1 **Conjugative IncF and IncI1 plasmids with *tet*(A) and class 1 integron conferring multidrug**
2 **resistance in F18$^+$porcine enterotoxigenic *E. coli.***
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28 **Abstract**

29 Enterotoxigenic *E. coli* (ETEC) bacteria are frequently causing watery diarrhea in newborn and
30 weaned pigs. Plasmids carrying genes of different enterotoxins and fimbrial adhesins and plasmids
31 conferring antimicrobial resistance are of prime importance in the epidemiology and pathogenesis
32 of ETEC. Recently, the significance of the porcine ETEC plasmid pTC was revealed, carrying
33 tetracycline resistance gene *tet*(B) with enterotoxin genes. In contrast the role of *tet*(A) plasmids in
34 transferring resistance of porcine ETEC is less understood. Objective of the present study was to
35 provide comparative analysis of antimicrobial resistance and virulence gene profiles of porcine
36 post-weaning enterotoxigenic *E. coli* (ETEC) strains representing pork producing areas in Central-
37 Europe and in the USA with special attention on plasmids carrying the *tet*(A) gene.
38

39 Antimicrobial resistance phenotype and genotype of 87 porcine ETEC strains isolated from cases of
40 post-weaning diarrhea in Austria, Czech Republic, Hungary and the Midwest USA was determined
41 by disk diffusion and by PCR. Central-European Strains carrying *tet*(A) or *tet*(B) were further
42 subjected to molecular characterization of their *tet* plasmids. Results indicated that >90% of the
43 ETEC strains shared a common multidrug resistant (MDR) pattern of sulfamethoxazole (91%),
44 tetracycline (84%) and streptomycin (80%) resistance. Tetracyclin resistance was most frequently
45 determined by the *tet*(B) gene (38%), while *tet*(A) was identified in 26% of all isolates with wide
46 ranges for both *tet* gene types between some countries and with class 1 integrons and resistance
47 genes co-transferred by conjugation. The virulence gene profiles included enterotoxin genes (*lt*, *sta*
48 and/or *stb*), as well as adhesin genes (*k88/f4, f18*). Characterization of two representative *tet*(A)
49 plasmids of porcine F18$^+$ ETEC from Central-Europe revealed, that the IncF plasmid (pES11732)
50 of the Czech strain (~120 kb) carried *tet*(A) in association with *catA1* for chloramphenicol
51 resistance. The IncI1 plasmid (pES2172) of the Hungarian strain (~138 kb) carried *tet*(A) gene and
52 a class 1 integron with an unusual variable region of 2,735 bp composed by two gene cassettes:
53 *estX-aadA1* encoding for streptothricin-spectinomycin/streptomycin resistance exemplifying
54 simultaneous recruitment, assembly and transfer of multidrug resistance genes by *tet*(A) plasmid of
55 porcine ETEC..
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57 By this we provided the first description of IncF and IncI1 type plasmids of F18$^+$ porcine
58 enterotoxigenic *E. coli* responsible for co-transfer of the *tet*(A) gene with multidrug resistance.
59 Additionally the unusual determinant *estX,* encoding for streptothricin resistane was first reported
60 here in porcine enterotoxigenic *E. coli*.

61

62 **Keywords**: enterotoxigenic *Escherichia coli*, tetA plasmid, multiresistance, antimicrobial
63 resistance, virulence

