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**Molecular epidemiology of the endemic multiresistance plasmid pSI54/04 of *Salmonella* Infantis in broiler and human population in Hungary**

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## 1 Abstract

2 *Salmonella* Infantis (SI) became endemic in Hungary where the PFGE cluster B,  
3 characterized by a large multiresistance (MDR) plasmid emerged among broilers leading to  
4 an increased occurrence in humans. We hypothesised that this plasmid (pSI54/04) assisted  
5 dissemination of SI. Indeed, Nal-Sul-Tet phenotypes carrying pSI54/04 occurred increasingly  
6 between 2011-2013 among SI isolates from broilers and humans. Characterization of  
7 pSI54/04 based on genome sequence data of the MDR strain SI54/04 indicated a size of ~277  
8 kb and a high sequence similarity with the megaplasmid pESI of SI predominant in Israel.  
9 Molecular characterization of 78 representative broiler and human isolates detected the  
10 prototype plasmid pSI54/04 and its variants of together with novel plasmid associations  
11 within the emerging cluster B. To test *in vitro* and *in vivo* pathogenicity of pSI54/04 we  
12 produced plasmidic transconjugant of the plasmid-free pre-emergent strain SI69/94. This  
13 parental strain and its transconjugant have been tested on chicken embryo fibroblasts (CEFs)  
14 and in orally infected day old chicks. The uptake of pSI54/04 did not increase the  
15 pathogenicity of the strain SI69/94 in these systems. Thus, dissemination of SI in poultry  
16 could be assisted by antimicrobial resistance rather than by virulence modules of the endemic  
17 plasmid pSI54/04 in Hungary.

18 **Keywords:** *S. Infantis*; plasmid; broilers; PFGE clone; molecular typing

## 19 1. Introduction

20 *Salmonella* Infantis has been the most prevalent serovar isolated from fresh poultry  
21 meat and broiler flocks all over in Europe (EFSA and ECDC, 2015) and in several other  
22 countries like Israel in 2007-2009 (Gal-Mor et al., 2010) and Japan 2000-2003 (Asai et al.,  
23 2006; Shahada et al., 2006). Concomitantly *S. Infantis* became the fourth most widespread  
24 serovar isolated from humans in the EU, behind *S. Enteritidis*, *S. Typhimurium* and  
25 monophasic *S. Typhimurium* 1,4,[5],12:i:- (EFSA and ECDC, 2015). Earlier *S. Infantis* has  
26 been frequently isolated from asymptomatic poultry (Asai et al., 2006; Shahada et al., 2006),  
27 and human patients in Japan (Murakami et al., 2007). It became the most dominant serovar in  
28 poultry and in the human population in Israel (Gal-Mor et al 2010).

29 Dissemination of multidrug resistant (MDR) *S. Infantis* in Belgian poultry carrying a  
30 *bla*<sub>TEM-52</sub> plasmid has been reported by Cloeckert et al. (2007). The first evidence for clonal  
31 spread of *S. Infantis* strains in broiler and in human populations with a large conjugative

32 MDR plasmid (>168 kb) carrying a class 1 integron (containing the *aadA1* gene cassette for  
33 streptomycin/spectinomycin resistance) and the *tet(A)* gene for tetracycline resistance came  
34 from Hungary (Nógrády et al., 2007). Emerging strains characteristically showing nalidixic  
35 acid-streptomycin-sulphonamide-tetracycline (NalStrSulTet) resistance belong to the  
36 dominant PFGE cluster B (Nógrády et al., 2007; 2008). The representative strain SI54/04 of  
37 this cluster B has been sequenced and its large MDR plasmid, earlier estimated as >168 kb,  
38 has been determined as ~277 kb (Olasz et al., 2015). Our further studies have shown that  
39 these MDR *S. Infantis* strains are also endemic among broilers in several European countries,  
40 especially in Austria and Poland and belong to the same or closely related clones as the  
41 Hungarian cluster B (Nógrády et al., 2012). Furthermore, they also carry the same or very  
42 similar large MDR plasmid with class 1 integron and *tet(A)* gene.

43 Here we aimed at studying the recent epidemiology of MDR *S. Infantis* in Hungary by  
44 molecular characterization of the strains and of the emerging ~277 kb MDR plasmid  
45 designated as pSI54/04. Plasmid pSI54/04 of the emerging Hungarian broiler strain SI54/04  
46 revealed high sequence similarity with the megaplasmid pESI of a human *S. Infantis* strain  
47 (Aviv et al., 2014) concerning its specific regions with functions in antimicrobial resistance,  
48 virulence, plasmid maintenance and transfer. Molecular typing of recent *S. Infantis* strains of  
49 broiler and human origin indicated that the PFGE cluster B carrying this MDR plasmid is still  
50 endemic and pSI54/04 might have contributed to the unprecedented spread of *S. Infantis*  
51 between 2000-2013 in the Hungarian broiler flocks and to a much lesser degree in humans.

