning ETEC strains representing the modern pig industry of different geographical regions including Hungary, Austria, the Czech Republic and the USA. Furthermore, the characterisation of genetic vectors for multidrug resistance and tetracycline resistance in this collection was also attempted, with special regard to *tet*(A).

Materials and methods

Bacterial strains

The porcine post-weaning ETEC strains studied here had been isolated and identified between 1987 and 1995 from cases of post-weaning diarrhoea, representing 8–12 farms/country in three neighbouring countries: Hungary (n = 16), Austria (n = 34) and the Czech Republic (n = 17). The Central European collection was complemented with 20 comparable ETEC strains originating from the Midwestern United States (Midwest USA). Selected properties of some Hungarian and some US strains have been reported previously (Fekete et al., 2003; Olasz et al., 2005). All ETEC strains were stored at –80 °C in Tryptic Soy Broth (TSB) complemented with 10% glycerol.

Detection of antimicrobial resistance phenotype and identification of tetracycline resistance genes

The antimicrobial resistance phenotype was tested by disc diffusion assay against 18 antimicrobial compounds (Oxoid) with clinical relevance. These were as follow (lower indices indicate disc concentration in μg/ml): amoxicillin (AMX_{25}), ampicillin (AMP_{10}), cefotaxime (CTX_{30}), chloramphenicol (CHL_{30}), enrofloxacin (ENR_5), erythromycin (ERY_{10}), florfenicol (FFC_{30}), gentamicin (GEN_{10}), kanamycin (KAN_{30}), nalidixic acid (NAL_{30}), rifampicin (RIF_5), spectinomycin (SPE_{100}), streptomycin (STR_{10}), sulphamethoxazole (SMX_{25}), tetracycline (TET_{30}), trimethoprim (TMP_5), and trimethoprim-sulphamethoxazole ($SXT_{1.25/23.75}$). Interpretation of the data was performed according to the Clinical and Laboratory Standard Institute (CLSI) guidelines and interpretive standards

(CLSI, 2013). ETEC strains with intermediate zone diameter values were considered susceptible. The *E. coli* reference strain ATCC 25922 was used as control. Multidrug resistance (MDR) of strains was defined as co-resistance to three or more antimicrobial classes.

ETEC strains of tetracycline resistant phenotype were subjected to PCR-based typing of the *tet* gene, using primers to detect the common *tet* genes of *Enterobacteriaceae*, as listed in Table 1.

Identification and characterisation of ETEC plasmids mediating tetracycline resistance

In order to characterise plasmids for tetracycline resistance, and to detect the possible co-transfer of tetracycline resistance genes and those encoding typical ETEC virulence factors *(sta*, *stb*, *elt, f18, k88/F4)*, a total of 8 *tet*(A) and 12 *tet*(B) representative ETEC strains from Austria, the Czech Republic and Hungary were selected for conjugation experiments.

Conjugations were performed by using the plasmid-free, rifampicin-resistant *E. coli* K12 J5-3 strain as recipient. Overnight LB broth cultures of the parental ETEC strains and the recipient cells were mixed at a ratio of 1:1 and plated onto Luria-Bertani (LB) agar plates. On the subsequent day the bacterial lawn was dissolved in 5 ml phosphate-buffered saline (PBS), and tenfold dilutions were made up to 10^{-7}. Selection of the transconjugants was carried out on LB agar plates complemented with the combination of tetracycline (50 μg/ml) and rifampicin (150 μg/ml). Conjugation frequency was calculated as a ratio between the number (CFU/ml) of transconjugants and that of the recipient strain J5-3. ETEC strain 2173 served as a reference for the transferability of the *tet*(B)-mediating plasmid pTC (Fekete et al., 2012).

Plasmid profile analysis and replicon typing

Parental and transconjugant strains representing successful transfer of the *tet*(A) and *tet*(B) genes were subjected to plasmid profile analysis. Plasmids were prepared according to the alkaline lysis method of Kado and Liu (1981). Separation of non-digested plasmids was performed in 0.7% agarose gel in a vertical system with TBE buffer at 180 V. The DNA was stained with 0.5 μg/ml of ethidium bromide. Plasmid sizes were estimated in comparison with plasmid markers (2.1–168 kb) isolated from *E. coli* strains V517 and MD112, respectively. Due to our main focus on plasmids carrying the *tet*(A) gene in $F18^+$ ETEC strains, further analyses were done on two *tet*(A)-positive monoplasmidic transconjugant strains (2172/11 and 11732/11), derived from a Hungarian and a Czech isolate, respectively. Plasmid incompatibility (Inc) groups were determined by PCR-based replicon typing (PBRT) using primers and conditions developed by Carattoli et al. (2005) and García-Fernández et al. (2009).