64 **Introduction**

65 Severe watery diarrhoea of newborn- and weaned pigs as well as of newborn calves are often
66 caused by enterotoxigenic *Escherichia coli* (ETEC) strains (Nagy and Fekete, 2005). On the other
67 hand the ETEC infection often leads to traveler's diarrhoea among humans and may cause
68 significant morbidity and mortality of children in the developing countries (Quadri et al., 2005).
69 Due to the pathogenic similarities of ETEC infections in animals and humans, ETEC has been the
70 subject of intensive studies in human and veterinary medicine over the past three decades.
71 In weaned pigs two main types of virulence factors are essential for development of the ETEC-
72 caused diarrhoea: bacterial attachment to the mucosal surface of the small intestine mediated by
73 fimbrial adhesins (*i.e.* K88/F4 and/or F18ac), and the enterotoxins - heat-labile toxin (LT) and/or
74 heat-stable toxins (STa, STb) - changing the absorptive function of the small intestine to secretion
75 leading to diarrhoea and loss of water and electrolytes.
76 Genes encoding the above virulence factors are located on ETEC virulence plasmids (Gyles,
77 1994; Fekete et al., 2012), however several other mobile virulence elements such as pathogenicity
78 islands (PAIs), bacteriophages and transposons may also contribute to transfer of virulence genes
79 between *E. coli* strains (Hacker et al., 1997). As an example, heat-stable enterotoxin genes *sta* and
80 *stb* are part of transposons: *sta* being carried by Tn*1681* (So and McCarthy, 1980), and *stb* was
81 described as a part of Tn*4521* (Hu and Lee, 1988). The spread of the heat labile toxin gene *lt* is
82 realized by IS-mediated transfer mechanisms (Schlör et al., 2000).
83 In our previous studies on the representative porcine post-weaning F18$^+$ ETEC strain 2173 we
84 described and sequenced the large conjugative plasmid pTC (*sta*$^+$, *stb*$^+$, *tetB*$^+$) as responsible for the
85 enterotoxigenicity and tetracycline resistance [*tet*(B)] of the host strain (Nagy et al., 1990; Fekete, et
86 al., 2012). Among *Enterobacteriaceae* the tetracycline resistance is encoded mainly by tetracycline
87 efflux proteins, removing the tetracycline from the bacterial cytoplasm most often ecoded by *tetA,*
88 *tetB, tetC, tetD, tetE* and *tetG* genes.. Tetracycline resistance genes are often located on mobile
89 elements, such as plasmids, transposons and/or conjugative transposons, which can sometimes be
90 transferred between bacterial species (Michalova et al., 2004; Chopra and Roberts,2001), Studies on
91 the tetracycline resistance mechanisms are justified by data about tetracyclines being the far most
92 frequently used antimicrobials in the EU (Schwarz and Chaslus-Dancla, 2001., Moulin, 2008).

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

101 **Materials and methods**

102 *Bacterial strains*

103 Porcine post-weaning enterotoxigenic *E. coli* (ETEC) strains studied here were isolated and
104 identified between 1987-1995 from cases of post weaning diarrhoea, representing 8-12
105 farms/country in three neighbouring countries: Hungary (n=16), Austria (n=34) and the Czech
106 Republic (n=17). The Central-European collection was complemented with 20 comparable ETEC
107 strains originating from the Midwest-USA. Selected properties of some Hungarian and some US
108 strains have been previously reported (Fekete et al. 2003, and Olasz et al., 2005) All ETEC strains
109 were stored at -80°C in Tryptic soy broth (TSB) complemented with 10% glycerol.

110 *Detection of antimicrobial resistance phenotype and identification of tetracycline resistance genes*

111 The antimicrobial resistance phenotype was tested by disc diffusion assay against 18
112 antimicrobial compounds (Oxoid) with clinical relevance. These were as follows (lower indexes
113 indicates disc concentration): amoxicillin ($AMX_{25}$), ampicillin ($AMP_{10}$), cefotaxime ($CTX_{30}$),
114 chloramphenicol ($CHL_{30}$), enrofloxacin ($ENR_5$), erythromycin ($ERY_{10}$), florfenicol ($FFC_{30}$),
115 gentamicin ($GEN_{10}$), kanamycin ($KAN_{30}$), nalidixic acid ($NAL_{30}$), rifampicin ($RIF_5$), spectinomycin
116 ($SPE_{100}$), streptomycin ($STR_{10}$), sulfamethoxazole ($SMX_{25}$), tetracycline ($TET_{30}$),
117 trimethoprim ($TMP_5$) and trimethoprim-sulfamethoxazole ($SXT_{1.25/23.75}$). Interpretation of the data
118 was performed according to Clinical and Laboratory Standard Institute (CLSI) guidelines and
119 interpretive standards (CLSI, 2013). ETEC strains with intermediate zone diameter values were
120 considered susceptible. The *E. coli* reference strain ATCC 25922 was used as control. Multidrug
121 resistance (MDR) was defined as co-resistance to three or more antimicrobial classes.

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

124 *Identification and characterisation of ETEC plasmids mediating tetracycline resistance*

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

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

138 *Plasmid profile analysis and replicon typing*

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

150 *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 Table 1 and Table 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).

*Characterization of class 1 integron carried by the tetA plasmid*

The variable region of the class 1 integron detected in the Hungarian mono-plasmidic *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 submitted to sequencing with primers listed in Table 1. Nucleotide sequences were analysed in comparison with the NCBI database using the BLASTN algorithm. The resulted fragment of 2,645 bp identified in the Hungarian strain 2172/11was deposited in the GenBank under accession number JQ313793.

