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Characterization of *Salmonella* isolates from municipal sewage, patients, foods, and animals in Greece using antimicrobial susceptibility testing and pulsed field gel electrophoresis

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Abstract

Aims: We aimed to compare *Salmonella* isolates from different sources using molecular and phenotypic methods, targeting better possibility of understanding the epidemiology of this organism in the Greek context with emphasis in municipal wastewater.

Materials and Methods: In this study, we used pulsed field gel electrophoresis (PFGE) in combination with antimicrobial susceptibility testing to analyze a total of 88 *Salmonella* Enterica isolates from municipal sewage (n=25), humans (n=36), animals (n=24), and foods (n=3) in Greece.

Results: The higher resistance rates were found to the following antimicrobials: streptomycin (59.1%), tetracycline (47.7%), nalidixic acid (46.6%), ampicillin (37.5%), and oxolinic acid (35.2%). Resistance to ciprofloxacin was not observed; 22 isolates (25%) were sensitive to all 9 antimicrobials, 36%, 25% and 12% of human, animal and wastewater origin, respectively, showing a significant difference. *Salmonella* ser. Hadar was the serovar with the highest resistance rates followed by *Salmonella* ser. Anatum and *Salmonella* ser. Typhimurium; *Salmonella* ser. Infantis strains were almost pansusceptible. Cluster analysis did not reveal close genetic relationship between human animal food and wastewater strains belonging to the same serovars. In most of the cases, distinct clusters were observed between human and non-human isolates indicating diversity and no epidemiological connection.

Conclusion: This study indicates that municipal wastewater would be of interest to further monitor the community's prevalence of subclinical or non-reported *S.* Enterica infections.

Keywords: *Salmonella*, wastewater, sewage, PFGE, antimicrobial resistance.

Introduction

Salmonella is the most important cause of food-borne outbreaks in the European Union (EU). In 2013, *Salmonella* was responsible for approximately 30% of all outbreaks reported to the European Food Safety Authority. It is a zoonotic pathogen with poultry, cattle, and pigs being the primary reservoirs [1]. Humans can acquire *Salmonella* infections through the consumption of contaminated foods as well as contaminated drinking water. A variety of food products have been described as vehicles for transmitting *Salmonella* infections to humans including beef, poultry, pork, eggs, cheese, and seafood as well as fruits and vegetables [2]. Although wastewater and drinking water are treated to eliminate pathogenic microorganisms and prevent waterborne transmission, numerous studies indicate that conventional wastewater treatment does not guarantee their complete elimination [3]. Several studies investigating salmonellosis outbreaks have linked the use of water contaminated with human feces or animal manure to outbreaks [4-6].

Most nontyphoidal *Salmonella* infections (NTS) result gastro-enteritis and do not require treatment; severe invasive infections can also occur, which then requires antibiotic treatment. Drugs for NTS infections include fluoroquinolones, trimethoprim-sulfamethoxazole, ampicillin, or third generation cephalosporins [7]. The emergence and spread of resistant bacteria and the subsequent failure of treatment of infections is significant and increasing Public Health problem. Resistance to antimicrobials used to treat invasive *Salmonella* is also increasing, including extended spectrum cephalosporins and quinolones [8,9].

DNA fingerprinting techniques such as pulsed-field gel electrophoresis (PFGE), which is currently

the gold standard for subtyping of salmonellae, are used in outbreak investigations to supplement phage typing data where further strain discrimination is required [10,11]. PFGE has been applied extensively in the epidemiological investigation and surveillance of *Salmonella* for the last two decades. Although PFGE is considered as time-consuming and labor-intensive [12], it is currently the most widely used molecular subtyping method for *Salmonella* and is routinely used in the United States and Europe.

We hypothesize that there is a detectable prevalence of *Salmonella* in human communities and that municipal wastewater reflects this prevalence. Furthermore, this prevalence may be influenced by the environment that surrounds communities such as animals and especially poultry.

The aim of this work was to study the epidemiology of this organism in the Greek context with emphasis in municipal wastewater. A total of 88 strains isolated from patients, animals, food products, and wastewater were characterized by (i) assessing phenotypic antimicrobial resistance profiles using disc diffusion method and (ii) by genotyping using PFGE after macrorestriction with *XbaI*, to evaluate the relationship of the strains.

Materials and Methods

Ethical approval

All samples were collected as per standard sample collection procedure without harming humans or animals.

