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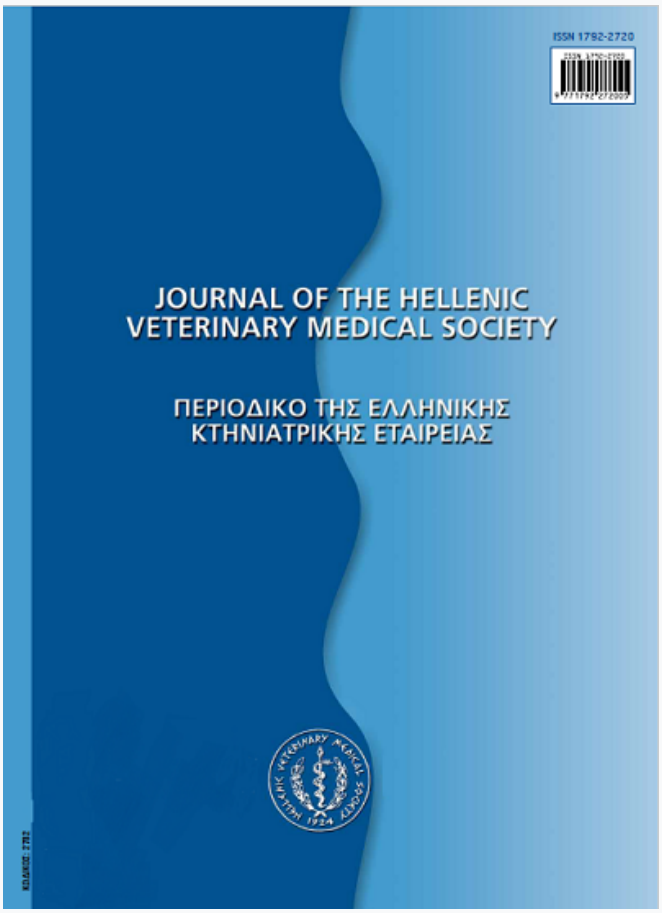

Antibiotic resistance and distribution of SodCI, sopE, sefA genes among Salmonella enteric serotype Enteritidis isolates from poultry

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Antibiotic resistance and distribution of *SodCI*, *sopE*, *sefA* genes among *Salmonella enteric* serotype Enteritidis isolates from poultry

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Αντιβιοανθεκτικότητα και κατανομή των γονιδίων *SodCI*, *sopE*, *sefA* σε στελέχη *Salmonella enterica* serotype *Enteritidis* ορνίθειας προέλευσης

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ABSTRACT. Present work aimed to examine the antibiotic resistance of the *Salmonella enterica* serotype Enteritidis (SE) isolates from poultry, to study the plasmid-mediated ampicillin resistance and to detect and determine the distribution of *sodCI*, *sopE* and *sefA* genes. Thirty-five SE isolates from one-day chicks, layers and broilers were studied for susceptibility/resistance to sixteen antimicrobial agents; 23 (65.7%) of them showed resistance to ampicillin, 5 (14.3%) to ampicillin and tetracycline, 4 (11.45%) to tetracycline and 1 (2.9%) isolate showed multi-drug resistance. Ampicillin (Amp^R) and ampicillin/tetracycline (Amp^RTe^R) resistance was easily transferred by conjugation, and all isolates except two possessed a common band. The molecular mass of the plasmid carrying ampicillin resistance was approximately determined at 41kb after DNA digestion with *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV and *Pst*I restriction enzymes and ligation of *Eco*RI fragments to pET29c. For the detection of TEM-1 or/and TEM-2 ß-lactamases, two pairs of primers were used in a polymerase chain reaction (PCR). The PCR products showed the presence of bla_{TEM-1} gene in all isolates. The presence of *SodCI*, *sopE*, *sefA* genes was also examined by PCR. Twenty-two (62,8%) isolates carried the *sodCI* gene, thirty-four (97,2%) isolates carried the *sopE* gene and all isolates carried the *sefA* fimbrial locus.

Keywords: *S.* enteritidis; plasmid analysis; *sodCI,* sopE and *sefA* genes; $bla_{TEM-1,}$ bla_{TEM-2} genes

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Περίληψη. Η παρούσα εργασία είχε ως στόχο να προσδιορίσει την αντιβιοανθεκτικότητα στελεχών *Salmonella enteric* serotype Enteritidis (SE) ορνίθειας προέλευσης, να μελετήσει την πλασμιδιο-εξαρτώμενη ανθεκτικότητα στην αμπικιλλίνη και να προσδιορίσει την κατανομή μεταξύ των στελεχών των γονιδίων *sodCI*, *sopE* και *sefA*. Τριάντα πέντε SE στελέχη που απομονώθηκαν από νεοσσούς ηλικίας μίας ημέρας, από όρνιθες ωοτοκίας και κρεοπαραγωγής μελετήθηκαν ως προς την ανθεκτικότητα/ευαισθησία σε δεκαέξι αντιβιοτικά. Είκοσι τρία στελέχη (65,7%) εμφάνισαν ανθεκτικότητα στην αμπικιλλίνη, πέντε στελέχη (14,3%) στην αμπικιλλίνη και την τετρακυκλίνη, τέσσερα στελέχη (11,45%) στην τετρακυκλίνη και ένα (2.9%) εμφάνισε πολυανθεκτικότητα. Η ανθεκτικότητα στην αμπικιλλίνη (Amp^R) και το συνδυασμό αμπικιλλίνης/τετρακυκλίνης (Amp^RTe^R) μεταφέρθηκε εύκολα με τη σύζευξη σε όλα τα στελέχη τα οποία – με εξαίρεση δύο- εμφάνισαν μία κοινή πλασμιδιακή ζώνη. Η μοριακή μάζα του πλασμιδίου που έφερε το γονίδιο ανθεκτικότητας στην αμπικιλλίνη προσδιορίστηκε στα 41kbμετά από πέψη του DNA με τα ένζυμα περιορισμού *Bam*HI, *Hind*III, *Eco*RI, *Eco*RV και *Pst*I. Η επαλήθευση του μεγέθους της μοριακής μάζας έγινε με την επίδεση (ligation) στο πλασμίδιο pET29c, των τμημάτων DNA τα οποία προέκυψαν μετά από πέψη με το ένζυμο περιορισμού *Eco*RI. Για την ανίχνευση των TEM-1 ή/και TEM-2 ß-λακταμασών, χρησιμοποιήθηκαν δύο εξειδικευμένα ζεύγη εκκινητών με τη μέθοδο της αλυσιδωτής αντίδρασης της πολυμεράσης (PCR). Τα προϊόντα της PCR έδειξαν την παρουσία του γονιδίου bla_{TEM-1} σε όλα τα στελέχη. Ελέγχθηκε επίσης η παρουσία των γονιδίων of *SodCI*, *sopE*, *sefA* με εξειδικευμένους εκκινητές της PCR. Είκοσι δύο (62,8%) στελέχη έφεραν το γονίδιο *sodCI*, τριάντα τρία (97,2%) το γονίδιο *sopE* και όλα τα στελέχη ήταν φορείς του γονιδίου *sefA*.