## Results

*Antimicrobial resistance phenotype of porcine ETEC strains*

According to the results of the antimicrobial susceptibility testing, an overwhelming majority of the 87 ETEC strains tested (94.3%) were considered as multidrug resistant (MDR), showing co-resistance to at least three antimicrobial classes. The distribution of antimicrobial resistance phenotypes among ETEC strains from Hungary, 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. Majority of the strains shared a common MDR backbone, most frequently being resistant to sulfamethoxazole (91%), tetracycline (84%), erythromycin (84%), and streptomycin (79%). Resistance to spectinomycin (44%), rifampicin (36%) and amoxicillin (32%) were 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 against these drugs, while kanamycin resistance was most frequently detected among the strains from the USA and Hungary (90% and 81%). Besides, Hungarian strains showed reduced resistance to gentamicin, chloramphenicol and ampicillin(13-

181 19%). ETEC strains from Austria showed the highest rate of chloramphenicol resistance (53%),
182 while 50% of the USA strains were resistant to ampicillin (Fig. 1).The mean prevalence of
183 resistance to trimethoprim and nalidixic-acid were relatively low (18% and 15%), while all strains
184 were susceptible to cefotaxime, enrofloxacin and florfenicol (Fig. 1).

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

186 ETEC strains with tetracycline resistant phenotype have been tested by PCR for the
187 identification of the *tet* gene types [*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G)] representing the most
188 frequently described efflux pump encoding genes in *Enterobacteriaceae*. The *tet*(B) was the most
189 frequently found gene conferring tetracycline resistance in 38%, of ETEC strains, while the *tet*(A)
190 gene was identified in 26% (Fig. 2). Only a few strains showed the coexistence of the above two *tet*
191 gene types. The combination of *tet*(A)/*tet*(B), *tet*(B)/*tet*(C) and of *tet*(B)/*tet*(D)genes averaged 3%,
192 1% and 1% respectively. In 31% of the strains the tetracycline resistant phenotype was not
193 confirmed by any of the *tet* genes tested (Fig. 2) indicating resistance mechanisms encoded by some
194 of the other less frequent tetracyclin resistance genes beside those listed in Table 1.
195 Distribution of the *tet* gene types varied according to geographical origin.. Hungarian and
196 Austrian strains were characterized by prevalence of 38% and 21% of the *tet*(A) gene respectively
197 and *tet*(B)gene (25% and 32% respectively). In contrast most strains from The Czech Republic
198 carried the *tet*(A) gene (59%). Predominance of the *tet*(B) gene (65%) caharctezied the USA strains
199 (Fig. 2). No ETEC strains tested carried *tet*(E) or *tet*(G) genes.
200

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

202 Out of the 8 *tet*(A) and 12 *tet*(B) ETEC strains the conjugative transfer of the *tet*(A) plasmids
203 was successful in 2 of the 8 *tet*(A) strains selected. One was an $F18^+$ ETEC strain from Hungary
204 (2172), and the other one $F18^+$ ETEC strain was from the Czech Republic (11732), resulting in two
205 *tet*(A)-positive transconjugant strains designated as 2172/11 and 11732/71 respectively. The
206 transfer of *tet*(B) plasmids resulted in 6 Austrian *tet*(B) strains out of a total of 12 *tet*(B) tested.
207 from which 8 were of Austrian origin (Table 3). The conjugation frequency of the *tet*(A) plasmids
208 ranged between $1.06\times10^{-5}$ and $2.37\times10^{-5}$, while the *tet*(B)plasmids were transferred with a
209 frequency of $2.83\times10^{-4}$ to the transconjugants of the Austrian strain AII.28. Conjugation frequency
210 of the control *tet*(B) plasmid pTC was $7.76\times10^{-4}$ and the difference between the conjugative
211 transfers of these *tet*(A) and *tet*(B) plasmids was obvious but statistically not significant (Fig. 3).