Sampling

The Veterinary Research Institute of Thessaloniki (VRI) receives samples during monitoring programs and also veterinary samples. Between 1998 and 2000 a total of 25 strains from municipal sewage samples were isolated from VRI and were sent to National Veterinary Reference Laboratory in Chalkis (NRL-Vet) for serotyping. These samples were isolated from sewage in the area of municipality of Thessaloniki (Table-1). During 2008, National Reference Centre for *Salmonella* received 597 clinical isolates from clinical microbiology laboratories all over Greece, 36 of these isolates were included in this study. These samples were randomly selected from patients that have been hospitalized for *Salmonella* in several Greek hospitals. Furthermore, a total of 24 isolates from poultry were isolated from the area of Thessaloniki in VRI between 1998 and 2000 and were sent to NRL-Vet for serotyping. Finally, three strains isolated from foods of animal origin (ready to eat chicken products) in 2010 were included in this study; these isolates were categorized as environmental (Table-1).

Table-1: *Salmonella* isolates included in this study.

***Salmonella* serovars**	***N***			
	Environment	**Animal**	**Human**	**Total**
Abonus	2	0	0	2
Alamo	1	0	0	1
Anatum	8	2	0	10
Blockley	3	4	8	15
Braenderup	1	0	0	1
Bredeney	1	3	0	4
Corvallis	1	0	0	1
Hadar	3*	9	9	21
Infantis	2	0	10	12
Orion	4	1	0	5
Typhimurium	1	5	9	15
Zerifin	1	0	0	1
Total	28	24	36	88

*Food isolates from 2010

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk-diffusion method, and the zones of inhibition obtained were interpreted according to the Clinical and Laboratory Standards Institute guidelines [13]. The strains were screened for resistance to 9 antibiotics. The antibiotic discs (µg, Bio-Rad, Marnes-La-Coquette, France) used were ampicillin 10 (A), cefoperazone 30 (Cf), ciprofloxacin 5 (Cp), gentamicin 10 (G), nalidixic acid 30 (Nx), oxolinic acid 10 (Ox), streptomycin 10 (S), tetracycline 30 (T), and trimethoprim 5 (Tm). Isolates were cultured in trypticase soy broth supplemented with 0·6% yeast extract and transferred to Mueller-Hinton agar (Difco Laboratories, Detroit, MI, USA). The results were interpreted following "The European Committee on Antimicrobial Susceptibility Testing" criteria, except from oxolinic acid as diameter of 19 mm or less was considered susceptible [14]. *Escherichia coli* ATCC 25922 and *Salmonella* ser. Enteritidis ATCC 13076 were used as control strains. Multidrug resistance was defined as previously proposed [15].

PFGE

Salmonella isolates belonging to serovars Anatum (*S.* Anatum) (n=10), Blockley (*S*. Bockley) (n=15), Infantis (*S*. Infantis) (n=12), Hadar (*S*. Hadar) (n=21) and Typhimurium (*S.* Typhimurium) (n=15), were studied using PFGE as previously described with slight modifications [11]. Cells were grown overnight on blood agar plates at 37°C. Each culture was suspended in TE buffer (100 mM Tris-HCl, 100 mM ethylenediaminetetraacetic acid, pH 8.0). To prepare the agarose plugs, 10 µl of proteinase K (2000 mg/dL stock) was added to 190 µl of the adjusted cell suspension. Then, 200 µl of melted 1% Certified Megabase agarose (Biorad, Hamstead, UK) was added to the 200 µl cell suspension/Proteinase K mixture and mixed gently. The mixture was immediately dispensed into wells of plug molds. The bacterial cells in the agarose plugs were lysed by treatment with a lysis solution containing 0.1 mg/ml Proteinase K (GIBCO-BRL, Gaithersburg, MD), 50 mM Tris–HCl (pH 8.0), 50 mM EDTA, and 1% N-lauroylsarcosine, for 2 h at 54°C. The plugs were digested with 40 U of restriction enzyme *XbaI* (Promega Corp., Madison, WI) for 4 h at 37°C. Digested fragments were resolved in a 1%

SeaKem Gold agarose gel (Lonza, Rockland, Maine) in 0.5 × Tris-Borate-EDTA (TBE) buffer using a contour-clamped homogeneous electric field (CHEF) apparatus (CHEF-DR III, Bio-Rad Laboratories, Richmond, CA). Electrophoresis was performed at 6 V/cm with 2.2-63.8 s linear ramp time for 19 h. Gels were cooled at 14°C throughout the run and then stained with ethidium bromide and destained with distilled water. Banding patterns were visualized by UV and photographed (Gel Doc 2000 Imager, Biorad). *Salmonella* ser. Braenderup H9812-PulseNet standard was used as a molecular weight marker after digestion with *XbaI* [16].