Λέξεις ευρετηρίασης: *S.enteritidis,* ανάλυση πλασμιδίου, γονίδια *sodCI,* sopE, *sefA* genes, bla_{TEM-1}, και bla_{TEM-2}

INTRODUCTION

Salmonella enterica serotype Enteritidis (SE) is one of the two most frequent etiological agents of human foodborne salmonellosis. Contaminated poultry facilities is the main vehicle of SE, ranging from 29% to 34% of all *Salmonella* infections (Sarna et al. 2002, CDSC 2004; Altekruse et al., 2006; Braden, 2006; Jain and Chen, 2006; Linam and Gerber, 2007; CDC 2013; Henriques et al., 2013).

Most SE isolates harbour serovar-associated factors as the virulence, the fibrial and the antibiotic resistance genes (Lu et al., 1999; Anğu-Küçüker et al., 2000; Bakshi et al., 2003). Virulence genes encode products that assist organisms to express the virulence in the host cells. Some genes as *Sod* and *Sop* are associated with the survival in the host system or in the actual manifestation of pathogenic processes and others as *Sef* genes, are involved in adhesion and invasion (Murugkar et al., 2003). As for *Sef* genes, the *sef14* fimbriae operon contains four structure genes (*sefABCD*) required for the translocation and biogenesis and the *sefA* gene, encodes the main subunit of the SEF14 fimbrial protein in SE (Turcotte and Woodward, 1993; Lopes et al., 2006; Zhu et al., 2010). Virulent serovars of *S. enterica* possess two different periplasmic [Cu, Zn] superoxide dismutases, the SodCI and the SodCII. The SodCI dismutase, encoded by *SodCI* gene, seems to be of higher importance as it is involved in the defence against oxidative stress, to which SE is exposed when residing intracellularily in macrophages or neutrophils. SopE is a SPI-1-dependent translocated protein that modulates host cell RhoGTPase function. It is located on a cryptic λ-like phage in serotypes Enteritidis, Dublin, Hadar and Gallinarum (Hopkins and Threfall, 2004). Although not studied in such a detail, *sodCI* gene has been reported to be highly associated to the virulence activity of *SE* strains, while *sopE* gene is relatively randomly distributed among *S. enterica* serovars (Karasova et al., 2009).

Antibiotics are used in livestock, including chickens, to treat or prevent disease and to promote growth. The correlation between the use of antibiotics and the emergence of resistance has been documented. Moreover, the increasing rate of antibiotic resistance has been reported from 1993 (Threfall et al., 1993) until recently (Smith et al., 2002; Maripandi and Al-Salamah, 2010; Melendez et al., 2010). A variety of antibiotics such as ampicillins, tetracyclines, sulfonamides and streptomycin, have been used for the treatment of salmonellosis leading to the emergence of resistant or multi-resistant strains (Glynn et al., 1998; Metzer et al., 1998; Buyd

et al., 2002; Snow et al., 2007; Yang et al. 2002). The resistance genes involved in *Salmonella* strains are usually plasmid encoded and they can be transmitted to other bacteria (Foley and Lynne, 2008). These conjugative plasmids have a high molecular weight and as they can disseminate genetic information within cells and species, are of great significance (Rychlik et al., 2006).

In Greece, the most detailed data about antibiotic resistance in humans have been published from '80s to '90s. The frequency of resistance SE isolates, started from a low level (7.9%) in 1987, increased to 30.4% in 1991 and reached a plateau or even decreased in 1997 (Vatopoulos et al., 1994; Tassios et al., 1997; Tassios at al., 1999; Markogiannakis et al., 2000). In 1998, Arvatitidou et al., found that chicken carcasses from the hospitals in Thessaloniki were contaminated by SE strains at a frequency up to 69% while the same period the ampicillin resistant isolates from poultry, were found up to 91.6%. The majority of the SE resistant isolates of poultry origin, were resistant to ampicillin or/and tetracycline (Zdragas, 2001). Similar high prevalence of ampicillin resistant SE isolates from poultry have been reported from many countries in Europe such as France (Llanes et al., 1999), the U.K. (Yates and Amyes, 2005; Papadopoulou et al., 2009), or worldwide as in Korea (Suh and Song, 2006) Pakistan (Mirmomeni et al., 2007) and Tynisia (Abbassi-Ghossi et al., 2011).

The aim of the present work was to examine the antibiotical resistance of the SE isolates from poultry, to study the plasmid-mediated ampicillin resistance and to detect and determine the distribution of *sodCI*, *sopE* and *sefA* genes.