212 All *tet*(B)-positive ETEC strains carried diverse plasmids ranging from ~10 to 200 kb in size,
213 and their derivative transconjugants showed different plasmid combinations as well.. In general,
214 large plasmids of ~ 120, 145 and 175 kb were the most likely transferable (Table 3). The co-transfer
215 of *tet*(B)-*aadA1-catA1* or of *tet*(B)-*catA1* genes and the corresponding phenotypes of tetracycline-
216 aminoglycoside-chloramphenicol resistance was detected by testing the antimicrobial resistance
217 patterns of the *tet*(B)-positive transconjugant strains originated from Austria (Table 3). All *tet*(B)
218 strains carried class 1 integrons which were also transferred by conjugation to all but one of the
219 transconjugants.The transfer of certain toxin genes was observed in two transconjugant strains only
220 (derivatives of AII.23 and AII.27), sharing the virulence patterns *sta-stb* and *stb-lt-astA*
221 respectively, while the adhesin genes *f18*and *k88/f4* were not transferable in the system used (Table
222 3). Together with the identification of antimicrobial resistance and virulence genes, parental and
223 transconjugant strains were tested for the 5' flanking region of the *stb* gene, as a marker for the
224 toxin specific locus (TSL) of the *tet*(B)-mediating plasmid pTC in porcine ETEC. Results indicated
225 the presence of this locus in four of the six *tet*(B) strains, and in one of the *tet*(A) strains without
226 being transferable in this system (Table 3).

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

228 The Hungarian strain 2172 and the Czech strain 11732 carrying the *tet*(A) plasmids were also
229 multi-plasmidic. Accordingly, plasmid replicon typing identified the coexistence of IncI1, IncF,
230 IncP and $colE_{Tp}$ type plasmids in both of them. However, only the *tet*(A) plasmid was transferred
231 from both of these strains. The *tet*(A) gene was transferred by a large plasmid of IncI1 type (~138
232 kb) of the Hungarian strain, 2172 (designated as pES2172) and of IncF type (~120 kb) of the Czech
233 strain 11732 (designated as pES11732) respectively (Table 3). The IncI1 plasmid was responsible
234 for the co-transfer of *tet*(A)-*aadA*-*strA*-*catA1*(tetracycline-aminoglycoside-chloramphenicol
235 resistance) genes in the Hungarian strain, while the transfer of *tet*(A)-*catA1*was mediated by a large
236 IncF plasmid of the Czech strain (Table 3). PCR analysis identified the *tet*(A) gene as part of the
237 Tn*1721* transposon in all parental and transconjugant strains.. The toxin genes *sta*, *stb* and the
238 adhesin gene *f18*, of these two ETEC strains were not transferable (Table 3).
239 In the Hungarian $F18^+$ ETEC strain, the *aadA1* gene was part of a class 1 integron, located also
240 on the IncI1 *tet*(A) plasmid (pES2172). The amplification of its variable region resulted in a 2,735
241 bp fragment composed by two gene cassettes. Adjacent to the integrase gene, the *estX* gene was
242 identified encoding resistance to streptothricin, downstream of which the
243 streptomycin/spectinomycin gene *aadA1*was detected.

244 **Discussion**

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

246 Most virulence and antimicrobial resistance genes of enterotoxigenic *Escherichia coli* (ETEC) are
247 located on large plasmids which makes these extra-chromosomal mobile genetic elements the
248 essential tools of evolution through horizontal gene transfer. Plasmids carrying genes of different
249 enterotoxins and/or fimbrial adhesins and plasmids conferring antimicrobial resistance are usually
250 different, but in some cases they appear as hybrid plasmids carrying both resistance and virulence
251 genes. Recently one such hybrid plasmid of porcine post-weaning ETEC (pTC) has been
252 characterized in detail (Fekete et al, 2012). This 90kb self-conjugative plasmid proved to be
253 characteristic to F18$^{+}$ porcine post-weaning ETEC carrying the tetracycline resistance encoding
254 *tet*(B) gene (Fekete et al., 2003; Olasz et al., 2005). However, the role of *tet*(A) plasmids in
255 transferring resistance and virulence of porcine ETEC seems to be much less understood. Therefore,
256 in these studies we aimed to perform a comparative analysis of antimicrobial resistance and
257 virulence gene profiles of porcine post-weaning enterotoxigenic. *E. coli* (ETEC) strains
258 representing pork producing areas in Central-Europe and in the USA in order to assess the
259 significance of tetracycline resistance and the role of the underlying *tet* gene types especially *tet*(A),
260 carried by conjugative plasmids.