Data and statistical analysis

Statistical comparison between different antimicrobial susceptibility rates was analyzed by the chi-square test (Microsoft Excel, 2011). Fingerprinting profiles were examined using the BioNumerics 6.1 software with the Dice coefficient and clustering was based on the unweighted pair group average method (UPGA) with 1.5% optimization and 1.5% position tolerance. A cut off value of 80% similarity was assigned for clustering isolates.

Results

Antimicrobial susceptibility testing

The antibiotic resistance of *Salmonella* strains to nine antimicrobial agents is shown in Table-2. Overall, the highest resistance rates were found to the following antimicrobials: Streptomycin (59.1%), tetracycline (47.7%), nalidixic acid (46.6%), ampicillin (37.5%), and oxolinic acid (35.2%) ($p<0.05$). Resistance to ciprofloxacin was not observed; 22 isolates (25%) were sensitive to all nine antimicrobials, 36% and 25%, respectively, for human and animal isolates, but only 12% from wastewater isolates, showing a significant difference. Interestingly, 15 out of 36 (41%) human and 13 out of 24 (54%) animal isolates were multiresistant but only 6 out of 25 of wastewater isolates.

S. Hadar was the serovar with the highest resistance rates ($p<0.05$) with 14 out of 21 (66.7%) strains being multiresistant. *S.* Blockley was also a serovar with high levels of multiresistance with 8 out of 15 (53.3%) isolates. Serovar Infantis was almost susceptible to all the antimicrobials tested, only 2 strains out of 12 (16.7%) showed resistance

Table-2: Antimicrobial resistance percentages of main *Salmonella* serovars.

Antimicrobial agents	Number (%) of resistant isolates					
	Anatum (n=10)	Blockley (n=15)	Hadar (n=21)	Infantis (n=12)	Typhimurium (n=15)	Total (n=88)
Ampicillin	1 (10)	5 (33.3)	15 (71.4)	0 (0)	9 (60)	33 (37.5)
Cefoperazone	0 (0)	3 (20)	11 (52.4)	0 (0)	1 (6.7)	16 (18.2)
Ciprofloxacin	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Gentamycin	1 (10)	0 (0)	0 (0)	0 (0)	4 (26.7)	5 (5.7)
Nalidixic acid	4 (40)	15 (100)	15 (71.4)	0 (0)	4 (26,7)	41 (46.6)
Oxalinic acid	4 (40)	6 (40)	14 (66.7)	0 (0)	5 (33.3)	31 (35.2)
Streptomycin	6 (60)	15 (100)	13 (61.9)	2 (16.7)	4 (26.7)	52 (59.1)
Tetracycline	4 (40)	13 (86.7)	13 (61.9)	0 (0)	9 (60)	42 (47.7)
Trimethoprim	0 (0)	0 (0)	0 (0)	0 (0)	2 (13.3)	4 (4.5)

Table-3: Antimicrobial resistance profiles of *Salmonella* isolates included in this study.

Resistance patterns	Total (n=88)	Ab (n=2)	Al (n=1)	An (n=10)	Bl (n=15)	Br (n=1)	Bre (n=4)	Cor (n=1)	Had (n=21)	Inf (n=12)	Or (n=5)	Tm (n=15)	Zer (n=1)
A	1	-	-	-	-	-	-	-	1	-	-	-	-
S	14	2	-	3	-	1	-	1	-	2	5	-	-
T	1	-	-	-	-	-	-	-	-	-	-	1	-
Tm	1	-	-	-	-	-	-	-	-	-	-	1	-
A-Nx	3	-	-	-	-	-	-	-	3	-	-	-	-
A-Tm	1	-	-	-	-	-	-	-	-	-	-	1	-
S-Nx	2	-	-	-	2	-	-	-	-	-	-	-	-
A-S-T	3	-	-	-	-	-	-	-	-	-	-	3	-
S-T-Nx	5	-	-	-	5	-	-	-	-	-	-	-	-
S-T-Ox	1	-	1	-	-	-	-	-	-	-	-	-	-
A-Nx-Ox-Cf	2	-	-	-	-	-	-	-	1	-	-	-	1
A-S-T-Nx	2	-	-	-	2	-	-	-	-	-	-	-	-
A-S-T-Tm	2	-	-	-	2	-	-	-	-	-	-	-	-
S-T-Nx-Ox	9	-	-	3	3	-	-	-	3	-	-	-	-
A-G-T-Nx-Ox	5	-	-	1	-	-	-	-	-	-	-	4	-
A-S-T-Nx-Ox	1	-	-	-	-	-	-	-	-	-	-	1	-
A-S-T-Nx-Ox-Cf	13	-	-	-	3	-	-	-	10	-	-	-	-