MATERIALS AND METHODS

Bacterial strains

Thirty-five SE isolates obtained from one-day old chicks with high or low mortality ratio, from layers with sporadic egg-peritonitis and from clinically healthy broilers by random sampling in slaughter-houses (G1 Group) or from healthy meat producer broilers, randomly selected from thirty flocks (Group 2). For the selective enrichment of all strains, the modified Rappaport-Vassiliadis broth was used (Vassiliadis, 1983). Isolation of the SE strains was performed according to ISO 6579-2002. Isolated strains were identified biochemically and checked serologically by Polyo A-S (Prolab) antiserum.

Antimicrobial resistance testing

Routine screening for antibiotic susceptibility was performed using Mueller–Hinton agar (Biorad-Marnes-la-Coquette. France) and the disk diffusion method according to the current recommendations of the CLSI (2009), against:Enrofloxacin (ENR), Tetracycline (TE) 30μg, Trimethroprim Sulphamethoxazole (SXT) 1,25/23,75μg, Erythromycin (E) 30μg, Cephalothin(CEF) 30μg, Nalidixic Acid (NA) 30μg, Kanamycin (K) 30μg, Ampicillin (AM) 10μg, Streptomycin (S) 10μg, Floumequin (UB) 30μg, Apramycin (APR) 30μg, Amoxycillin (AMX) 30μg, Ceftriaxone (CRO) 30μg, Neomycin (N) 30ui, Gentamicin (GM) 10μg, Penicillin (P) 10ui. Minimal Inhibitory Concentration (MIC) was performed to resistant strains according to the current recommendations of the Clinical Laboratory and Standards Institute (CLSI, 2009) in a series of twofold antibiotic concentrations in Brain Heart Infusion Broth (Scharlau).

Conjugation

SE isolates, *E. coli* N99 and TOP10F´ recipient cells were grown in LB broth to logarithmic phase. Conjugation experiments were carried out in Brain Heart Infusion (BHI) broth by the filter method (Ekateriniadou et al., 1994) at a 1:10 donor/recipient ratio. In E. coli TOP10F´ cells culture, 50μg/ml S was added. Transconjugants were selected on MacConkey agar containing 50μg/ml AM or 50μg/ml AM + 30μg/ml TE, after 24-48h incubation at 37°C. The transfer frequency was expressed as the number of the transconjugant colonies per donor colony.

Plasmid isolation

Single colonies of the donors and the transconjugants were transferred in 70 ml of BHI and LB broth respectively with 50μg/ml AM or 50μg/ml AM + 30μg/ml TE and were incubated overnight. Plasmid DNA was extracted by the Concert™ High Purity – Plasmid Midiprep System kit (Life Technologies,

GibcoBRL). The isolated DNA was electrophoresed in 0.7% agarose gels at 70 V for 6 h or in 0.7% agarose gels at 90 V for 4 h, stained with ethidium bromide, and analyzed under UV illumination by the TEX-20M (Life Technologies, GibcoBRL) system (Balis et al., 1996).

Plasmid analysis by Restriction Fragment Length Polymorphism - RFLP

The approximate moleculat mass of the plasmid was determined by RFLP analysis and confirmed by ligation. Plasmid DNA from ten SE AM^R isolates was digested with *BamH*I**,** *Eco*R**I,** *Hind*III**,** *Eco*RV and *Pst*I (Brown et al., 1993; Llanes et al., 1999). DNA fragments were subjected to electrophoresis in 1% w/v agarose gel with (λ) *Hind*III and 1Kb DNA ladder as markers. Restriction endouneclease analysis was repeated three times for *Eco*RV and *Eco*RI to better determine the plasmid molecular mass. To confirm the estimated plasmid molecular mass, the *Eco*RI fragments of the AM^R plasmid DNA, at a concentration of 120 fmol each, were ligated to 30 fmol of plasmid pET29c (Invitrogen) previously digested with *Eco*RI. In all reactions, 1 Unit of T4 DNA ligase (TAKARA BIO Inc) was used. After 16h incubation at 15°C, the product was transformed to *E. coli* **c**ompetent cells (Sambrook and Russell, 2001).

Detection of bla_{TEM-1} and bla_{TEM-2} genes

The presence of bla_{TEM-1} and bla_{TEM-2} genes was examined by PCR. Oligonucleotide primers were used to amplify a 291-bp fragment for the bla_{TEM-1} gene and a 489-bp fragment for the bla_{TEM-2} *gene* (Table 1). Amplification reaction mixtures were prepared at a final volume of 25 μl containing 1x PCR Taq Polymerase Buffer (Invitrogen), 2.5 mmol MgCl2 (Invitrogen), 0.2 mmoldNTPs, 1 μmol of each primer, 100 ng of genomic DNA and 1.5 U of Taq polymerase (Invitrogen). The temperature cycling for amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) as follows: initial denaturation 95°C for 5 min followed by 30 cycles at 95°C for 1 min denaturation, 48°C for 30sec annealing, 72°C for 30sec extension and 5 min final extension at 72°C. The resulting amplification products were separated by the electrophoresis in 1.5% w/v agarose gel, stained with ethidium bromide and visualized under UV light.

Identification of *sodCI, sopE* and *sefA* genes

The presence of plasmid-mediated virulence genes was examined by PCR. Oligonucleotide primers were used to amplify a 912-bp fragment for the *sodCI* gene**,** a 722-bp fragment for the *sopE* gene and a 498-bp fragment for the *sefA* fimbrial locus (Table

Table 1. Primers used in PCR for the detection of sodCI, sopE, sefA, blaTEM1, blaTEM1/2 ß-lactamases genes in S. enteritidis