## 52 2. Materials and methods

### 53 2.1. Strains of *Salmonella Infantis* and antimicrobial resistance phenotyping

54 A collection of 186 epidemiologically unrelated strains of *S. Infantis* was established,  
55 by choosing isolates with differing geographic origin, covering as much of Hungary as  
56 possible for the years of 2011-2013, containing approximately equal numbers of broiler and  
57 human isolates without causative links between them (Table S1). This basic collection was set  
58 up in order to represent the antimicrobial resistance profiles of *S. Infantis* in Hungarian broiler  
59 flocks and in humans. It provided a basis for comparative epidemiological and molecular  
60 studies for Hungary, keeping in mind that about 90 % of poultry meat consumed is produced  
61 in this country. Broiler strains (n=91) were provided by the Food and Feed Safety Directorate  
62 of the National Food Chain Safety Office (NEBIH), as part of the national *Salmonella*  
63 monitoring program, representing overwhelmingly the two major broiler breeds Ross 308 and

64 Cobb 500. Human strains of *S. Infantis* (n=95) were provided by the National Center for  
65 Epidemiology, representing sporadic clinical cases. Strains were stored at -80°C in LB (Luria-  
66 Bertani) broth containing 10% glycerol.

67 Antimicrobial susceptibility testing of *S. Infantis* strains was performed by disc  
68 diffusion against the listed antimicrobial compounds: ampicillin (Amp), cefotaxime(Ctx),  
69 chloramphenicol (Chl), ciprofloxacin (Cip), kanamycin (Kan), nalidixic acid (Nal),  
70 sulfonamide compounds (Sul), tetracycline (Tet) and trimethoprim (Tmp). Results were  
71 interpreted according to Clinical and Laboratory Standard Institute (CLSI) guidelines and  
72 interpretive standards (CLSI, 2013). *S. Infantis* isolates with intermediate zone diameter  
73 values to respective antimicrobials were considered susceptible. *E. coli* ATTC 25922 was  
74 used as a reference strain.

## 75 2.2. Molecular characterization: pulsed-field gel electrophoresis, plasmid profiling and 76 antimicrobial resistance genotyping

77 A reduced number of broiler (n=31) and human (n=47) strains of *S. Infantis* were  
78 selected for molecular analysis (Table S1). Selection of the strains was based on their  
79 antimicrobial resistance phenotype to represent the diversity of the existing resistance  
80 patterns. As a reference, two sequenced strains of broiler origin, SI69/94 and SI54/04 were  
81 also included (Olasz et al., 2015). They represented the pre-emergent plasmid-free  
82 pansensitive isolates from the 1990s and the emerging MDR strains from the 2000s carrying  
83 the plasmid pSI54/04 respectively (Nógrády et al., 2007; Nógrády et al., 2008; Olasz et al.,  
84 2015).

85 PFGE analysis was carried out according to the CDC PulseNet standardized *Salmonella*  
86 protocol using *Salmonella* Braenderup H9812 as a molecular standard. PFGE-generated DNA  
87 profiles were entered into the Fingerprinting II Software (Bio-Rad Laboratories, Ventura, CA,  
88 USA). Cluster analysis was performed by the unweighted pair-group method (UPGMA) with  
89 arithmetic means. DNA sequence relatedness was calculated on the basis of the Dice's  
90 coefficient. A 1.0% position tolerance and 1.5% optimization setting were applied.

91 Plasmid preparation was carried out using the alkaline lysis method of Kado and Liu  
92 (1981), and plasmids were separated in 0.75% agarose gel in a vertical system. The  
93 approximate sizes of plasmids were estimated by comparing them with the reference plasmids  
94 of *E. coli* V517 (2.0–53.7 kb) and *E. coli* MD112 (168 kb) using the Quantity One software  
95 (Bio-Rad Laboratories).

96 Resistance gene patterns of the above selected strains were identified by AMR05 PCR-  
97 microarray (<http://alere-technologies.com/en/products/lab-solutions/amr-ve-genotyping.html>),  
98 designed to detect among others several plasmidic genes conferring resistance to  
99 aminoglycosides,  $\beta$ -lactams (including extended spectrum  $\beta$ -lactams), quinolones and  
100 tetracyclines, as well as genes associated to class 1 and class 2 integrons (Batchelor et al.,  
101 2008). Array spots were read with ArrayTube Reader ATR03 and the signals were detected  
102 and analyzed using IconoClust 2 software, with the positive threshold values set at  $\geq 0.4$ .

### 103 2.3. Sequence analysis of specific regions and PCR-typing of plasmid pSI54/04

104 The deposited genome contigs of the emerging strain SI54/04 (Olasz et al., 2015) were  
105 used for the extraction of specific regions of plasmid pSI54/04 with functions in antimicrobial  
106 resistance, virulence, plasmid maintenance and transfer. As a reference for the assembly of  
107 these plasmidic regions, the corresponding contigs (ASRF01000099 - ASRF01000108) of the  
108 published megaplasmid pESI from an Israeli MDR strain of human *S. Infantis* were used  
109 (Aviv et al., 2014). Bioinformatic analysis was performed by Geneious 9.0.5 software  
110 package (Biomatters Ltd), for pairwise alignment the inbuilt application Geneious Alignment  
111 was used under default settings.

