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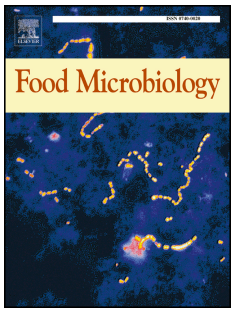

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Molecular epidemiology of the endemic multiresistance plasmid pSI54/04 of *Salmonella* Infantis in broiler and human population in Hungary

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Abstract

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

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

1. Introduction

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

Dissemination of multidrug resistant (MDR) S. Infantis in Belgian poultry carrying a $bla_{\text{TEM-52}}$ plasmid has been reported by Cloeckaert et al. (2007). The first evidence for clonal spread of *S.* Infantis strains in broiler and in human populations with a large conjugative

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

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

2. Materials and methods

2.1. Strains of Salmonella Infantis and antimicrobial resistance phenotyping

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

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

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

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

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

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

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

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

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

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

In order to identify pSI54/04 and its possible variants, we developed a PCR typing system with primers designed to identify marker genes from each specific regions of the prototype plasmid pSI54/04. Primers and the corresponding target genes are presented in Table 1. Primers were tested for specificity against the NCBI nucleotide database by using BLAST. Broiler and human strains of *S.* Infantis that carried a large plasmid of >168 kb were subjected to pSI54/04 typing by using the above PCR system. The emerging strain SI54/04 and the plasmid-free pre-emergent strain SI69/94 were included as positive and negative controls. Simplex PCR reactions were performed in a final volume of 25 µl, containing 0.6 u of PCRBIO Taq DNA polymerase, 5 µl of 5× PCRBIO Reaction buffer, 400 nM of each primer (Sigma-Aldrich), and 2 µl of template DNA. Amplifications were performed in a BIO-RAD iCycler PCR system, and reaction conditions were uniform for all set of primers: 94 °C for 3 min, 35× 94 °C for 30 s, 60 °C for 1 min, 72 °C for 30 s and a final extension at 72 °C for 10 min. Detection of genes *tet*(A), *intI1* and *sul1* considered as prime markers for pSI54/04, were performed by the above PCR-microarray (Batchelor et al., 2008). Plasmid incompatibility of pSI54/04 was determined by PCR-based replicon typing (PBRT) developed by Carattoli et al. (2005).

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

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

2.5. Infection of chicken embryo fibroblasts

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

The day before infection, CEFs were seeded into 36 mm Petri dishes (Nunc) and grown for 18 hours at 37°C under 5% CO2. The invasiveness of *S.* Infantis strains in CEFs was tested as described by Barrow et al. (1989). Briefly, on the second day of growth, semi-confluent cell cultures were washed 3× with HBSS (Sigma-Aldrich) and MEM was replaced with DMEM (Sigma-Aldrich) with 5% fetal calf serum and 1% D-mannose. CEFs were

infected for 2 h at 37°C and 5% CO2 with overnight bacterial cultures at a multiplicity of infection (MOI) equal to 10. After the incubation CEFs were washed 3× with HBSS and incubated for 1.5 h at 37 °C and 5% CO2 in MEM (Sigma-Aldrich) containing kanamycin 250 μg/ml to eliminate extracellular bacteria. Finally, CEFs were digested with 0.025% trypsin (Sigma-Aldrich) and 1% Tween 20 (Sigma-Aldrich) in 0.01M NaH_2PO_4 (pH: 8) for 30 min at 37 °C. Intracellular *S.* Infantis counts were determined by plating 10× dilutions on BTB plates. Infection with each of the strains was performed in two different experiments, with two replicates each.

2.6. Infection of day old broiler chicks with S. Infantis

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

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

2.7. Statistical analysis

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

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

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

3. Results

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

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

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

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

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

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

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

PCR typing detected the genes *tet*(A) (tetracycline resistance), *merA* (mercury resistance), *intI1-aadA1-sul1* (class 1 integron), *tehA* (tellurit resistance) as appropriate marker genes for the antimicrobial- and heavy metal resistance regions of the prototype plasmid pSI54/04. Furthermore the genes *irp1-fyuA* (yersiniabactin), *htrE-faeI-pefC* (fimbrial proteins) were assigned as markers representing major virulence regions of this MDR plasmid. All these marker genes of pSI54/04 were shown by PCR to be present in the pSI54/04 transconjugant of the pre-emergent strain SI69/94 (designated as HP1834) but none of them, were present in the strain SI69/94. Same PCR typing results were obtained on the pSI54/04 transconjugant of *E. coli* K12 strain J5-3 (results not shown).