Primer	Target gene	Sequence 5′ → 3′	Amplified Fragment Size (bp)
VL1F		CTTGCAAACATATACCTGC	912
VL1R	*sodCI*[31]	GACTATCTGAATGCTTA	
VL2F		TCAGGGAGTGTTTTGTATATATTTA	722
VL2R	*sopE*[31]	GTGACAAAAATAACTTTATCTCCCC	
VL3F		ATGCGTAAATCAGCATCTGCAGTAG	498
VL3R	*sefA*[31]	TTA GTT TTG ATA CTG CTG AAC GTA	
TEM1F		GCA CGA GTG GGT TAC ATC GA	291
TEM1R	bla_{TEM1}[60]	GGT CCT CCG ATC GTT GTC AG	
TEM1/2F		GAG TACT CA CCA GTC ACA GAA AAC	489
TEM1/2R	$bla_{TEM1/2}$[29]	TTAGTTTTGATACTGCTGAACGTAG	

Table2. Antibiotic resistance of S. enteritidis strains

a/a	Isolates	Antibiogramme	M.I.C.
	Group 1*		
1.	3, 4, 6, 8, 16E, 17/E, 358, 162	AM	50μg/ml AM
2.	9, 6x, 334, 32, 34,	AM	100μg/ml AM
3.	10, 11E, 162, x_1s_1	AM/TE	50μg/ml AM, 30μg/mlTE
4.	x/15	AM/TE	100μg/ml AM, 30μg/mlTE
5.	197, 4x, 29, 294	TE	30μg/mlTE
6.	35	AM/GM/SXT/TE	AM/AMX/GM/SXT/TE
7.	146, 371	Sensitive (blank)	-
	Group 2**		
1.	$1G_2$ - $10G_2$	AM	50μg/ml AM

* Trirty-five isolates from one-day old chicks with a high mortality ratio, one-day old chicks with a low mortality ratio, layers with sporadic egg-peritonitis and clinically healthy broilers by random sampling in slaughter-houses.
**Ten isolates from thirty flocks with healthy meat producer broilers, randomly selected.

1). Amplification reaction mixtures were prepared at a final volume of 25 μl containing 1x PCR Taq Polymerase Buffer (Invitrogen), 2.5 mmol MgCl2 (Invitrogen), 0.2 mmoldNTPs, 1 μmol of each primer, 100 ng of genomic DNA and 2.5 U of Taq polymerase (Invitrogen). The temperature cycling for amplification was performed in a PTC-200 Peltier Thermal Cycler (MJ Research) as follows: initial denaturation 95°C for 5 min followed by 30 cycles at 95°C for 1 min denaturation, 58°C for 40sec annealing, 72°C for 40sec extension and 5 min final extension at 72°C. The resulting amplification products were separated by the electrophoresis in 1.5% w/v agarose gel, stained with ethidium bromide and visualized under UV light.

RESULTS

All the thirty-five isolates of current study were identified serologically and biochemically as *Salmonella enteric* serotype Enteritidis (SE). Thirteen to twenty-five isolates of G1 group (52%) were resistant to ampicillin, five isolates (20%) to ampicillin and tetracycline, four isolates (16%) to tetracycline, one isolate (4%) appeared multi-drug resistance and two isolates (8%) were susceptible. All ten isolates (100%) from G2 group were resistant to ampicillin (results are shown in Table 2). It is also significant that six of the twenty-five (17.1%) SE isolates from the G1 group carried resistance to more than one antimicrobial.

From the MIC test it was pointed out that resistance to ampicillin was five or ten times higher than the disk concentration (50μg/ml or 100μg/ml instead of 10μg/ml). Ampicillin or ampicillin/tetracycline resistance was transferred to *E. coli* N99 and *E. coli*

SE 4SE 10 *SE X_1S_1* *Se162* *SE 17/E* *SE1Δ*
AM^R AM^RTE^R AM^RTE^R AM^R AM^RTE^R AM^R

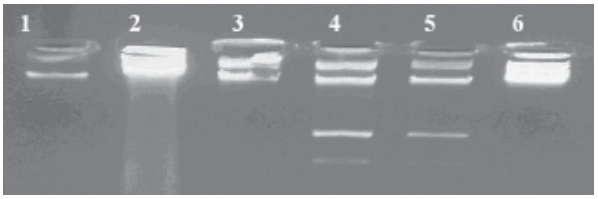

Figure1. Analysis of antimicrobial resistance plasmidDNA

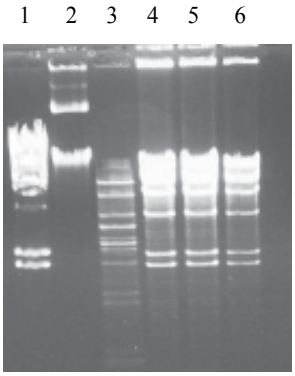

Figure 2a. Plasmid DNA from SE AMRafter treatment with EcoRV, EcoRI restriction enzymes. Lane 1:λ/HindIII DNA, lane 2: uncut pDNA, lanes 3-6: digestion with EcoRV, EcoRI, EcoR1, EcoR1, respectively

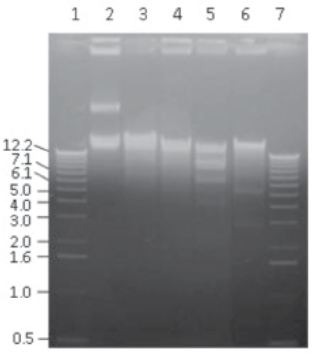

Figure 2b.Analysis of plasmid DNA after treatment with BamHI, PstI, EcoRI and HindIII restriction enzymes. Lanes 1 and 7: 1Kb DNA Ladder, lane 2: uncut pDNA, lanes 3-6: digestion with BamHI, PstI, EcoRI and HindIII, respectively.

TOP10F΄ recipients at a frequency from $1x10^{-4}$up to $2.5x10^{-7}$. DNA isolation and agarose gel electrophoresis of both SE donors and *E. coli* recipients showed that all isolates possessed a common band of the same molecular mass. Two isolates have more than one band (162 and 17/E Amp^RTe^R). These extra bands could be different conformations of the same plasmid. The results are shown in Figure 1.