112 In order to identify pSI54/04 and its possible variants, we developed a PCR typing  
113 system with primers designed to identify marker genes from each specific regions of the  
114 prototype plasmid pSI54/04. Primers and the corresponding target genes are presented in  
115 Table 1. Primers were tested for specificity against the NCBI nucleotide database by using  
116 BLAST. Broiler and human strains of *S. Infantis* that carried a large plasmid of  $>168$  kb were  
117 subjected to pSI54/04 typing by using the above PCR system. The emerging strain SI54/04  
118 and the plasmid-free pre-emergent strain SI69/94 were included as positive and negative  
119 controls. Simplex PCR reactions were performed in a final volume of 25  $\mu$ l, containing 0.6 u  
120 of PCR BIO Taq DNA polymerase, 5  $\mu$ l of 5 $\times$  PCR BIO Reaction buffer, 400 nM of each  
121 primer (Sigma-Aldrich), and 2  $\mu$ l of template DNA. Amplifications were performed in a BIO-  
122 RAD iCycler PCR system, and reaction conditions were uniform for all set of primers: 94  $^{\circ}$ C  
123 for 3 min, 35 $\times$  94  $^{\circ}$ C for 30 s, 60  $^{\circ}$ C for 1 min, 72  $^{\circ}$ C for 30 s and a final extension at 72  $^{\circ}$ C  
124 for 10 min. Detection of genes *tet(A)*, *intI1* and *sulI* considered as prime markers for  
125 pSI54/04, were performed by the above PCR-microarray (Batchelor et al., 2008). Plasmid  
126 incompatibility of pSI54/04 was determined by PCR-based replicon typing (PBRT) developed  
127 by Carattoli et al. (2005).

128 2.4. Conjugative transfer of plasmid pSI54/04 and *S. Infantis* strains used for pathogenicity  
129 testing and the setup of *in vitro* and *in vivo* experiments

130 To model the scenario of a possible transfer of pSI54/04 into a plasmid-free strain, the  
131 plasmid pSI54/04 was conjugated from the wild type (wt) strain SI54/04 into SI69/94 wt. The  
132 donor strain SI54/04 was resistant to both nalidixic acid and rifampicin (Nal-Rif). As the  
133 recipient strain SI69/94 was also resistant to Rif, the plasmid pST76-C having a  
134 thermosensitive pSC101ts replication system and *catA1* gene, was transiently introduced by  
135 electroporation. This plasmid confers chloramphenicol (Chl) resistance for the  
136 SI69/94:pST76-Chl transformants grown at 30°C, but can easily be eliminated at 42°C (Pósfai  
137 et al., 1997). The transfer of plasmid pSI54/04 into SI69/94:pST76-Chl transformants was  
138 performed by conjugation. Transconjugant colonies were selected on LB plates supplemented  
139 with TetStrChl, incubated overnight at 30°C. Plasmid pST76-C then was cured from four  
140 StrSpcTetChl transconjugants by spreading onto LB/StrSpcTet plates incubated overnight at  
141 42°C. Single colonies were tested for the loss of Chl resistance marker and one resulting  
142 SI69/94:pSI54/04 transconjugant (StrSpcTet), was designated as HP1834 and selected for  
143 subsequent *in vitro* and *in vivo* pathogenicity studies using the emerging strain SI54/04  
144 carrying pSI54/04 and the pre-emergent plasmid-free strain SI69/94 as controls. For testing *in*  
145 *vitro* and *in vivo* pathogenicity, chicken embryo fibroblasts and day old chicks were infected  
146 with the above three strains marked for these purposes with nalidixic acid (Nal) resistance.

147 2.5. Infection of chicken embryo fibroblasts

148 Chicken embryo fibroblast (CEF) cell cultures were purchased from the Virology  
149 Laboratory, Veterinary Diagnostic Directorate of the National Food Chain Safety Office of  
150 Hungary, where freshly prepared cultures of CEFs are routinely used for virus isolation. CEFs  
151 were prepared from 12-day old chicken embryos of the Leghorn breed and maintained in  
152 MEM (Sigma-Aldrich) with 5% fetal calf serum (FCS) until infection. Handling of chicken  
153 embryos was performed in accordance with the relevant Hungarian legislation (Animal  
154 Protection and Welfare Act No. 103/2002).

155 The day before infection, CEFs were seeded into 36 mm Petri dishes (Nunc) and grown  
156 for 18 hours at 37°C under 5% CO<sub>2</sub>. The invasiveness of *S. Infantis* strains in CEFs was  
157 tested as described by Barrow et al. (1989). Briefly, on the second day of growth, semi-  
158 confluent cell cultures were washed 3× with HBSS (Sigma-Aldrich) and MEM was replaced  
159 with DMEM (Sigma-Aldrich) with 5% fetal calf serum and 1% D-mannose. CEFs were

160 infected for 2 h at 37°C and 5% CO<sub>2</sub> with overnight bacterial cultures at a multiplicity of  
161 infection (MOI) equal to 10. After the incubation CEFs were washed 3× with HBSS and  
162 incubated for 1.5 h at 37 °C and 5% CO<sub>2</sub> in MEM (Sigma-Aldrich) containing kanamycin  
163 250 µg/ml to eliminate extracellular bacteria. Finally, CEFs were digested with 0.025%  
164 trypsin (Sigma-Aldrich) and 1% Tween 20 (Sigma-Aldrich) in 0.01M NaH<sub>2</sub>PO<sub>4</sub> (pH: 8) for  
165 30 min at 37 °C. Intracellular *S. Infantis* counts were determined by plating 10× dilutions on  
166 BTB plates. Infection with each of the strains was performed in two different experiments,  
167 with two replicates each.