Detection of antimicrobial resistance and virulence genes

Parental and transconjugant strains were tested by PCR for the presence of antimicrobial resistance genes related to mobile genetic elements and typical ETEC virulence factors, including the flanking regions of the *stb* gene specific to the pTC plasmid of F18$^+$ ETEC 2173 (Fekete et al., 2012). Genes *intI1*, *qacEΔ1* and *sul1* as parts of the 3' conserved region of the class 1 integron were also tested by PCR. Primers used for the above PCR testing are presented in Tables 1 and 2. To reveal whether the *tet*(A) gene is located on the transposon Tn*1721*, the primer TetAR3: 5'-GGCATAGGCCTATCGTTTCCA-3' was used (Hartman et al., 2003).

Characterisation of class 1 integron carried by the tet*(A) plasmid*

The variable region of the class 1 integron detected in the Hungarian monoplasmidic *tet*(A) transconjugant strain 2172/11 was amplified with primers 5'CS-F1 and 3'CS-R (Table 1). In order to identify the gene cassette array of the variable region, the PCR product was purified with Qiagen PCR Purification Kit (Qiagen) and sequenced with the primers listed in Table 1. Nucleotide sequences were analysed in comparison with the NCBI database using the BLASTN algorithm. The resulting fragment of 2,735 bp identified in the Hungarian strain 2172/11 was deposited in GenBank under accession number JQ313793.

Results

Antimicrobial resistance phenotype of porcine ETEC strains

Based upon the results of antimicrobial susceptibility testing, an overwhelming majority of the 87 ETEC strains tested (94.3%) were considered to be multidrug resistant (MDR), showing co-resistance to at least three antimicrobial classes. The distribution of antimicrobial resistance phenotypes among ETEC strains from Hungary, the Czech Republic, Austria and the USA is presented in Fig. 1. In general, the prevalence of resistance was lower in ETEC strains from the Central European countries, as compared to those from the USA. The majority of the strains shared a common MDR backbone, most frequently being resistant to sulphamethoxazole (91%), tetracycline (84%), erythromycin (84%), and streptomycin (79%). Resistance to spectinomycin (44%), rifampicin (36%) and amoxicillin (32%) was also detected independently of the geographical origin of the strains (Fig. 1). Concerning resistance to kanamycin and gentamicin, ETEC strains from the Czech Republic were highly susceptible to these drugs, while kanamycin resistance was most frequently detected among strains from the USA and Hungary (90% and 81%, respectively). Besides, Hungarian strains showed reduced resistance to gentamicin, chloramphenicol, and ampicillin (13–19%).

Table 1

Primers used for the detection of antimicrobial resistance genes and for the characterisation of class 1 integrons

Antimicrobial family and genes	Primer	Sequence (5'→3')	Amplicon (bp)	Method	Reference
Tetracyclines					
tet(A)	tetA f	GGCCTCAATTTCCTGACG	372	PCR	Guillaume et al., 2000
	tetA r	AAGCAGGATGTAGCCTGTGC			
tet(B)	tetB f	GAGACGCAATCGAATTCGG	228	PCR	Guillaume et al., 2000
	tetB r	TTTAGTGGCTATTCTTCCTGCC			
tet(C)	tetC f	TCCTTGCATGCACCATTCC	635	PCR	Guillaume et al., 2000
	tetC r	AACCCGTTCCATGTGCTCG			
tet(D)	tetD f	GGATATCTCACCGCATCTGC	436	PCR	Guillaume et al., 2000
	tetD r	CATCCATCCGGAAGTGATAGC			
tet(E)	tetE f	TCCATACGCGAGATGATCTCC	442	PCR	Guillaume et al., 2000
	tetE r	CGATTACAGCTGTCAGGTGGG			
tet(G)	tetG f	GCTCGGTGGTATCTCTGCTC	468	PCR	Frech and Schwarz, 2000
	tetG r	AGCAACAGAATCGGGAACAC			
Aminoglycosides					
aacC2	aacC2 f	GGCAATAACGGAGGCAATTCGA	698	PCR	Frana et al., 2001
	aacC2 r	CTCGATGGCGACCGAGCTTCA			
aacA4	aac(6')Ib f	GTTACTGGCGAATGCATCACA	217	PCR	Frana et al., 2001
	aac(6')Ib r	TGTTTGAACCATGTACACGGC			
aadB	aadB1 fw	GTTGGACTATGGATTCTTAGC	248	PCR	This study
	aadB1 rv	GCCTGTAGGACTCTATGTG			
aadA	aadA fw	GTACGGCTCCGCAGTGGATGG	193	PCR	This study
	aadA rv	GATGATGTCGTCATGCACG		PCR/SQ	
strA	strA fw	CCTGGTGATAACGGCAATTC	546	PCR	Rosengren et al., 2009
	strA rev	CCAATCGCAGATAGAAGGC			
strB	strB fw	ATCGTCAAGGGATTGAAACC	509	PCR	Rosengren et al., 2009
	strB rev	GGATCGTAGAACATATTGGC			