Plasmid analysis of the ten SE AM^R isolates after endonuclease digestion with *Bam*HI showed three bands, with *Hind*III two bands, with *Eco*RI seven bands, with *EcoR*V thirteen bands and with *Pst*I one band. Plasmid DNA from the same isolates was also digested with *BamH*I, *EcoR*I, *Hind*III, *EcoR*V and *Pst*I after ligation to compare the plasmid molecular mass. The results of the digestion are shown in Figures 2a and 2b. The restriction endonuclease analysis revealed that ampicillin resistance was encoded by a 41 kb plasmid in all isolates.

Identification of the o$bla_{\text{TEM-1}}$ and $bla_{\text{TEM-2}}$ genes associated with resistance to ampicillines was accomplished using PCR. Only $bla_{\text{TEM-1}}$ gene was detected in all isolates.

As for the *sodCI***,** *sopE* and *sefA* genes, twenty-two (62.8%) isolates harboured the *sodCI* gene and among them fourteen (40%) belonged to the G1 group and eight (22.8%) to the G2 group. Thirty-four (97.2%) isolates carried the sopE gene and among them twenty-four (68.6%) belonged to G1 group and ten (28.6%) to the G2 group. All isolates carried the *sefA* gene.

DISCUSSION

Animals are the consumers of almost half of Europe's production of antimicrobials. The prevalence of *S.* Enteritidis (SE) strains in poultry farming has as a result the appearance of this serotype in human food chain (Altekruse et al., 2006). The most recent data in European Union(www.efsa.europa.eu/en/efsajournal/2597.pdf) indicate that *S.* Enteritidis (SE) is the most frequent serovar observed in confirmed human salmonellosis. In Greece, Arvanitidou et al, (1998) reported SE as the predominant serotype. Past decade, the most common *Salmonella* serotypes, from broiler flocks in EU were Enteritidis, Infantis, Mbandaka, Typhimurium and Hadar (van de Giessen et al., 2006; www.efsa.europa.eu/en/efsajournal/2597.pdf). In the United States, during the Surveillance for Foodborne Disease Outbreaks 2009–2010, among the 225 confirmed *Salmonella* outbreaks with a serotype reported, Enteritidis was the most common serotype with 76 outbreaks (34%). In parallel with the increased gastroenteritis cases in humans of high-risk groups, the problem of the resistance to antibiotics has raised.

This study describes phenotypic and genotypic characteristics of thirty five isolates. All isolates were identified as *Salmonella enterica* serotype Enteritidis (SE). The results from the antibiotic resistance tests were similar to earlier studies in Greece (Balis et al., 1996,; Iordanidis and Georgopoulou, 1998; Vatopoulos et al., 1994) and in other countries (Dogru

et al., 2010; Hur et al. 2011). It is remarkable that ampicillin resistant SE strains were also isolated from healthy broilers. *S.* Enteritidis was one of the predominant serotypes carrying tetracycline and ampicillin resistance in Zdragas et al., (2012). Although healthy carriers of ampicillin resistant SE isolates transfer the resistance to other animals and they could contribute to the resistance transfer from animals to humans being a threat still their role is not absolutely clarified.

The restriction endonuclease analysis revealed that ampicillin resistance was encoded by a ca. 41 kb plasmid in all isolates while earlier studies showed that the ampicillin resistance has been correlated to plasmids with a molecular mass varying from 54.44 kb to 160 kb (Balis et al., 1996; Vatopoulos et al., 1994).The last ten years some studies – especially in Europe, resulted that while human and poultry strains possess many common structural features, they represent two distinct populations (Suh et al. 2006). This hypothesis seems to be enhanced from the fact that antibiotic resistance is transferred from plasmids with different molecular mass depending on the origin (human or animal). In Southern Italy, where SE isolates resistant to ampicillin and ampicillin/tetracycline have been found at a high frequency, the molecular mass of the isolated plasmid DNAs were estimated to be from 40kb to 128kb (Nastazi et al., 2000; Villa et al., 2002). In Taiwan, the size of the isolated plasmids ranged from 3.6 to 100 kb including a plasmid of 36kb (Chu et al., 2009). Present findings from India revealed that SE isolates possessed more than one plasmid. The plasmids size ranged between 0.43 and 115 MDa (Maripandi and Al-Salamah, 2010).

In Iran, six different plasmid patterns were detected among 49 isolates and a 68-kb plasmid w as found in 98% of isolates (Morshed and Peighambari, 2010). The fact that in Greece also,there are plasmids of different molecular mass is probably a first indication that human and poultry strains possess plasmids of two distinct populations.

The distribution of virulence factors used by *Salmonella* to induce enteropathogenesis has not been fully explored. Although all the serovars of *S. enterica* are considered as potentially pathogenic, the fact that there are significant differences between virulence genes, even among the isolates of the same serovar, makes more crucial to obtain as much data as we can. The *sodCI* gene appears to be functionally important as it is carried by selected strains belonging to some of the most highly pathogenic serotypes (Fang et al., 1999). Many investigations have demonstrated the contribution of the gene to the ability of *Salmonella* to cause disease in the host. It has been showed that the differences in their virulence are associated to the presence or absence of *sodCI* gene; a number of mutations in thegene confer different reductions in virulence for mice. The presence of *sodCI* gene in the genome of SE may influence its persistence in poultry flocks (Karasova et al., 2009).

The *sopE* gene, it has been detected in different phage types of SE and that may contribute for the expression of *Salmonella* invasion by stimulating membrane ruffling; it has been found to be encoded by a temperate phage. The presence of *sopE* gene has been associated with many *Salmonella* species such as *S. typhimurium*, *S.typhi*, *S.enteritidis*, *S.dublin*, *S.hadar*, *S. gallinarum* and it is associated with epidemic disease in both humans and animals (Hopkins and Threlfall, 2004; Rahman et al., 2004; Smith et al., 2010; Dione et al., 2011). In present study the great majority of the isolates (34/36 or 97.2%) harbored the *sopE* gene.