T=Tetracycline, Nx=Nalidixic acid, A=Ampicillin, S=Streptomycin, Tm=Trimethoprim, Cf=Cefoperazone, G=Gentamicin, Ox=Oxolinic acid, Ab=Abonus, Al=Alamo, An=Anatum, Bl=Blockley, Br=Braederup, Bre=Bredeney, Cor=Corvalis, Had=Hadar, Inf=Infantis, Or=Orion, Tm=Typhimurium, Zer=Zerifin

to streptomycin. For *S.* Typhimurium and *S.* Anatum, 5 out of 14 (35.7%) and 4 out of 10 isolates (40%) respectively were multiresistant. 17 different patterns were observed in the 66 resistant isolates; of them, 43 showed resistance to more than two antibiotics (Table-3). The predominant resistance pattern was S and was found in 14 strains followed by A-S-T-Nx-Ox-Cf in 13, S-T-Nx-Ox in nine strains and S-T-Nx and A-G-T-Nx-Ox resistance patterns in five strains, respectively.

PFGE patterns

S. Anatum

PFGE of *XbaI* digested chromosomal DNA from 10 isolates gave stable reproducible patterns consisting of 11-16 fragments. Seven patterns were identified. Two environmental isolates with R-type S-T-Nx-Ox shared the *XbAn01* pulsotype, two animal isolates (susceptible to all antibiotics) shared *XbAn07* pulsotype. Using a cut off value of 80% similarity five clusters were observed. The most common cluster (cluster D) consisted of 4 out of 10 isolates, two from animal and two from environmental origin (Figure-1).

S. Blockley

Macrorestriction with *XbaI* yielded 14 different patterns consisting of 11-17 fragments. Three major clusters (A, C, and D) were observed using a cut off value of 80% similarity. All human isolates belonged to clusters C and D and all the animal and environmental isolates belonged to different clusters. Correlation between specific pulsotypes and R-types was not observed (Figure-2).

S. Hadar

PFGE patterns of *XbaI*-digested chromosomal DNA of 21 *S.* Hadar isolates consisting of 10-15 fragments are shown on Figure-3. About 15 patterns were observed belonging to six clusters. Clusters C and D were predominant; C consisting of six out of nine human isolates sharing R-type (A-S-T-Nx-Ox-Cf) and D consisting of eight out of nine animal isolates, sharing different R-types. The three environmental strains isolated from foods in 2010 shared the same pulsotype (XbHd14) and R-type (A-Nx).

S. Infantis

Digestion of the 12 *S.* Infantis strains with restriction enzyme *XbaI* demonstrated 11 patterns consisting of 10-15 fragments. 11 pulsed-field profiles were obtained, one main cluster (C) with five human isolates all susceptible to antimicrobials and seven individual types (Figure-4).

S. Typhimurium

About 10 PFGE types were demonstrated after digestion of 15 *S.* Typhimurium strains consisting of 11-16 fragments. These types were classified into five clusters, cluster A consisted of five animal strains; four strains XbTyph01 (A-G-T-Nx-Ox R-type all isolated in 2000) and one XbTyph02 (susceptible, isolated in 1998). The human strains belonged to clusters

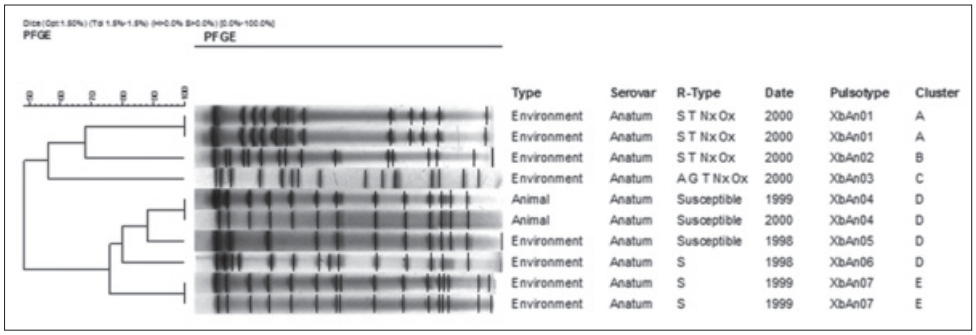

Figure-1: Dendrogram for *Salmonella* ser. Anatum.