261 Antimicrobial resistance phenotype and genotype of a representative collection of 87 porcine
262 ETEC strains isolated from cases of post-weaning diarrhoea in Austria, Czech Republic, Hungary
263 and the Midwest USA, indicated the existence of a common resistance backbone of ETEC strains in
264 these two distant geographic regions: with an average multidrug resistance to sulfamethoxazole
265 (91%), tetracycline (84%) and streptomycin (80%). Although the USA strains have shown a
266 generally higher frequency of resistance to the clinically relevant antimicrobials tested, the
267 occurence of tetracycline resistance was the highest (100%) among the ETEC strains from the USA,
268 confirming the data of Boerlin et al (2005), from Ontario, Canada in the North-American region..
269 Regarding tetracycline it must be borne in mind that approx. 50-66% of antimicrobial substances
270 used in animal production in the EU was tetracycline (Schwarz and Chaslus-Dancla, 2001, Moulin
271 et al, 2008), while >80% of growing swine in the USA receive tetracylin or tylosin (Landers et al,
272 2012). No wonder that the reported prevalence of resistance genes are high in the EU and in the US.
273 Regarding the two main types of genes encoding tetracycline resistance. Hungarian and Austrian
274 strains were characterized by somewhat similar prevalence of the *tet*(A) and *tet*(B) gene (38% vs
275 21%) and (25% vs 32%) respectively, while most strains from The Czech Republic carried the

276 *tet*(A) gene (59%). In contrast, the predominance of the type *tet*(B) gene (65%) was found in the
277 USA strains. These data about differences between countries and regions regarding types of *tet*
278 genes are confirmatory with earlier pu blications of Olasz et al. (2005) and of Boerlin et al.,(2005).
279 Their data are supported here by indicating an important role of tetracycline resistance plasmids in
280 the epidemiology of porcine post weaning ETEC. It is acknowledged however that distribution of
281 *tet*(A) and *te*t(B) types of porcine ETEC on the same area could change over time (Maynard et al.,
282 2003). Therefore, these data should form a comparative background for similar studies on recent
283 postweaning ETEC isolates from these countries. Identification of the genes encoding the three
284 main types of tetracylin resistance mechanisms (efflux, ribosomal protection, and enzymatic
285 inactivation) and of the so called unknown types should also be the aim of a future molecular
286 analysis for >30 *tet* gene types that could come theoretically into question. (Roberts, 2005).
287

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

298

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

306

307 *Characteristics of tet(A) plasmids of F18⁺ ETEC strains of Central-European origin*

308 Although *tet*(A) and *tet*(B) are the two most frequently occurring efflux pump encoding resistence
309 genes of porcine ETEC, the role of *tet*(A) plasmids in transferring resistance of porcine F18$^+$ ETEC
310 is less understood as that of the *tet*(B) plasmids (Fekete et al., 2003., Olasz et al., 2005). One of the
311 reasons is that *tet*(A) seems to be less frequent than *tet*(B) in porcine ETEC (Boerlin et al., 2005,
312 Maynard et al. 2003). Other reasons could be the differences in the incompatility and conjugative
313 forces of the plasmids involved..The large self conjugative *tet*(A) plasmids of IncI1 type (~138 kb)
314 of the Hungarian ETEC (pES2172) and of IncF type (~120kb) of the Czech ETEC (pES11732)
315 seem to deserve attention as representative *tet(*A) plasmids of F18$^+$ porcine ETEC in Central
316 Europe. Both carried *tet*(A) as part of Tn*1721* as expected. Besides both self conjugative plasmids
317 carried further resistance genes as well. The plasmid (pES2172) was responsible for the co-transfer
318 of *tet*(A)-*aadA*-*strA*-*catA1*(tetracycline-aminoglycoside-chloramphenicol resistance) genes,, while
319 the plasmid (pES11732) mediated the transfer of *tet*(A)-*catA1*. Literature data about replicon types
320 of self conjugative multidrug resistant *tet*(A) plasmids of porcine F18$^+$ ETEC have been missing..
321 Earlier we have determined replicon types of *f18* plasmids of porcine ETEC (*f18ab)* and VTEC
322 (*f18ac*) using basic replicon DNA probes and found both kinds of fibmrial plasminds possessing the
323 FIc type of replicons (Fekete et al., 2002). Recently Johnson et al (2011) provided comparative
324 genomic analysis of IncI plasmids of porcine ETEC and suggested the existence of a conserved
325 IncI1 plasmid backbone with a single locus for the acquisition of accesory genes associated with
326 antimicrobial resistance. However, they did not study IncI or IncF plasmids of ETEC carrying
327 tetracylin resistance genes. In our present study we provided data for the first time about IncI1 and
328 IncF replicon types of multidrug resistance *tet*(A) plasmids of F18$^+$ porcine ETEC.