β-lactams					
bla$_{CTX-M}$	CTX-M f	CGATGTGCAGTACCAGTAA	585	PCR	Batchelor et al., 2005
	CTX-M r	TTAGTGACCAGAATCAGCGG			
bla$_{TEM}$	TEM f	CATTTTCGTGTCGCCCTTAT	793	PCR	Hopkins et al., 2007
	TEM r	TCCATAGTTGCCTGACTCCC			
bla$_{SHV}$	SHV f	ATTTGTCGCTTCTTTACTCGC	1018	PCR	Yagi et al., 2000
	SHV r	TTTATGGCGTTACCTTTGACC			
Phenicols					
catA1	catI f	AGTTGCTCAATGTACCTATAACC	680	PCR	Rosengren et al., 2009
	catI r	TTGTAATTCATTAAGCATTCTGCC			
floR	floR f	CGCCGTCATTCCTCACCTTC	888	PCR	Rosengren et al., 2009
	floR r	GATCACGGGCCACGCTGTGTC			
cmlA	cmlA f	TTGCAACAGTACGTGACAT	293	PCR	Rosengren et al., 2009
	cmlA r	ACACAACGTGTACAACCAG			
Class 1 integron-related					
intI1	intI1 f	GGGTCAAGGATCTGGATTTCG	483	PCR	Mazel et al., 2000
	intI1 r	ACATGGGTGTAAATCATCGTC			
qacEΔ1	qac F	GGCTGGCTTTTTCTTGTTATCG	273	PCR	Mazel et al., 2000
	qac R	TGAGCCCCATACCTACAAAGC		PCR/SQ	
sul1	sul1 f	TGGTGACGGTGTTCGGCATTC	789		Sáenz et al., 2004
	sul1 r	GCGAGGGTTTCCGAGAAGGTG			
Variable region					
	3CS-R	GGAATTCGACCTGATAGTTTGGCTGTG		PCR	
	sqpr 1 fw	CCTTGCCCTCCCGCACGATG		SQ	This study
	sqpr 2 rv	CACCACACCGCAGACGACATT		SQ	This study
	sqpr 3 fw	TGGCGAATCAACTCAGGTACTG		SQ	This study
	sqpr 4 fw	CAGAGGTAGTTGGCGTCATC		SQ	This study
	sqpr 5 fw	AAGGATGTCGCTGCCGACTG		SQ	This study

SQ: primers used for sequencing

Table 2

Primers used for the detection of virulence genes and corresponding flanking regions

Gene/ Region	Primer	Sequence (5'→3')	Amplicon (bp)	Reference
sta	sta fw sta rev	TTTCTGTATTATCTTTCCCC ATTACAACAAAGTTCACAGC	167	Alexa et al., 1997
stb	stb fw stb rev	TCTTCTTGCATCTATGTTCG TCTCTAACCCCTAAAAAACC	138	Alexa et al., 1997
stbfl 5'	is1 rev stb rev	ACAGCGACTTCCGTCCCAGCC TCTCTAACCCCTAAAAAACC	987	Alexa et al., 1997
lt	lt fw lt rev	TTACGGCGTTACTATCCTCTCTA GGTCTCGGTCAGATATGTGATTC	274	Alexa et al., 1997
f18	f18 fw f18 rev	GTGAAAAGACTAGTGTTTATTTC CTTGTAAGTAACCGCGTAAGC	511	Imberechts et al., 1994
k88	k88 fw k88 rev	GGTGATTTCAATGGTTCGGTC AATGCTACGTTCAGCGGAGCG	764	Alexa et al., 1997
fedA	f18 fw f18 rev	GTGAAAAGACTAGTGTTTATTTC CTTGTAAGTAACCGCGTAAGC	511	Imberechts et al., 1994
fanA	fanA fw fanA rev	AATACTTGTTCAGGGAGAAA AACTTTGTGGTTAACTTCCT	230	Boerlin et al., 2005
fasA	fasA fw fasA rev	GTAACTCCACCGTTTGTATC AAGTTACTGCCAGTCTATGC	409	Boerlin et al., 2005
east1	astA fw astA rev	TCGGATGCCATCAACACAGT GTCGCGAGTGACGGCTTTGTAAG	125	Boerlin et al., 2005
paa	paa fw paa rev	GGCCCGCATACAGGCCTTG TCTGGTCAGGTCGTCAATACTC	282	Boerlin et al., 2005
aidA-I	AIDA fw AIDA rev	ACAGTATCATATGGAGCCA TGTGCGCCAGAACTATTA	585	Boerlin et al., 2005
sepA	sepA fw sepA rev	TAAAACCCGCCGCCTGAGTA TGCCGGTGAACAGGAGGTTT	611	Boerlin et al., 2005

ETEC strains from Austria showed the highest rate of chloramphenicol resistance (53%), while 50% of the US strains were resistant to ampicillin (Fig. 1). The mean prevalence of resistance to trimethoprim and nalidixic acid was relatively low (18% and 15%, respectively), while all strains were susceptible to cefotaxime, enrofloxacin, and florfenicol (Fig. 1).