#### 168 2.6. Infection of day old broiler chicks with *S. Infantis*

169 For testing *in vivo* pathogenicity, 6 newly hatched broiler chickens (SPF, Ross308) per  
170 group were orally inoculated with 0.5 ml of the above three Nal resistant strains of *S. Infantis*.  
171 The infectious dose was ~10<sup>7</sup> CFU/animal and the infected chickens were killed humanely at  
172 5 days post infection. The control group consisted of 6 non-infected chickens killed on day 4  
173 of life, when the *Salmonella*-free status of the control group was also confirmed (ISO 6579).

174 During sample processing, the cecal content and the spleen as well as 0.2g of the liver  
175 were removed aseptically from each bird. Cecal content was re-suspended in Rappaport  
176 Vassiliadis (RV) enrichment broth (1:10) containing nalidixic acid (50 µg/ml). From these  
177 suspensions, 10× dilutions were made from which 10 µl aliquots were plated onto nalidixic  
178 acid-selective BTB agar plates in order to determine the *Salmonella* colonization in the cecum  
179 (CFU/g). Liver and spleen samples were homogenized by using a STOMACHER 80 (Seward,  
180 Labsystem, UK) tissue homogenizer, in RV (1:50) supplemented with nalidix acid, and were  
181 further processed similar to the cecal samples in order to determine the organ invasion  
182 (CFU/g of *Salmonella* in the parenchymal organs). *In vivo* pathogenicity was tested in two  
183 independent experiments. The specific experiments were approved by the Food Chain Safety  
184 and Animal Health Directorate of the Pest County Government Office, Budapest (No. IV-I-  
185 001/229-4/2012).

#### 186 2.7. Statistical analysis

187 Statistical analysis concerning the prevalence of antimicrobial resistance phenotypes  
188 between 2011 and 2013, was performed by using the built-in regression analysis tool of the  
189 Analysis ToolPak for Excel. Determination coefficient ( $r^2$ ) values higher than 0.7 were



190 considered as indicating strong relation between the year of isolation and prevalence.  
191 Resistance patterns present in less than 5% of the strains were not included in the analysis.

192 Statistical analysis of the pathogenic potential was performed by the Student's t-test.  
193 Analysis aimed to detect differences in CEF invasion and cecal colonization abilities of the *S.*  
194 *Infantis* strains tested. Significant differences were considered at  $p < 0.05$ .

### 195 3. Results

#### 196 3.1. Multiresistance phenotype of Nal-Sul-Tet is predominant in recent isolates of *S. Infantis*

197 Antimicrobial susceptibility testing of the 186 epidemiologically unrelated strains of *S.*  
198 *Infantis* from broilers and humans showed that 69 (75.8%) broiler and 57 (60.0%) human  
199 strains were resistant against multiple antimicrobial compounds representing at least three  
200 different antimicrobial classes.

201 In general a large diversity of antimicrobial resistance patterns were observed for the  
202 tested strains of *S. Infantis*, but only four of these patterns reached a mean prevalence of  $>5\%$   
203 with nalidixic acid resistance in common (Fig. 1). The MDR pattern of nalidixic acid-  
204 sulfonamide-tetracycline (Nal-Sul-Tet) was predominant both in broiler and in human  
205 collections with a mean prevalence of 69.3% and 50.8% respectively in the three years period.  
206 In the prevalence of the above dominant resistance pattern an increasing trend was observed  
207 between 2011 and 2013, ranging from 64.5% to 71.9% among broiler isolates ( $r^2=0.80$ ) and  
208 from 35.7% to 65.6% among isolates from humans ( $r^2=0.99$ ) (Fig. 1).

209 The closely related resistance pattern of Nal-Sul, however was decreasing overtime  
210 particularly in humans ( $r^2=0.93$ ). In contrast to this, the prevalence of nalidixic acid resistance  
211 showed differing tendency between 2011-2013, and was increasing in broilers (from 6.5% to  
212 18.8%) but decreasing in humans (from 21.4% to 9.4%) ( $r^2=0.80-0.97$ ). Trimethoprim  
213 resistance (Tmp) was detected only in *S. Infantis* from broilers, in association with the  
214 multiresistance pattern Nal-Sul-Tet (12.9%). None of the broiler strains but 23.6% of the  
215 human strains were susceptible to all antimicrobials tested (Fig. 1).