329 It is of further interest that in the IncI1 *tet*(A) plasmid (pES2172) the *aadA1* gene was part of a
330 class 1 integron, with a 2,735 bp fragment composed by two gene cassettes. Adjacent to the
331 integrase gene, the unusual *estX* gene was identified encoding resistance to streptothricin,. The
332 aminoglycosid antibiotic streptothricin has not been licenced for use in veterinary medicine but it
333 was known as a growth promoter in the former German Democratic Republic (Witte,1997: Roberts,
334 2005). Genes encoding resistance to streptothrichin acetiltransferase, (*sat1* and *sat2*), as well as the
335 putative esterase (*estX*) have been described to occur on class 2 integrons of avian pathogenic and
336 commensal *E.coli* of turkeys in Italy (Piccirillo, 2014) of commensal *E. coli* from healthy chicks in
337 Korea (Dessie et al, 2013), and of urinary pathogenic *E. coli* (UPEC) of swine and dog in Germany
338 (Kadlecz 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), teherfore its occurence 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 representing Central-Europe and the USA revealed that almost all ETEC were mutidrug resistant, sharing a common pattern of sulfamethoxazole  tetracycline and streptomycin resistance. By PCR and sequencing on tetracycline resistance genes and on associated integrons as well as on 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. Additionaly the unusal resistance determinant *estX,* encoding for streptothricin resistance was first described here in a porcine enterotoxigenic *E. coli.*