Distribution of the tet *gene types and their combinations among the ETEC strains*

ETEC strains with tetracycline-resistant phenotype were tested by PCR to identify the *tet* gene types [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G)] represent-

ing the most frequently described efflux pump encoding genes in *Enterobacteriaceae*. The *tet*(B) was the most frequently found gene conferring tetracycline resistance in 38% of ETEC strains, while the *tet*(A) gene was identified in 26% of the strains (Fig. 2). Only a few strains showed the co-existence of the above two *tet* gene types. The combination of *tet*(A)/*tet*(B), *tet*(B)/*tet*(C) and of *tet*(B)/*tet*(D) genes averaged 3%, 1% and 1%, respectively. In 31% of the strains the tetracycline-resistant phenotype was not confirmed by any of the *tet* genes tested (Fig. 2), indicating resistance mechanisms encoded by some of the less frequent tetracycline resistance genes other than those listed in Table 1.

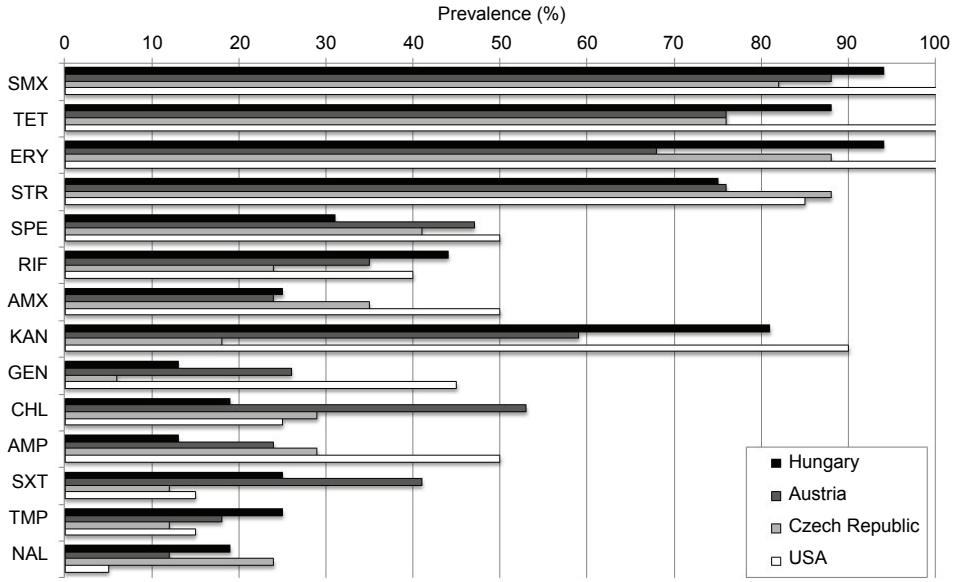

Fig. 1. Distribution (%) of antimicrobial resistance phenotypes among ETEC strains from Hungary, Austria, the Czech Republic and the USA. Antimicrobial compounds are abbreviated as follows: SMX, sulphamethoxazole; TET, tetracycline; ERY, erythromycin; STR, streptomycin; SPE, spectinomycin; RIF, rifampicin; AMX, amoxicillin; KAN, kanamycin; GEN, gentamicin; CHL, chloramphenicol; AMP, ampicillin; SXT, trimethoprim-sulphamethoxazole; TMP, trimethoprim; NAL, nalidixic acid

Distribution of the *tet* gene types varied according to geographical origin. Hungarian and Austrian strains were characterised by a prevalence of 38% and 21% of the *tet*(A) gene and a prevalence of 25% and 32% of the *tet*(B) gene, respectively. In contrast, most strains from the Czech Republic carried the *tet*(A) gene (59%). The US strains were characterised by the predominance of the *tet*(B) gene (65%) as shown in Fig. 2. No ETEC strains tested carried *tet*(E) or *tet*(G) genes.

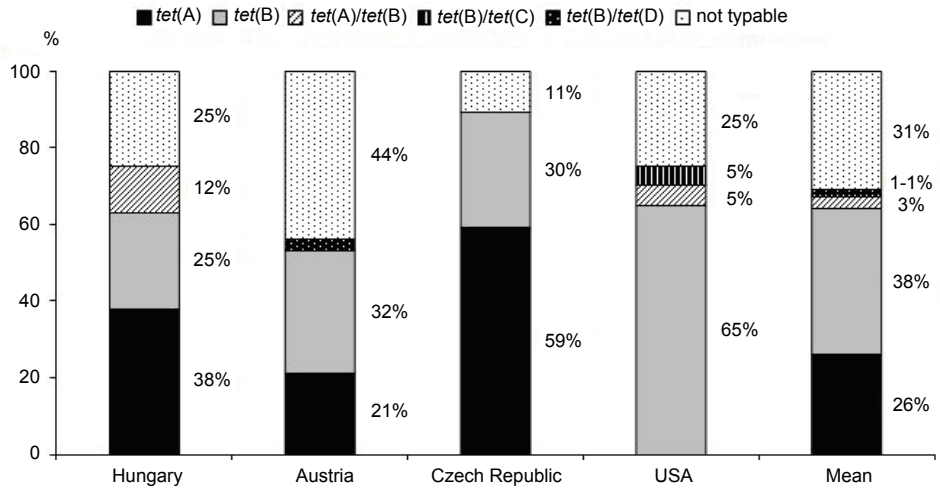

Fig. 2. Distribution (%) of *tet* gene types and their combinations among ETEC strains from Hungary, Austria, the Czech Republic and the USA