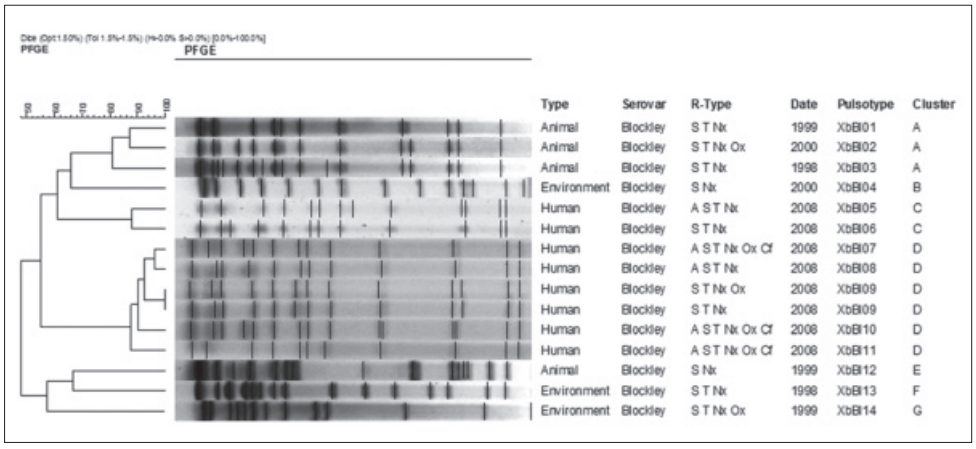

Figure-2: Dendrogram for *Salmonella* ser. Blockley.

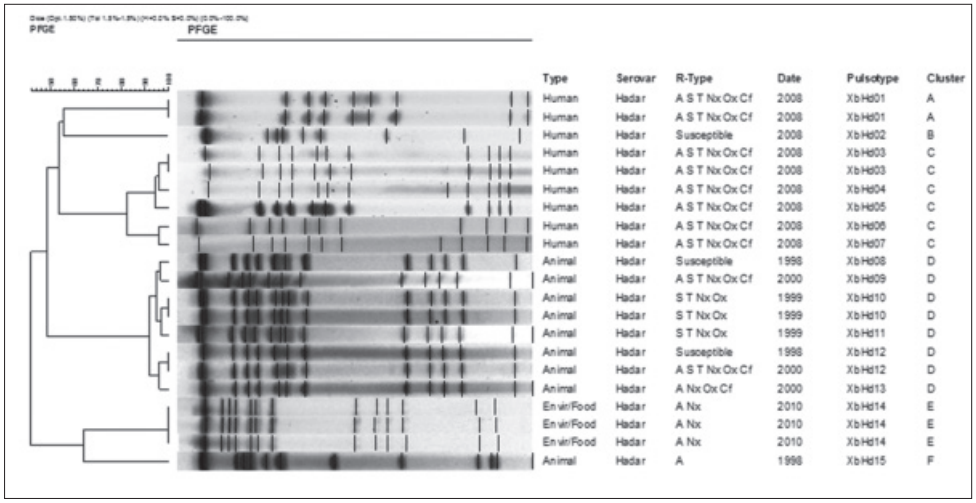

Figure-3: Dendrogram for *Salmonella* ser. Hadar.