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491

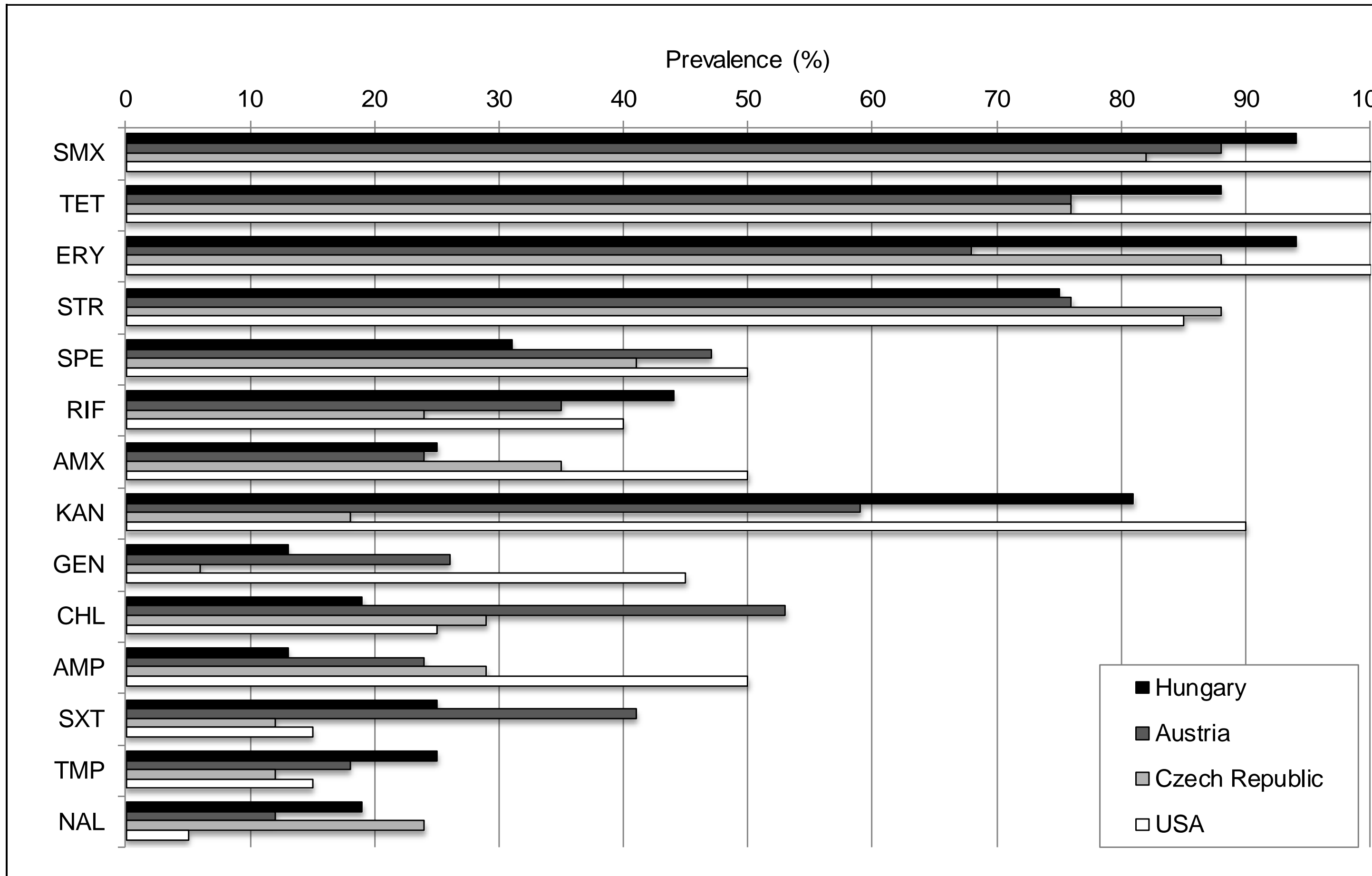
492 **Figure legends**

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

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

501 **Fig. 3.** Conjugation frequency of tetracycline resistance plasmids *tet*(A) *andtet*(B) in ETEC strains.
502 Conjugation frequency was calculated as the ratio between the number of transconjugant CFUs
503 (grown on tetracycline and rifampicin) and the number of recipient J5-3 CFUs (which were resistant
504 only to rifampicine). The strain 2173 served as a pTC conjugative transfer control. The CFUs were
505 calculated in two independent experiments with three parallel samples each. Distribution of
506 conjugation frequencies were compared to the 2173 using two-tailed Student's t-test.
507

508 **Figure 1.**

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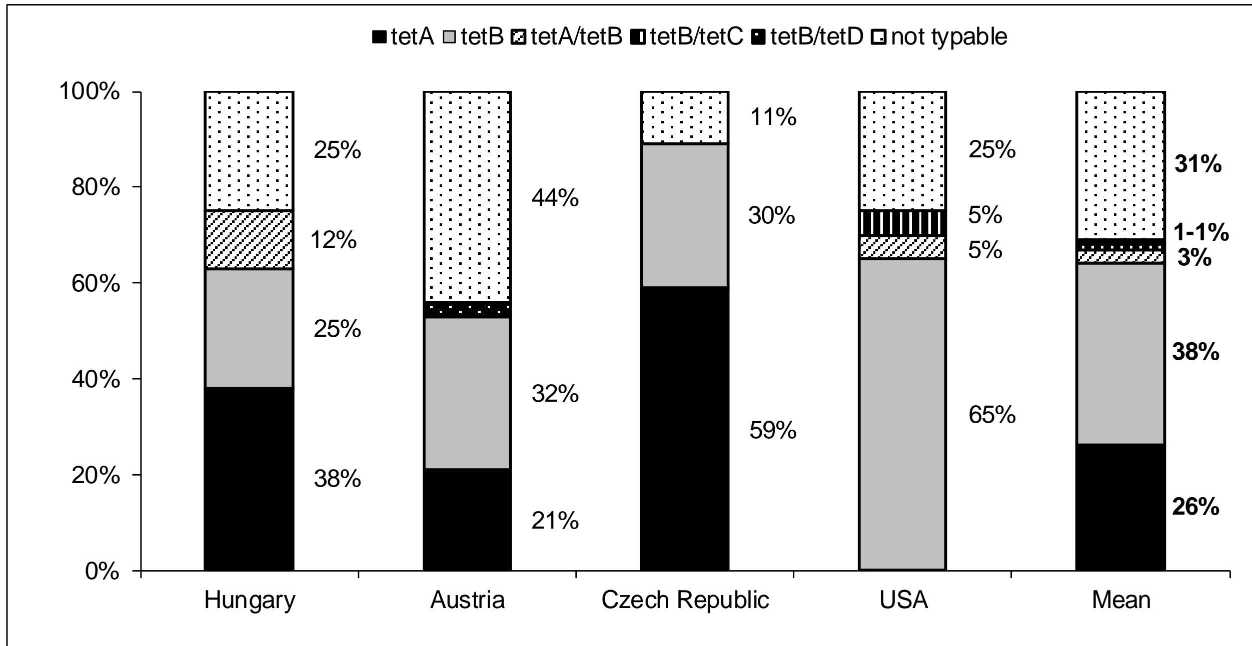
512 **Figure 2.**