#### 216 3.2. Molecular epidemiology of the plasmid pSI54/04 of emerging *S. Infantis*

217 PFGE analysis of selected strains ( $n=78$ ) of broiler and human origin revealed a  
218 diversity of clonal lineages, but three clones (B2, B1 and A1, in decreasing order) were  
219 regarded as major clones. The rest of them were represented by one isolate each. Strains from

220 clones B2 and B1 demonstrated the most prevalent MDR phenotype of Nal-Sul-Tet (Fig. S1,  
221 Table S2). A 54.8% of the broiler and 61.7% of the human strains were grouped into the  
222 PFGE clone B2 (17 and 29 strains respectively). Clone B1 was also common in broilers and  
223 humans (22.6% and 12.8%, with 7 and 6 strains) while clone A1 comprising the pansensitive  
224 isolates (susceptible to all antimicrobials tested) was identified in the human group only  
225 (12.8%, with 6 strains). There were further 12 minor clones identified, with one isolate each  
226 (Fig. S1, Table S2). Resistance to ampicillin was associated with a number of MDR  
227 phenotypes, and related strains were grouped into multiple clones (Table S2). Plasmid profile  
228 analysis resulted in the identification of large plasmids in 68 (32 broiler and 36 human) out of  
229 the 78 strains, indicating that these 68 strains may carry the ~277 kb plasmid pSI54/04 or its  
230 variants. This was confirmed and extended by PCR typing using the primers designed to  
231 identify representative genes from each specific region of pSI54/04.

232 PCR typing detected the genes *tet(A)* (tetracycline resistance), *merA* (mercury  
233 resistance), *intI1-aadA1-sulI* (class 1 integron), *tehA* (tellurite resistance) as appropriate  
234 marker genes for the antimicrobial- and heavy metal resistance regions of the prototype  
235 plasmid pSI54/04. Furthermore the genes *irp1-fyuA* (yersiniabactin), *htrE-faeI-pefC* (fimbrial  
236 proteins) were assigned as markers representing major virulence regions of this MDR  
237 plasmid. All these marker genes of pSI54/04 were shown by PCR to be present in the  
238 pSI54/04 transconjugant of the pre-emergent strain SI69/94 (designated as HP1834) but none  
239 of them, were present in the strain SI69/94. Same PCR typing results were obtained on the  
240 pSI54/04 transconjugant of *E. coli* K12 strain J5-3 (results not shown).

241 From the 68 strains of *S. Infantis* subjected for pSI54/04-typing, the co-existence of the  
242 above resistance and virulence marker genes of pSI54/04 prototype plasmid was identified in  
243 a total of 54 strains (23 of 32 from broilers and 31 of 36 from humans). The pSI54/04  
244 prototype was mostly related to PFGE clone B2, and was associated with the predominant  
245 MDR phenotype Nal-Sul-Tet (Table 2, Fig. S1). We found that the specific virulence pattern  
246 of pSI54/04 was stable in all strains tested, but the resistance regions were more variable.  
247 Deletion of consecutive genes *tet(A)-merA-intI1-aadA1-sulI* (variant 1) were detected in  
248 some broiler and human strains belonging mostly to clone B1. The rest of the variants  
249 occurred in some individual isolates and were characterized by the deletion of *tet(A)*, *intI1* or  
250 *aadA1* genes. In case of multiplasmidic strains, the plasmid pSI54/04 was associated with  
251 plasmids carrying *bla*<sub>TEM-1</sub> and *qnrS* genes conferring resistance to ampicillin and  
252 ciprofloxacin (Table 2). In one of the pSI54/04 negative strains representing clone A5 a large  
253 MDR plasmid of ~180 kb was identified, mediating resistance to Amp-Chl-Kan-Sul-Tet-Tmp

254 associated with *intI1*, *sull*, *aadA4*, *catA1*, *strB*, *sul2*, *tet(B)*, *bla*<sub>TEM-1</sub> resistance genes (Table  
255 S2).

256 Concerning the above functional regions and genes assigned for PCR typing of plasmid  
257 pSI54/04, we found in 89.4-100% sequence similarity between the Hungarian broiler MDR  
258 plasmid pSI54/04 and the Israeli human megaplasmid pESI of *S. Infantis*. The PCR-based  
259 replicon typing indicated that plasmid pSI54/04 belongs to the incompatibility group IncP.

260 *3.3.pSI54/04 did not increase pathogenic potential of S. Infantis in chicken fibroblasts and in*  
261 *day old chicks*

262 For testing pathogenic potential of pSI54/04, the two wild type strains, the plasmidic  
263 SI54/04 and the plasmid-free SI69/94 were compared with the plasmidic transconjugant of  
264 SI69/94 designated as HP1834 in terms of CEF invasion and cecal colonization in day old  
265 chicks. *In vitro* infection of CEFs resulted in a significantly ( $p < 0.04$ ) reduced invasion for  
266 both plasmidic strains SI54/04 and HP1834 (4.08 and 4.84  $\log_{10}$  CFU/ml) in comparison with  
267 the plasmid-free strain SI69/94 (4.98  $\log_{10}$  CFU/ml). In orally infected day old broiler chicks  
268 these three *S. Infantis* strains were colonizing the intestine in high numbers (7.22-7.86  $\log_{10}$   
269 CFU/g) without any significant differences but with somewhat reduced colonization by the  
270 plasmidic strains SI54/04 and HP1834 in comparison with the plasmid-free strain SI69/94  
271 (Fig. 2). None of the three strains proved to be invasive for parenchymal organs.