As for the SEF14 fimbriae, although they have not a demonstrable role in the pathogenesis or virulence of SE, may be important in the attachment to the host epithelium in the early stages of infection (Ogunniyim et al., 1997). In our study, all isolates carried the *sefA* fimbrial locus giving probably higher abilities to the attachment to the host epithelium.

CONCLUDING REMARKS

In our study the prevalence and combinations of SE antimicrobial resistance were determined in isolates from chickens, which contribute the majority of food-borne salmonellosis found in humans. From the thirty-five isolates of the current study, thirty three (94.3%) appeared resistance to antimicrobials and from them twenty nine (82.9%) to ampicillin while ten (28.6%) to streptomycin.

Plasmid DNA of poultry origin carrying ampicillin resistance was identified and analyzed for the first time in Greece. DNA analysis showed one band, common

in all isolates, possessing the antimicrobial resistance genes. The plasmid isolation, ligation and RFLP analysis, revealed that the ampicillin resistance is encoded by a ca. 41 kb plasmid in all isolates. The fact that from poultry and human strains identified plasmids of different molecular mass possibly indicates that in Greece, the above strains possess plasmids of two distinct populations. Moreover, according to recent unpublished data the prevalence of the AM^R isolates is gradually reducing due to the limited use of this antibiotic in poultry industry, resulting to the improvement of current situation.

The possession of virulence-associated genes in these SE isolates and especially in the isolates from the healthy broilers, suggests that they could cause serious disease and give rise to public health problems if they were dispersed in the human population.

REFERENCES

Abbassi-Ghossi I, Jaquani A, Aissa RB, Martinez-Urtaza J, Boudabous A, Gtari M (2011) Antimicrobial resistance and molecular analysis of non-typhoidal salmonella isolates from human in Tunisia. Pathol Biol 59: 207-212.

Altekruse SF, Bauer N, Chanlongbutra A, DeSagun R, Naugle A, Schlosser W, Umholtz R, White P (2006) *Salmonella Enteritidis* in broiler chickens, United States, 2000–2005. Emerg Infect Dis 12: 1848–1852.

Anğu-Küçüker M, Tolun V, Helmuth R, Rabsch W, Büyükbaba-Boral O, Törümküney-Akbulut D. Susever S, Anğ O (2000) Phage types, antibiotic susceptibilities and plasmid profiles of *Salmonella typhimurium* and *Salmonella Enteritidis* strains isolated in Istanbul, Turkey. Clin Microbiol & Infect 6 (11): 593-599.

Antunes P, Mourão J, Machadoc J, Peixe L (2011) First description of qnrS1-IncN plasmid in a ST11 *Salmonella Enteritidis* clinical isolate from Portugal Diagnostic Microbiol Inf Dis 69:463–465.

Arvanitidou M, Tsakris A, Sofianou D, Katsouyannopoulos V (1998) Antimicrobial resistance and R-factor transfer of *salmonellae* isolated from chicken carcasses in Greek hospitals. In. J Food Microbiol 14(40): 197-201.

Bakshi CS, Sing VP, Malik M, Singh RK, Sharma B (2003) 55 kb plasmid and virulence-associatedgenes are positively correlated with *Salmonella enteritidis* pathogenicity in mice and chickens. Vet Res Commun 27(6): 425-32

Balis E, Vatopoulos AC, Kanelopoulou M, Mainas E, Hatzoudis G, Kontogianni V, Malamou-Lada H, Kitsou-Kyriakopoulou S, Kalapothaki V (1996) Indications on In Vivo Transfer of an Epidemic R Plasmid from *Salmonella Enteritidis* to *Escherichia coli* of the Normal Human Gut Flora. J Clin Microbiol 34(4): 1977-979.

Boyd D, Cloechaert A, Chaslus-Dancla E, Mulvey MR (2002) Characterization of Variant *Salmonella* Genomic Island 1 Multidrug Resistance Regions from Serovars *Typhimurium* DT104 and *Agona*. Antimicrob Agents Chemother 46(6): 1714-22.

Braden CR (2006) *Salmonella enterica* serotype *Enteritidis* and eggs: a national epidemic in the United States. Clin Infect Dis 43: 512–517.

Brown DJ, Threlfall EJ, Hampton MD, Rowe B (1993) Molecular characterization of plasmids in *Salmonella Enteritidis* phage types.Epidemiol Infect 110(2): 209-216.

C.D.S.C. (Communicable Disease Surveillance Centre), Health Protection Agency (2004) *Salmonella Enteritidis* in humans faecal & unknown reports England and Wales, 1981 – 2003 report. CDSC 4: 204-208.

Centers for Disease Control and Prevention.Surveillance for foodborne disease outbreaks--United States, 2009-2010 (2013) MMWR Morb Mortal Wkly Rep CDC 62(3): 41-7.

Chu C, Wong DW, Wang MH, Lin HH, Chen YS, Tien N, Shih MC, Chen TH, Chiu CH (2009) Genotyping, Plasmid Analysis, and Antimicrobial Susceptibility of *Salmonella enterica* Serotype *Enteritidis* Isolates from Humans and Chickens in Central Taiwan.J Formos Med Assoc 108 (10): 765-771.

Clinical and Laboratory Standards Institute, CLSI (2008) Development of In Vitro Susceptibility Testing Criteria and Quality Control Parameters; Approved Guideline, 3nd Edition M23-A3: 28 (27).

Clinical and Laboratory Standards Institute, CLSI (2009) Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically; Approved Standard- Eight Edition M7-A8: 26 (2).

Dione MM, Ikumapayi U, Saha D, Mohammed NI, Adegbol RA, Geerts S, Ieven M, Antonio M (2011) Antimicrobial resistance and virulence genes of non-typhoidal Salmonella isolates in The Gambia and Senegal.J Infect Dev Ctries 5(11): 765-775.

Dogru KA, Ayaz NA, Cencay YE (2010) Serotype identification and antimicrobial resistance profiles of Salmonella spp. isolated from chicken carcasses. Trop Anim Health Prod 42: 893–897.