Plasmid profiles and self-conjugative plasmids of tetracycline-resistant ETEC strains

Out of the 8 *tet*(A) and 12 *tet*(B) ETEC strains the conjugative transfer of the *tet*(A) plasmids was successful in 2 of the 8 *tet*(A) strains selected. One was an $F18^+$ ETEC strain from Hungary (2172), and the other was an $F18^+$ ETEC strain from the Czech Republic (11732), resulting in two *tet*(A)-positive transconjugant strains designated as 2172/11 and 11732/71, respectively. The transfer of *tet*(B) plasmids resulted in 6 Austrian *tet*(B) strains out of a total of 12 *tet*(B) tested, of which 8 were of Austrian origin (Table 3). The conjugation frequency of the *tet*(A) plasmids ranged between 1.06×10^{-5} and 2.37×10^{-5}, while the *tet*(B)plasmids were transferred with a frequency of 2.83×10^{-4} to transconjugants of the Austrian strain AII.28. The conjugation frequency of the control *tet*(B) plasmid pTC was 7.76×10^{-4}. The difference between the conjugative transfers of these *tet*(A) and *tet*(B) plasmids was obvious but statistically not significant (Fig. 3).

All *tet*(B)-positive ETEC strains carried diverse plasmids ranging from ~10 to 200 kb in size, and their derivative transconjugants showed different plasmid combinations as well. In general, large plasmids of ~120, 145 and 175 kb in size were the most likely transferable (Table 3). The co-transfer of *tet*(B)-*aadA1*-*catA1* or of *tet*(B)-*catA1* genes and the corresponding phenotypes of tetracycline-aminoglycoside-chloramphenicol resistance were detected by testing the antimicrobial resistance patterns of the *tet*(B)-positive transconjugant strains originating from Austria (Table 3). All *tet*(B) strains carried class 1 integrons which were also transferred by conjugation to all but one of the transconjugants.

Table 3

Plasmid profiles of selected tetracycline resistant parental ETEC strains and of their transconjugants

Strain	O-type	Country	*tet* type	Resistance gene patterns	Integron type	Virulence genes	stb 5' flanking*	Plasmid sizes (~kb)
2172	O141	Hungary	*tetA*	*aadA1, strA*	*intI1*	*sta, stb, f18*	+	174, 138, 38
2172/11 tc			*tetA*	*aadA1, strA*	*intI1*		–	138 (IncI1)
11732	O141	Czech Republic	*tetA*	*aadA1, strA, catA1*		*sta, f18*	–	138, 106, 60, 5, 4
11732/71 tc			*tetA*	*catA1*			–	106 (IncF)
AII.23	O138	Austria	*tetB*	*aadA1, aadB, strA, catA1*	*intI1*	*sta, stb, east1, f18, k88*	–	174, 145, 120, 106, 60, 47, 15, 4
AII.23/2 tc			*tetB*	*catA1*		*sta, stb*	–	174, 120
AII.23/3 tc			*tetB*	*aadA1, catA1*	*intI1*		–	174, 145, 120
AII.25	O138	Austria	*tetB*	*aadA1, catA1*	*intI1*	*sta, stb, f18*	+	174, 145, 120, 97, 50, 15
AII.25/1 tc			*tetB*	*catA1*	*intI1*		–	174, 145, 120
AII.27	nt	Austria	*tetB*	*aacC2, aadA1, catA1*	*intI1, intI2*	*stb, lt, east1, f18*	–	200, 135, 52, 49
AII.27/2 tc			*tetB*	*aadA1, catA1*	*intI1*	*stb, lt, east1*	–	200, 120
AII.28	O139	Austria	*tetB*	*aadA1, catA1*	*intI1*	*sta, stb, east1, f18, k88*	+	174, 145, 120, 15
AII.28/2 tc			*tetB*	*aadA1, catA1*	*intI1*		–	174, 145
AII.29	O138	Austria	*tetB*	*aadA1, aadB, catA1*	*intI1*	*sta, stb, f18*	+	174, 145, 120, 15
AII.29/1 tc			*tetB*	*aadA1, catA1*	*intI1*		–	174, 145, 120
AII.34	O138	Austria	*tetB*	*aacC2, aadA1, catA1*	*intI1*	*sta, stb, f18*	+	174, 145, 120, 15
AII.34/5 tc			*tetB*	*aadA1, catA1*	*intI1*		–	174, 145, 120

*stb 5' flanking positive PCR result indicates the presence of toxin-specific locus (TSL) characteristic of pTC-like plasmids

The transfer of certain toxin genes was observed in two transconjugant strains only (derivatives of AII.23 and AII.27), sharing the virulence patterns *sta-stb* and *stb-lt-astA*, respectively, while the adhesin genes *f18* and *k88/f4* were not transferable in the system used (Table 3). Together with the identification of antimicrobial resistance and virulence genes, parental and transconjugant strains were tested for the 5' flanking region of the *stb* gene, as a marker for the toxin-specific locus (TSL) of the *tet*(B)-mediating plasmid pTC in porcine ETEC. The results indicated the presence of this locus in four of the six *tet*(B) strains and in one of the *tet*(A) strains without being transferable in this system (Table 3).