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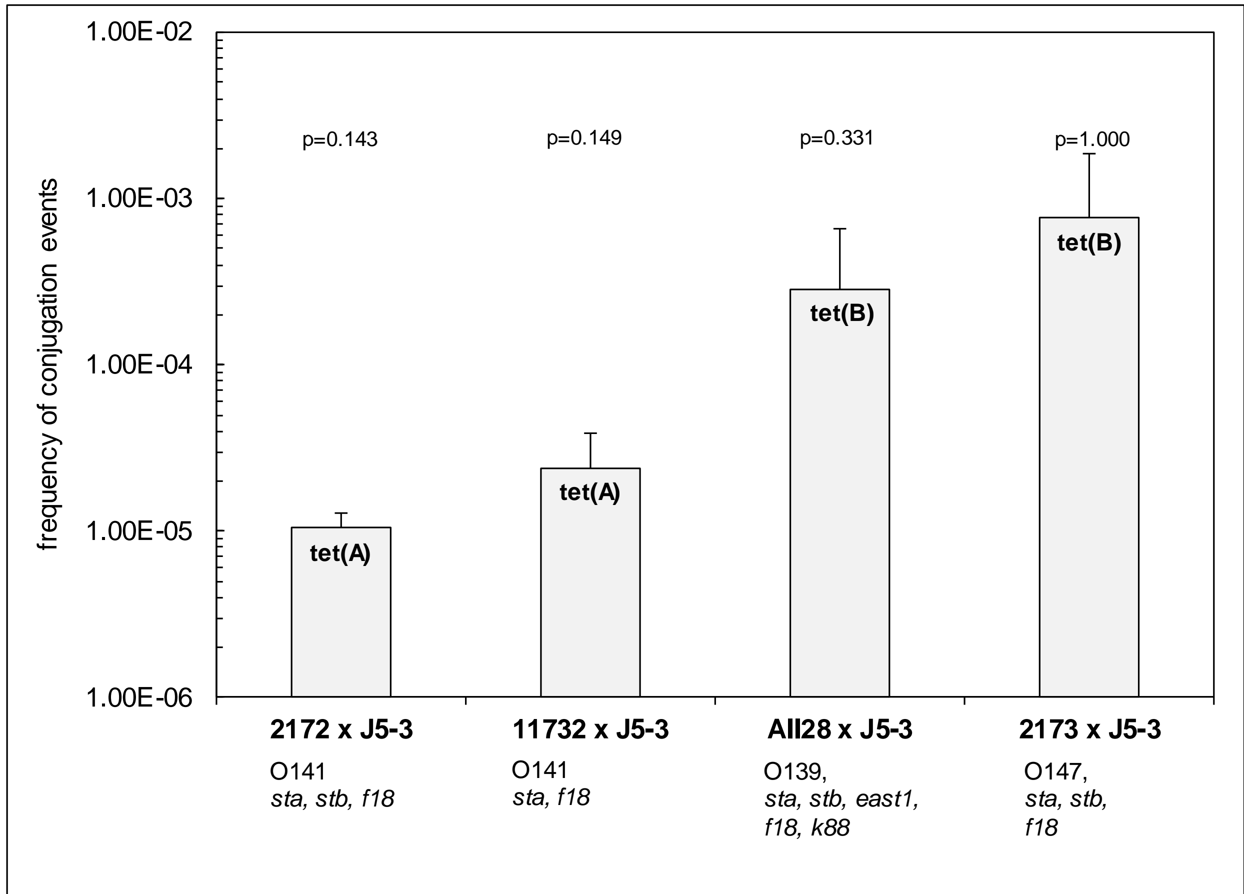
514 **Figure 3.**

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520 **Table 1.** Primers used for the detection of antimicrobial resistance genes and for the
521 characterization 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, 200( |
| | 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., 2003 |
| | 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 | 5CS-F1 | ATGTTACGCAGCAGGGC | variable | PCR/SQ | Libisch et al., 2004 |
| | 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 |

522
523 SQ: primers used for sequencing

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

525

526 **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 | The 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, |
| 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 |

527
528 *sstb 5' flanking positive PCR result indicate the presence of toxin specific locus (TSL) characteristic to pTC-like plasmids