Ekateriniadou LV, Papoutsopoulou SV, Kyriakidis DA (1994) High production of xanthan gum by a strain of *Xanthomonas campestris* conjugated with *Lactococcus lactis*. Bacteriology Letters 16 (5): 517-522.

Fang FC, Degroote MA, Foster JW, Baumler AJ, Ochsner U, Testerman T, Bearson S, Giard JC, Xu Y, Cambell G, Laessing T (1999) Virulent *Salmonella typhimurium* has two periplasmic Cu, Zn-superoxide dismutases. Proc Natl Acad Sci 96: 7502–7507.

Foley SL, Lynne AM (2008) Food animal-associated *Salmonella* challenges: Pathogenicity and antimicrobial resistance. J Anim Sci 86 (E. Suppl.): E173–E187.

Glynn MK, Bopp C, Dewitt W, Dabney P, Mokhtar M, Angulo FJ (1998) Emergence of multidrug-resostance*Salmonella enterica* serotype *typhimurium DT104* infections in the United States. N Engl J Med 338(19): 1333-8.

Henriques A, Sereno R, Almeida A (2013).Reducing Salmonella horizontal transmission during egg incubation by phage therapy. Foodborne Pathog Dis.

Hopkins KL and Threlfall EJ (2004) Frequency and polymorphism of sopE in isolates of Salmonella enterica belonging to the ten most prevalent serotypes in England and Wales. J Medical Microbiol 53: 539–543

Jain S and Chen J (2006) Antibiotic resistance profiles and cell surface components of salmonellae. J Food Prot 69: 1017–1023.

Hur J, Kim JH, Park JO, Lee YJ, Lee JH (2011) Molecular and virulence characteristics of multi-drug resistant *Salmonella Enteritidis* strains isolated from poultry. Vet J 189:306–311.

Iordanidis P and Georgopoulou I (1998) Study of the resistance in *Salmonella* strains against antimicrobial drugs. JHVMS 49(2): 134-136.

Karasova D, Havlickova H, Sisak F, Rychlik I (2009) Deletion of sodCI and spvBC in Salmonella enterica serovar Enteritidis reduced its virulence to the natural virulence of serovars Agona, Hadar and Infantis for mice but not for chickens early after infection. Vet Microbiol 139: 304-309.

Linam WM and Gerber MA (2007) Changing epidemiology and prevention of Salmonella infections. Pediatr Infect Dis J 26: 747–748.

Llanes EC, Kirchgesner V, Plesiat P (1999) Propagation of TEM- and PSE- Type β- Lactamases among Amoxicillin-Resistant *Salmonella* spp. Isolated in France. Antim Ag Chem 43(10): 2430-36.

Lopes VC, Velayudham BT, Halvorson DA, Nagaraja KV (2006) Preliminary evaluation of the use of the sefAfibrial gene to elicit immune response against *Salmonella enterica* serotype *Enteritidis* in chickens. Avian Dis 50(2): 185-190.

Lu S, Manges AR, Xu Y, Fang FC, Riley LW (1999) Analysis of Virulence of Clinical Isolates of *Salmonella Enteritidis* In Vivo and In Vitro. Infect & Immun 67: 5651-5657.

Maripandi A and Al-Salamah AA (2010) Multiple-Antibiotic Resistance and Plasmid Profiles of *Salmonella Enteritidis* Isolated from Retail Chicken Meats. American Journal of Food Technology 5(4): 260-268.

Markogiannakis A, Tassios PT, Lambiri M, Ward LR, Kourea-Kremastinou J, Legakis NJ, Vatopoulos AC (2000) Multiple clones within multigrug-resistant *Salmonella enterica serotype typhimurium* phage type DT104. The Greek Nontyphoidal Salmonella Study Group. J Clin Microbiol 38(3): 1269-71.

Melendez SN, Hanning I, Han J, Nayak R, Clement AR, Wooming A, Hererra P, Jones FT, Foley SL, Ricke, SC (2010) *Salmonella enterica* isolates from pasture-raised poultry exhibit antimicrobial resistance and class I integrons. Appl Microbiol 109: 1957–1966.

Metzer E, Agmon V, Andersen N, Cohen D (1998) Emergence of multidrug-resostance*Salmonella enterica serotype typhimurium* DT104 among *Salmonellae* causing enteridites in Israel. Epidemiol Infect 121(3): 555-9.

Mirmomeni MH, Hosseinzadeh-Colagar A, Ghazaey S (2007) Molecular study of *Salmonella Enteritidis* in poultry by PCR, plasmid curing, antibiotic resistance and protein pattern analysis. Pakistan J Biol Sci 10: 1562-1570.

Morshed R and Peighambari SM (2010) Drug resistance, plasmid profile and random amplified polymorphic DNA analysis of Iranian isolates of *Salmonella Enteritidis*.New Microbiologic 33: 47-56.

MurugkarHV, Rahman H, Dutta PK (2003) Distribution of virulence genes in *Salmonella* serovars isolated from man & animals. Indian J Med Res 117: 66-70.

Nastasi A, Mammina C, Cannova I (2000) Antimicrobial Resistance in *Salmonella enteritidis*, Southern Italy, 1990 – 1998. Emerg Infect Dis 6(4): 401-403.

Ogunniyi AD, Kotlarski I, Morona R, Manning PA (1997) Role of SefA subunit protein of SEF14 fimbriae in the pathogenesis of *Salmonella enterica serovar Enteritidis*. Infect Immun 65: 708-717.

Papadopoulou C, Davies RH, Carrique-Mas JJ, Evans SJ (2009) Salmonella serovars and their antimicrobial resistance in British turkey flocks in 1995 to 2006. Avian Pathology38: 349-357.

Rahman H, Streckel W, Prager R, Tschape H (2004) Presence of sopE gene & its phenotypic expression among different serovars of Salmonella isolated from man & animals. Indian J Med Res 120: 35-38.