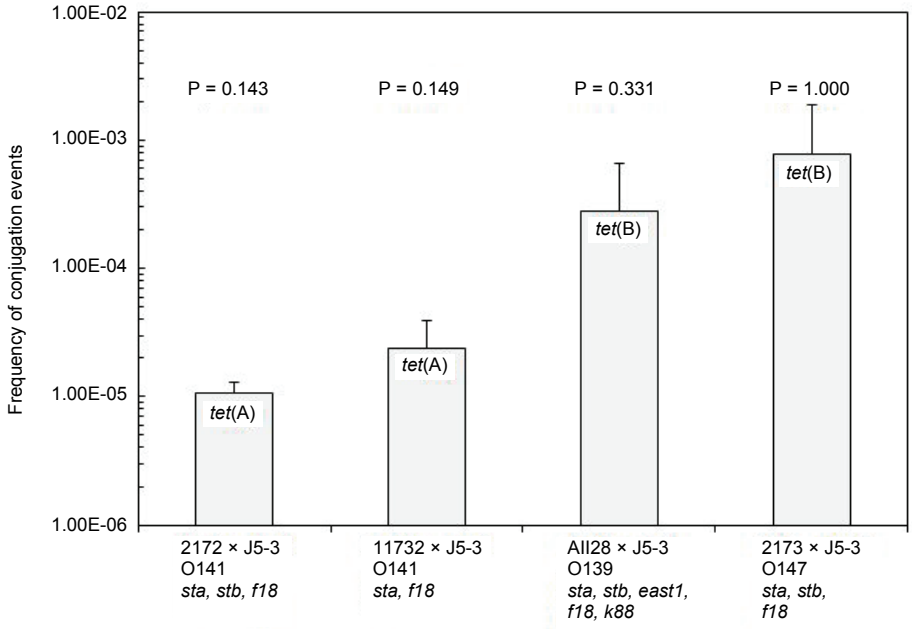

Fig. 3. Conjugation frequency of tetracycline resistance plasmids *tet*(A) and *tet*(B) in ETEC strains of serogroups O141, O139, and O147 with different virulence genes of the donor strains. Conjugation frequency was calculated as the ratio between the number of transconjugant CFUs (grown on tetracycline and rifampicin) and the number of recipient J5-3 CFUs (which were resistant only to rifampicine). The strain 2173 served as a pTC conjugative transfer control. The CFUs were calculated in two independent experiments with three parallel samples each. Distribution of conjugation frequencies were compared to strain 2173 using two-tailed Student's *t*-test

IncI1 and IncF plasmid-mediated transfers of tet*(A) gene and associated class 1 integron*

The Hungarian strain 2172 and the Czech strain 11732 carrying the *tet*(A) plasmids were also multi-plasmidic. Accordingly, plasmid replicon typing identified the coexistence of IncI1, IncF, IncP and colE_{Tp} type plasmids in both of

them. However, only the *tet*(A) plasmid was transferred from both of these strains. The *tet*(A) gene was transferred by a large plasmid of IncI1 type (~138 kb) of the Hungarian strain, 2172 (designated as pES2172) and of IncF type (~120 kb) of the Czech strain 11732 (designated as pES11732), respectively (Table 3). The IncI1 plasmid was responsible for the co-transfer of *tet*(A)-*aadA-strA-catA1* (tetracycline-aminoglycoside-chloramphenicol resistance) genes in the Hungarian strain, while the transfer of *tet*(A)-*catA1* was mediated by a large IncF plasmid of the Czech strain (Table 3). PCR analysis identified the *tet*(A) gene as part of the Tn*1721* transposon in all parental and transconjugant strains. The toxin genes *sta*, *stb* and the adhesin gene *f18* of these two ETEC strains were not transferable (Table 3).

In the Hungarian F18$^+$ ETEC strain, the *aadA1* gene was part of a class 1 integron, located also on the IncI1 *tet*(A) plasmid (pES2172). The amplification of its variable region resulted in a 2,735 bp fragment composed by two gene cassettes. Adjacent to the integrase gene, the *estX* gene was identified encoding resistance to streptothricin, downstream of which the streptomycin/spectinomycin gene *aadA1*was detected (Fig. 4).