#### 272 4. Discussion

273 According to the Commission Regulation (EC) No 1003/2005, *S. Infantis* is part of the  
274 community target for the reduction of the prevalence of certain *Salmonella* serovars in  
275 breeding flocks of *Gallus gallus* as one of the five most frequent *Salmonella* serovars in  
276 human salmonellosis. The prevalence of the most critical serovars *S. Enteritidis* and *S.*  
277 *Typhimurium* have been essentially reduced due to the effective prevention systems aimed  
278 primarily to these two serovars. Simultaneously however, *S. Infantis* became widespread in  
279 the broiler flocks of several European countries (EFSA and ECDC, 2015), and outside Europe  
280 (Asai et al., 2006; Shahada et al., 2006; Gal-Mor et al., 2010). The increased prevalence of *S.*  
281 *Infantis* among broilers could partly be a consequence of decreasing prevalence of *S.*  
282 *Enteritidis* due to reduction of intestinal inflammatory responses and/or changes in chicken  
283 microbiome potentially favourable for colonization by *S. Infantis* (Videnska et al., 2013; Mon  
284 et al., 2015).

285 However, in most countries there have been a switch in the epidemiology of *S. Infantis*  
286 strains around the late 1990s early 2000s associated with the increased incidence of  
287 multiresistant (MDR) strains and clonal lineages. In Hungary, results of N6gr6dy et al. (2007;  
288 2008) reported the emergence of the multiresistant PFGE cluster B of *S. Infantis* in human  
289 and broiler populations, characterized by a large (>168 kb) conjugative MDR plasmid  
290 carrying class 1 integron and *tet(A)* gene. This was followed by very similar reports from  
291 Israel (Gal-Mor et al. 2010), Japan (Shahada et al., 2008; 2010) and from several EU  
292 countries with additional appearance and dissemination of  $\beta$ -lactam-resistant isolates  
293 (Cloeckaert et al., 2007; Dionisi et al., 2011; N6gr6dy et al., 2012). Although these reports  
294 were indicating the role of large MDR plasmids in the emergence and spread, the molecular  
295 characterization of such plasmids encoding putative antimicrobial resistance, virulence and/or  
296 survival functions of the *S. Infantis* strains was lacking until recently. Such determinants of  
297 the megaplasmid pESI characterizing emergent MDR strains of *S. Infantis* of human origin  
298 have been reported from Israel (Aviv et al., 2014).

299 Due to the high prevalence of *S. Infantis* in broiler carcasses and in broiler flocks in  
300 Hungary (EFSA, 2007; 2010), we have been focusing on epidemiologic markers of *S. Infantis*  
301 possibly related to this increase of multiresistance. Therefore we aimed to provide molecular  
302 characterization of plasmid pSI54/04 endemic in *S. Infantis* from broilers in Hungary, and  
303 distribution of associated antimicrobial resistance and virulence determinants within the  
304 endemic clones of Hungarian isolates of *S. Infantis* of broiler and human origin. In order to  
305 understand the background for the exceptionally high prevalence of *S. Infantis* in broiler  
306 flocks of Hungary, we also wanted to explore the pathogenic significance of this large  
307 conjugative MDR plasmid.

308 Results on molecular typing of pSI54/04 typing based on the selected marker genes for  
309 resistance and virulence indicated that the pSI54/04 prototype plasmid was mostly related to  
310 PFGE clones B2 and B1 and was associated with the predominant MDR phenotype Nal-Sul-  
311 Tet. The deletion of consecutive genes *tet(A)-merA-intI1-aadA1-sul1* (variant 1) were  
312 detected in some broiler and human strains belonging mostly to clone B1. In case of three  
313 multiplasmidic strains representing clone B1 and B2, the plasmid pSI54/04 was associated  
314 with smaller plasmids (~45 kb) carrying *bla*<sub>TEM-1</sub> and/or *qnrS* genes indicating potentially new  
315 emerging PFGE subclones. However these seemed to be different from those described by  
316 Kehrenberg et al. (2006), Dionisi et al. (2011) or Kameyama et al. (2012) for *S. Infantis* of  
317 broiler origin in Germany, Italy and Japan respectively.

318 Concerning specific functional regions and genes assigned as resistance and virulence  
319 markers, plasmid pSI54/04 of Hungarian broiler origin has shown a high sequence similarity  
320 with the Israeli megaplasmid pESI of human origin (Aviv et al., 2014). PBRT indicated IncP  
321 incompatibility for pSI54/04, although it seems that the functional incompatibility group of  
322 certain MDR plasmids of *S. Infantis* is difficult to determine (Aviv et al., 2014; Dionisi et al.,  
323 2016).

324 The source of human infections by *S. Infantis* has been mainly related to poultry meat.  
325 The carcass prevalence of *S. Infantis* was 85% in Hungary (EFSA, 2010) and it was around  
326 50% in poultry processing plants in Israel (Gal-Mor et al., 2010). In spite of this, the reported  
327 incidence of human infections caused by *S. Infantis* in Hungary and Israel were quite  
328 different: 4.4 cases of *S. Infantis* infection/100,000 in Hungary in contrast to 14.7/100,000 in  
329 Israel, representing 8.6% and 34% of *Salmonella* isolates respectively (Anonymous, 2016;  
330 Gal-Mor et al., 2010). One explanation for this discrepancy could theoretically be a higher  
331 pathogenicity of the *S. Infantis* strains prevalent in Israel (Aviv et al., 2014), but several other  
332 factors such as differences in the infectious disease surveillance systems, as well as  
333 differences in climatic conditions could also play their role.