Rychlik ,IGregorova D, Hradecka H (2006) Distribution and function of plasmids in *Salmonella enterica*, Review Vet Microbiol 112: 1–10.

Sakaridis I, Soultos N, Iossifidou E, Koidis P, Ambrosiadis I (2011) Prevalence and antimicrobial resistance of salmonella serovars from chicken carcasses in Northen Greece. Journal of Food Safety 31: 203-210.

Sambrook J and Ruussell DW (2001) Molecular Cloning: a laboratory manual. 3rd ed. N.Y., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press.

Sarna M, Dowse G, Evans G, Guest C (2002) An outbreak of *Salmonella typhimurium PT135* gastroenteritis associated with a minimally cooked dessert containing raw eggs. Commun dis Intell 26: 32-37.

Smith KP, George J, Cadle KM, Kumar S, Aragon SJ, Hernadez RL, Jones SE, Floyd JL, Varela MF (2010) Elucidation of Antimicrobial Susceptibility Profiles and Genotyping of Salmonellaenterica Isolates from Clinical Cases of Salmonellosis in New Mexico in 2008. World J Microbiol Biotechnol 26: 1025-1031.

Smith LD, Harris DH, Johnston AJ, Silbergeld EK, Morris JJr (2002) Animal antibiotic use has an early but important impact on the

emergency of antibiotics resistance in human commensal bacteria. PNAS 99: 6434-6439.

Snow LC, Davies RH, Christiansen KH, Carrique-Mas JJ,Wales AD, O'Connor JL, Cook AJC, Evans SJ (2007) Survey of the prevalence of *Salmonella* species on commercial laying farms in the United Kingdom. Vet Rec 161: 471-476.

Suh DK and Song JC (2006) Analysis of *Salmonella enterica serotype Enteritidis* isolated from human and chicken by repetitive sequence-PCR fingerpringing, antibiotic resistance and plasmid profiles. J Ve Sci 7: 37-41.

Tassios PT, Markogiannakis A, Vatopoulos AC, Katsanikou E, Velonakis EN, Koures-Kremastinou J, Legakis NJ (1997) Molecular epidemiology of antibiotic resistance of *Salmonella enteritidis* during a 7-year period in Greece.J Clin Microbiol35 :1316-21.

Tassios PT, Gazouli M, Tzelepi E, Milch H, Kozlova N, Sidorenko S, Legakis NJ, Tzouvelekis LS (1999) Spread of *Salmonella typhimurium* clone resistant to expanded – spectrum cephalosporins in three European countries. J Clin Microbiol 37: 3774-7.

Threlfall EJ, Rowe B, Ward LR (1993) A comparison of multiple drug resistance in salmonellae from humans and animals in England and Wales, 1981 and 1990.Epidemiol Infect 111: 189-197.

Threlfall EJ, Fisher IST, Berghold C, Gemer-Smidt P, Tschape H, Corrnican M, Luzzi I, Schnieder F, Wannet W, Machado J, Edwards G (2003) Antimicrobial drug resistance in isolates of *Salmonella enterica* from cases of salmonellosis in humans in Europe in 2000: results of international multi-centre surveillance. Eurosurveillance 8: 41-45.

Turcotte C and Woodward MJ (1993) Cloning, DNA nucleotide sequence and distribution of the gene encoding the SEF14 fibrial antigen of *Salmonella Enteritidis*. J General Microbiol 139: 1477-1485.

Van de Giessen AW, Bouwknegt M, Dam-Deisz WD, van Pelt W, Wannet WJ, Visser G (2006) Surveillance of *Salmonella* spp. and *Campylobacter* spp. in poultry production flocks in The Netherlands. Epidemiol Infect 134: 1266-75.

Vassiliadis P (1983) The Rappaport – Vassiliadis enrichment media for the isolation of *Salmonellas*: an overview. J ApplBact 37: 411-418.

Vatopoulos AC, Mainas E, Balis E, Threlfall EJ, Kanelopoulou M, Kalapothaki V, Malamou-Lada H, Legakis NJ (1994) Molecular epidemiology of ampicillin-resistant clinical isolates of *Salmonella enteritidis*.J Clin Microbiol 32 : 1322-1325.

Villa L, Mammina C, Miriagou V, Tzouvelekis LS, Tassios PT, Nastasi A, Carattoli A (2002) Multidrug and broad-spectrum cephalosporin resistance among *Salmonella enterica* serotype *Enteritidis* clinicalisolates in Southern Italy. J Clin Microbiol 40 : 2662-2665.

Yang SJ, Park KY, So SH, Kyoung Min-No, Besser TE, Yoo HS, Kim SH, Lee BK, Park YH (2002) Antimicrobioal resistance in *Salmonella enterica serovars Enteritidis* and *typhimurium* isolated from animals in Korea: copmarison of phenotypic and genotypic resistance characterization. Vet Microbiol 86: 295-301.

Yates C and Amyes S (2005) Extended-spectrum **β**-lactamases in non-typhoid *Salmonella* spp. isolated in the U.K. are now a reality: why the late arrival? J Antimicrob Chemoth 56: 262-264.

Zdragas A (2001).Serological study of poultry salmonellosis with emphasis to those that caused by *Salmonella Enteritidis*. PhD Thesis, Thessaloniki.

Zdragas A, Mazaraki K, Vafeas G, Giantzi V, Papadopoulos T, Ekateriniadou L (2012) Prevalence, seasonal occurrence and antimicrobial resistance of Salmonella in poultry retail products in Greece. Lett App lMicrobiol 55(4):308-13.

Zhu CH, Wu J, Chen WW, Hassan HM, Zhu GO (2010) Difference and variation of the sef14 operon gene clusters in Salmonella pullorum. J Basic Microbiol 50: Suppl 1, S120-3. www.efsa.europa.eu/en/efsajournal/2597.pdf, pp14