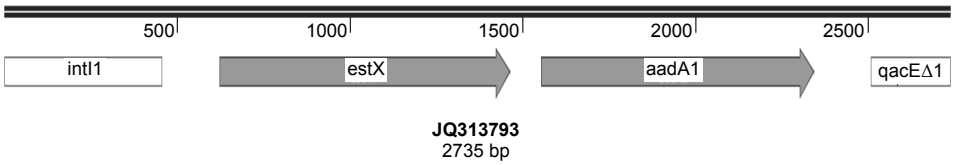

Fig. 4. Gene cassette array *estX* and *aadA1* genes of the *tet*(A) plasmidic (pES2172) class 1 integron detected in the Hungarian F18$^+$ ETEC strain 2172. Transparent boxes indicate partially sequenced genes *intI1* and *qacEΔ1*

Discussion

Antimicrobial resistance phenotypes and genotypes of ETEC carrying tet*(A) or* tet*(B) plasmids*

Most virulence and antimicrobial resistance genes of ETEC are located on large plasmids which makes these extra-chromosomal mobile genetic elements the essential tools of evolution through horizontal gene transfer. Plasmids carrying genes of different enterotoxins and/or fimbrial adhesins and plasmids conferring antimicrobial resistance are usually different, but in some cases they appear as hybrid plasmids carrying both resistance and virulence genes. Recently one such hybrid plasmid of porcine post-weaning ETEC (pTC) has been characterised in detail (Fekete et al., 2012). This 90-kb self-conjugative plasmid proved to be characteristic of F18$^+$ porcine post-weaning ETEC carrying the tetracycline resistance encoding *tet*(B) gene (Fekete et al., 2003; Olasz et al., 2005). However, the

role of *tet*(A) plasmids in transferring resistance and virulence of porcine ETEC seems to be much less understood. Therefore, in these studies we aimed to perform a comparative analysis of antimicrobial resistance and virulence gene profiles of porcine ETEC strains representing pork-producing areas in Central Europe and in the USA in order to assess the significance of tetracycline resistance and the role of the underlying *tet* gene types, especially *tet*(A), carried by conjugative plasmids.

The antimicrobial resistance phenotype and genotype of a representative collection of 87 porcine ETEC strains isolated from cases of post-weaning diarrhoea in Austria, the Czech Republic, Hungary and the Midwest USA indicated the existence of a common resistance backbone of ETEC strains in these two distant geographic regions with an average multidrug resistance to sulphamethoxazole (91%), tetracycline (84%), and streptomycin (80%). Although the US strains showed a generally higher frequency of resistance to the clinically relevant antimicrobials tested, the occurrence of tetracycline resistance was the highest (100%) among the ETEC strains from the USA, confirming the data of Boerlin et al. (2005) from Ontario, Canada in the North American region. Regarding tetracycline it must be borne in mind that approx. 50–66% of the antimicrobial substances used in animal production in the EU was tetracycline (Schwarz and Chaslus-Dancla, 2001; Moulin et al., 2008; EMA, 2014), while > 80% of growing swine in the USA receive tetracycline or tylosin (Landers et al., 2012). It is no wonder that the reported prevalence of resistance genes are high in the EU and in the USA.

Regarding the two main types of genes encoding tetracycline resistance, Hungarian and Austrian strains were characterised by a somewhat similar prevalence of the *tet*(A) and *tet*(B) gene (38% vs. 21% and 25% vs. 32%, respectively), while most strains from the Czech Republic carried the *tet*(A) gene (59%). In contrast, in the US strains the predominance of the type *tet*(B) gene (65%) was found. These data about differences between countries and regions regarding the types of *tet* genes confirm the findings reported in earlier publications (Olasz et al., 2005; Boerlin et al., 2005). Their data are supported here by indicating an important role of tetracycline resistance plasmids in the epidemiology of porcine post-weaning ETEC. It is acknowledged, however, that the distribution of *tet*(A) and *te*t(B) types of porcine ETEC in the same area could change over time (Maynard et al., 2003). Therefore, these data should form a comparative background for similar studies on recent post-weaning ETEC isolates from these countries. Identification of the genes encoding the three main types of tetracycline resistance mechanisms (efflux, ribosomal protection, and enzymatic inactivation) and of the so-called unknown types should also be the aim of a future molecular analysis for > 30 *tet* gene types that could come theoretically into question (Roberts, 2005).

An interesting difference between the two main types of *tet* plasmids was observed between the frequency of their conjugative transfers. The transfer of *tet*(A) plasmids was > 1 $\log_{10}$ less frequent as compared to the *tet*(B) plasmids. Although this difference was statistically not significant, it could be biologically important enough to be one of the contributing factors – besides plasmid incompatibility – to the wide-scale dissemination of *tet*(B) plasmids in the USA. Furthermore, in contrast to *tet*(A) strains, relatively more *tet*(B) strains (2/8 vs. 6/12) provided transferable plasmids, and they showed co-transfers with virulence plasmids or with virulence genes on the *tet*(B) plasmids. In contrast, *tet*(A) strains transferred only their *tet*(A) plasmids without virulence genes. However, they carried resistance genes against one or more other antibiotics, thereby assisting further selection and spread of multidrug resistance without specific selective pressures.