From the 68 strains of *S*. Infantis subjected for pSI54/04-typing, the co-existence of the above resistance and virulence marker genes of pSI54/04 prototype plasmid was identified in a total of 54 strains (23 of 32 from broilers and 31 of 36 from humans). The pSI54/04 prototype was mostly related to PFGE clone B2, and was associated with the predominant MDR phenotype Nal-Sul-Tet (Table 2, Fig. S1). We found that the specific virulence pattern of pSI54/04 was stable in all strains tested, but the resistance regions were more variable. Deletion of consecutive genes *tet*(A)-*merA*-*intI1-aadA1-sul1* (variant 1) were detected in some broiler and human strains belonging mostly to clone B1. The rest of the variants occurred in some individual isolates and were characterized by the deletion of *tet*(A), *intI1* or *aadA1* genes. In case of multiplasmidic strains, the plasmid pSI54/04 was associated with plasmids carrying $bla_{\text{TEM-1}}$ and *qnrS* genes conferring resistance to ampicillin and ciprofloxacin (Table 2). In one of the pSI54/04 negative strains representing clone A5 a large MDR plasmid of ~180 kb was identified, mediating resistance to Amp-Chl-Kan-Sul-Tet-Tmp

associated with *intI1*, *sul1*, *aadA4*, *catA1*, *strB*, *sul2*, *tet*(B), bla_{TEM-1} resistance genes (Table S2).

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

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

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

4. Discussion

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

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

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

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

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

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

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

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

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

5. Conclusion

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

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Conflict of interest statement

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)
merA	mercuric ion reductase	antimicrobial resistance	GATGTCTCCTACGCCAAGGG	CCTATAGCTGGGTCTTCGCG	842
tehA	tellurit resistance protein tehA	heavy metal resistance	CAAGGAGCTGTGTGGTTTGC	GTATTATCCCCTGGAGCCGC	642
repA	DNA replication protein	plasmid maintenance	CACTTTGTCCTTTCGCGGTG	TAAGGTCTGGCTCGGGATCA	616
irp1	iron aquisition yersiniabactin synthesis enzyme	iron aquisition	GAAACGCCACACTTCACCAC	AGCACGTTGTCCATGCAGTA	581
fyuA	iron aquisition outermembrane yersiniabactin receptor (FyuA, Psn, pesticin receptor)	iron aquisition	TGGGAAATACACCACCGACG	* CGCAGTAGGCACGATGTTGTA	541
pefC	plasmid encoded fimbriae	adhesion	CGGCATCATTCCCGAGTACA	AGGCCGGGATTTATGTCAGC	587
faeI	K88 minor fimbrial subunit faeI precursor	adhesion	TGCAGGTCGTCATACAACCC	AATCCCTGGGTCTGGAAGGT	365
htrE	outer membrane usher protein htrE 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)
pSI54/04 prototype	*tet*(A),	*merA*,	*intI1*,	*aadA1*,	*sul1*,	*tehA*	*irp1*,	*fyuA*,	*htrE*,	*faeI*,	*pefC*	190-225	-	B2(43), B1(4), B3, A1, L1, M1, N1, O1, P1	broiler (23) human (31)
												225	$bla_{\text{TEM-1}}$	B2	broiler (1)
												180	$bla_{\text{TEM-1}}$, *qnrS*	B1	human (1)
variant 1			-			*tehA*	*irp1*,	*fyuA*,	*htrE*,	*faeI*,	*pefC*	175-200	-	B2(2), B1(6), B10	broiler (6) human (3)
variant 2	-	*merA*,	*intI1*,	*aadA1*,	*sul1*,	*tehA*	*irp1*,	*fyuA*,	*htrE*,	*faeI*,	*pefC*	225	-	B1	broiler (1)
variant 3	*tet*(A),	*merA*,	-	*aadA1*,	*sul1*,	*tehA*	*irp1*,	*fyuA*,	*htrE*,	*faeI*,	*pefC*	200	$bla_{\text{TEM-1}}$	B1	human (1)

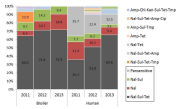
Plasmids carrying the gene $bla_{\text{TEM-1}}$ were ~45 kb large in size.

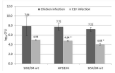
Supporting information legends

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.





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