334 Testing pathogenicity of the emerging plasmidic *S. Infantis* strain SI54/04 of broiler  
335 origin in comparison with a pre-emergent strain SI69/94 and its pSI54/04 transconjugant on  
336 CEFs and on orally infected day old chicks, showed reduced *in vitro* invasion and *in vivo*  
337 colonization by the pSI54/04 transconjugant strain. This reduction of pathogenic potential as  
338 compared to the plasmidless pre-emerging strain SI69/94 indicated that the pathogenicity of  
339 *S. Infantis* could not be influenced by the plasmid pSI54/04 in chicken host, in contrast to the  
340 results about pathogenic significance of pESI in a mouse model (Aviv et al. 2014). One  
341 reason for a possible differing pathogenic potential of the Hungarian and the Israeli strains  
342 could also be in their differing host adaptation (Alvarez-Ordóñez et al., 2015; Langridge et al.,  
343 2015). In case of *S. Infantis*, the adaptation to the chicken host could have resulted in a more  
344 attenuated host-pathogen interaction in chicken models. The degrees of pathogenicity for  
345 different hosts (i.e. for human, mouse or chicken) may also differ between clusters of *S.*  
346 *Infantis* as it has been reported by Yokoyama et al. (2015).

347 As the Hungarian strain SI54/04 of *S. Infantis* used for pathogenicity studies have been  
348 of broiler origin, the choice of using primary CEF cells and day old broiler chick models for  
349 *in vitro* and *in vivo* pathogenicity testing of our *S. Infantis* strains was appropriate. These  
350 systems have already been useful in our earlier studies on poultry strains of *Salmonella*  
351 *Enteritidis* as well (Imre et al., 2015; Szmolka et al., 2015). On the other hand, it could also be

352 hypothesized that the predominant *S. Infantis* strains in Israel could have more efficiently  
353 utilized the specific virulence determinants of the plasmid pESI in a mouse model and in  
354 immortalized cell lines thereby enhancing pathogenicity and stress tolerance of the recipient  
355 strains (Aviv et al., 2014). However, elucidation of the role of these factors would need a  
356 systematic comparative study in the same *in vitro* and *in vivo* models, using the same parental  
357 *S. Infantis* strains and their transconjugants containing pSI54/04 and pESI respectively,  
358 produced by the same methods and verified for both plasmids by detailed molecular analysis.  
359 Such detailed comparative studies on these two plasmids and their carrier *S. Infantis* strains  
360 were outside the scope of our molecular epidemiology approach.

## 361 5. Conclusion

362 These results of molecular epidemiologic studies on the plasmid pSI54/04 are extending  
363 the earlier reports from Hungary, Israel and from Japan on the significance of large MDR  
364 plasmids in dissemination and persistence of emerging *S. Infantis* clones. Our findings  
365 indicate that the plasmid pSI54/04 contributes to the multiresistance of endemic clones of *S.*  
366 *Infantis* in Hungary. Concerning specific regions with functions in antimicrobial resistance,  
367 virulence, plasmid maintenance and transfer plasmid pSI54/04 shares a high sequence  
368 similarity with the plasmid pESI endemic in *S. Infantis* from humans in Israel, thus, it could  
369 also be referred to as a pESI-like plasmid. The transfer of pSI54/04 to a pre-emergent *S.*  
370 *Infantis* of broiler origin did not increase the pathogenic potential of this strain in the *in vitro*  
371 and *in vivo* chicken models. Thus, plasmid pSI54/04 assisted dissemination of *S. Infantis* by  
372 antimicrobial resistance determinants rather than by the virulence genes. Our studies also  
373 highlighted that the emergence of plasmids *bla*<sub>TEM-1</sub> and *qnrS* mediating  $\beta$ -lactam- and  
374 fluoroquinolone resistance, that have already been described in other countries, may become  
375 more prevalent in the Hungarian broiler and human populations in the future.

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#### 384 **Conflict of interest statement**

385 The authors have no financial or commercial conflicts of interest to declare.

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499

500 **Figure legends**

501 **Fig. 1.** Prevalence of associated antimicrobial resistance phenotypes of recently isolated *S.*  
502 *Infantis* of broiler and human origin. A total of 186 *S. Infantis* strains from the above sources,  
503 isolated between 2011-2013 were clustered according to the antimicrobial resistance patterns.  
504 Most frequent resistance phenotypes with a prevalence of >5% are presented within the  
505 frame.

506 **Fig. 2.** *In vitro* and *in vivo* pathogenic potential of emerging plasmidic and the pre-emergent  
507 plasmid-free strains of *S. Infantis*. For testing pathogenic potential of pSI54/04, the two  
508 reference wild strains, the plasmidic SI54/04 and the plasmid-free SI69/94 were compared  
509 with the plasmidic transconjugant of SI69/94 (designated as HP1834) in terms of CEF  
510 invasion and cecal colonization in day old chicks.