Besides antimicrobial resistance and virulence genes, parental and transconjugant strains were tested for the 5' flanking region of the *stb* gene as a marker for the toxin-specific locus (TSL) of the *tet*(B)-mediating plasmid pTC. As expected, the results indicated the presence of this locus in the majority of *tet*(B) strains, confirming the results of Fekete et al. (2003) and Olasz et al. (2005) but, surprisingly, in one of the *tet*(A) strains (2172) the 5' flanking region was detected as well. However, this indicator gene of TSL was present in a non-*tet(*A) plasmid (most likely in the 174-kb plasmid) of this strain, which could be the subject of further studies about TSL associations.

Characteristics of tet*(A) plasmids of F18$^+$ ETEC strains of Central European origin*

Although *tet*(A) and *tet*(B) are the two most frequently occurring efflux pump encoding resistance genes of porcine ETEC, the role of *tet*(A) plasmids in transferring resistance of porcine F18$^+$ ETEC is less understood as that of the *tet*(B) plasmids (Fekete et al., 2003; Olasz et al., 2005). One of the reasons is that *tet*(A) seems to be less frequent than *tet*(B) in porcine ETEC (Maynard et al., 2003; Boerlin et al., 2005). Other reasons could be the differences in the incompatibility and conjugative forces of the plasmids involved. The large self-conjugative *tet*(A) plasmids of IncI1 type (~138 kb) of the Hungarian ETEC (pES2172) and of IncF type (~120 kb) of the Czech ETEC (pES11732) seem to deserve attention as representative *tet(*A) plasmids of F18$^+$ porcine ETEC in Central Europe. Both carried *tet*(A) as part of Tn*1721* as expected. In addition, both conjugative plasmids carried further resistance genes as well. The plasmid pES2172 was responsible for the co-transfer of *tet*(A)-*aadA*-*strA*-*catA1* (tetracycline-aminoglycoside-chloramphenicol resistance) genes, while the plasmid pES11732 mediated the transfer of *tet*(A)-*catA1*. Literature data about replicon types of self-conjugative multidrug resistant *tet*(A) plasmids of porcine F18$^+$ ETEC have been missing. Earlier we determined replicon types of *f18* plasmids of porcine ETEC *(f18ab)* and VTEC *(f18ac)* using basic replicon DNA probes

and found both kinds of fimbrial plasmids to possess the FIc type of replicons (Fekete et al., 2002). Recently, Johnson et al. (2011) have provided comparative genomic analysis of IncI plasmids of porcine ETEC and suggested the existence of a conserved IncI1 plasmid backbone with a single locus for the acquisition of accessory genes associated with antimicrobial resistance. However, they did not study IncI or IncF plasmids of ETEC carrying tetracycline resistance genes. In our present study we provided data for the first time about IncI1 and IncF replicon types of multidrug resistance *tet*(A) plasmids of $F18^+$ porcine ETEC.

It is of further interest that in the IncI1 *tet*(A) plasmid pES2172 the *aadA1* gene was part of a class 1 integron, with a 2,735 bp fragment composed of two gene cassettes. Adjacent to the integrase gene, the unusual *estX* gene was identified to encode resistance to streptothricin. The aminoglycoside antibiotic streptothricin has not been licensed for use in veterinary medicine but it was known as a growth promoter in the former German Democratic Republic (Witte, 1997; Roberts, 2005). Genes encoding resistance to streptothricin acetyltransferase (*sat1* and *sat2*), as well as the putative esterase *(estX)*, have been described to occur on class 2 integrons of avian pathogenic and commensal *E. coli* of turkeys in Italy (Piccirillo et al., 2014), of commensal *E. coli* from healthy chicks in Korea (Dessie et al., 2013), of urinary pathogenic *E. coli* (UPEC) of swine and dogs in Germany (Kadlec and Schwarz, 2008), and in commensal porcine *E. coli* as well as on class 2 integrons of *Aeromonas* and *E. coli* from a slaughterhouse wastewater plant without specification of slaughtered animals in Portugal (Moura et al., 2007). The *estX* gene has been reported as part of class 1 integron in two *E. coli* strains isolated from diarrhoeal swine without definition of their pathotype (Cocchi et al., 2007), therefore its occurrence in a porcine ETEC is a novel observation.

In summary, our comparative analysis of antimicrobial resistance and virulence gene profiles of porcine post-weaning enterotoxigenic *E. coli* (ETEC) isolated within less than a decade in Central Europe and the USA revealed that almost all ETEC were multidrug resistant, sharing a common pattern of sulphamethoxazole-tetracycline-streptomycin resistance. By PCR and sequencing of tetracycline resistance genes and associated integrons as well as of further antimicrobial resistance and virulence genes we provided the first description of IncF and IncI1 type plasmids of $F18^+$ porcine enterotoxigenic *E. coli* carrying *tet*(A) and multidrug resistance. Additionally, the unusual resistance determinant *estX,* coding for streptothricin resistance, was first described here in a porcine enterotoxigenic *E. coli.*

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