511

## Tables

**Table 1.** Specific regions and marker genes of prototype plasmid pSI54/04 used as targets for PCR-based plasmid detection and typing.

Gene	Description	pSI54/04 target region	Forward primer sequence	Reverse primer sequence	Amplicon (bp)
<i>merA</i>	mercuric ion reductase	antimicrobial resistance	GATGTCTCCTACGCCAAGGG	CCTATAGCTGGGTCTTCGCG	842
<i>tehA</i>	tellurite resistance protein <i>tehA</i>	heavy metal resistance	CAAGGAGCTGTGTGGTTTGC	GTATTATCCCCTGGAGCCGC	642
<i>repA</i>	DNA replication protein	plasmid maintenance	CACTTTGTCCTTTCGCGGTG	TAAGGTCTGGCTCGGGATCA	616
<i>irp1</i>	iron acquisition yersiniabactin synthesis enzyme	iron acquisition	GAAACGCCACACTTCACCAC	AGCACGTTGTCCATGCAGTA	581
<i>fyuA</i>	iron acquisition outer membrane yersiniabactin receptor (FyuA, Psn, pesticin receptor)	iron acquisition	TGGGAAATACACCACCGACG	* CGCAGTAGGCACGATGTTGTA	541
<i>pefC</i>	plasmid encoded fimbriae	adhesion	CGGCATCATTCCCGAGTACA	AGGCCGGGATTTATGTCAGC	587
<i>faeI</i>	K88 minor fimbrial subunit <i>faeI</i> precursor	adhesion	TGCAGGTCGTCATACAACCC	AATCCCTGGGTCTGGAAGGT	365
<i>htrE</i>	outer membrane usher protein <i>htrE</i> precursor	adhesion	TTCCCGTCTCCTCTGCTGTA	ACTGGCTGCCTACCGGTATA	414

\* Schubert et al., 1998

**Table 2.** Prototype plasmid pSI54/04 and its variants with differing gene patterns in antimicrobial- and heavy metal resistance regions. Gene pattern and the plasmidic environment of pSI54/04 and its variants are presented in relation with the PFGE clone.

pSI54/04-like plasmids	Resistance genes of pSI54/04					Virulence genes of pSI54/04					pSI54/04 range (~ kb)	Associated plasmidic resistance genes	PFGE clone (n)	Origin (n)	
	<i>tet(A)</i>	<i>merA</i>	<i>intI1</i>	<i>aadA1</i>	<i>sul1</i>	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>					<i>pefC</i>
pSI54/04 prototype	<i>tet(A)</i>	<i>merA</i>	<i>intI1</i>	<i>aadA1</i>	<i>sul1</i>	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>	<i>pefC</i>	190-225	-	B2(43), B1(4), B3, A1, L1, M1, N1, O1, P1	broiler (23) human (31)
variant 1	-	-	-	-	-	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>	<i>pefC</i>	225	<i>bla</i> <sub>TEM-1</sub>	B2	broiler (1)
variant 2	-	<i>merA</i>	<i>intI1</i>	<i>aadA1</i>	<i>sul1</i>	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>	<i>pefC</i>	180	<i>bla</i> <sub>TEM-1</sub> , <i>qnrS</i>	B1	human (1)
variant 3	<i>tet(A)</i>	<i>merA</i>	-	<i>aadA1</i>	<i>sul1</i>	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>	<i>pefC</i>	175-200	-	B2(2), B1(6), B10	broiler (6) human (3)
	<i>tet(A)</i>	<i>merA</i>	-	<i>aadA1</i>	<i>sul1</i>	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>	<i>pefC</i>	225	-	B1	broiler (1)
	<i>tet(A)</i>	<i>merA</i>	-	<i>aadA1</i>	<i>sul1</i>	<i>tehA</i>	<i>irp1</i>	<i>fyuA</i>	<i>htrE</i>	<i>faeI</i>	<i>pefC</i>	200	<i>bla</i> <sub>TEM-1</sub>	B1	human (1)

Plasmids carrying the gene *bla*<sub>TEM-1</sub> were ~45 kb large in size.

**Supporting information legends**

**Table S1.** List of the 186 isolates of *S. Infantis* of broiler and human origin included in this study. Strains are grouped according to the resistance phenotype. Grey background indicates strains selected for further molecular characterization (n=78).

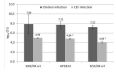
**Table S2.** Antimicrobial resistance pheno- and genotype of *S. Infantis* of broiler and human origin in major and minor PFGE clones. Grey background indicates strains carrying pSI54/04 plasmid or its variants.

**Fig. S1.** Distribution of PFGE clones of *S. Infantis* of broiler and human origin.



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**Highlights**

to the this manuscript entitled ‘Molecular epidemiology of the endemic multiresistance plasmid pSI54/04 of *Salmonella* Infantis in broiler and human population in Hungary’

- plasmid pSI54/04 contributes to multiresistance in recent isolates of *S. Infantis*
- pSI54/04 and plasmid pESI of *S. Infantis* in Israel share high sequence similarity
- variants of pSI54/04 differ in antimicrobial- and heavy metal resistance regions
- transfer of pSI54/04 did not increase pathogenic potential of *S. Infantis*