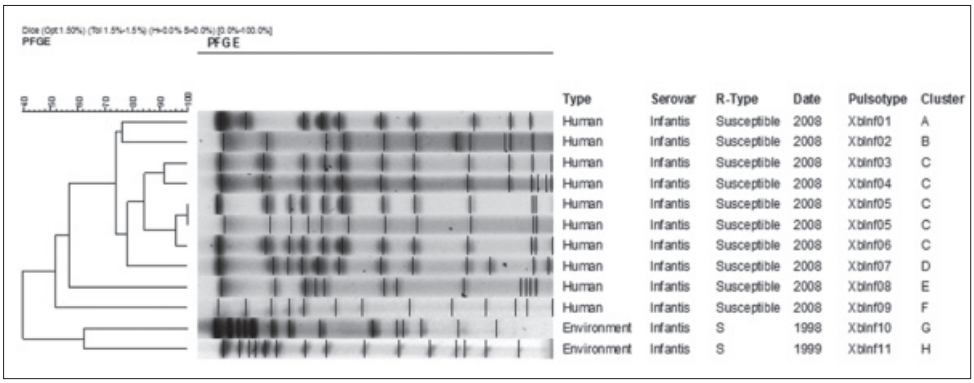

Figure-4: Dendrogram for *Salmonella* ser. Infantis.

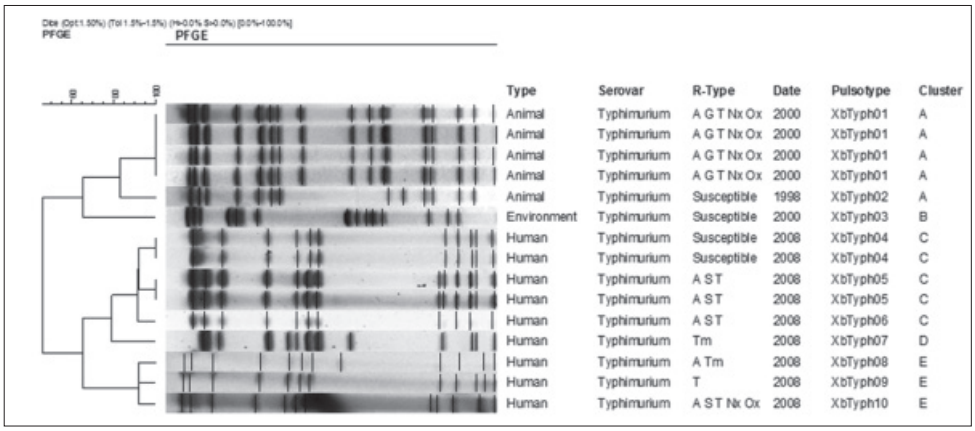

Figure-5: Dendrogram for *Salmonella* ser. Typhimurium.

C (five strains), D (one strain) and E (three strains) (Figure-5).

Discussion

To our knowledge, this is the first study that describes the population structure of *Salmonella* isolates based on PFGE types and drug resistance surveillance comparing human, animal and wastewater isolates in Greece. Even though the isolates do not cover the current period, we believe that the general condition has not changed substantially over the last years and the main conclusions are still an ongoing issue. However, this work underlines the need of establishing a monitoring system in wastewater facilities in Greece.

This study has shown that a wide variety of serovars and antimicrobial resistance patterns were observed in municipal wastewater. Several of the serotypes isolated in this study, including *S*. Typhimurium, *S*. Hadar, *S*. Infantis, *S*. Blockley and *S*. Anatum, have frequently been implicated in outbreaks or sporadic cases of human illness [17]. In Greece, as elsewhere in the world, *S.* Enteritidis and *S*. Typhimurium are the most common causes

of human salmonellosis [18-21]. The main serovars found in both animal and human fecal samples from Greece included *S*. Hadar (from poultry), *S*. Typhimurium (from all the studied animal species) and *S.* Infantis. According to our study, the most common serovar detected in the wastewater samples was *S.* Anatum, which is a rare serovar and has been isolated in wastewater also in Spain [22]. Interestingly *S.* Enteritidis, the most common serovar in human and poultry in Greece and worldwide, has not been detected from wastewater samples in our study as in other studies [23]. In contrast, this serovar has been isolated in Portugal [24] and also in Spain from sea, river and fresh reservoirs [25].

The higher resistance levels were observed to ampicillin, streptomycin, tetracycline, and nalidixic acid, similar to those reported in EU in 2012, where varying levels of resistance were observed among NTS infections acquired in the different geographical regions [19]. Concerning human strains acquired from different countries within EU, the highest levels of resistance are being observed to ampicillin (27.8%), streptomycin (23.9%), sulfonamides (29.2%), and tetracyclines (30.2%). Resistance to ciprofloxacin was not observed; in line to our results, low rates of resistance to ciprofloxacin (<3%) were observed from strains of human and animal origin in EU during 2007-2012. Interestingly resistance levels to ciprofloxacin are higher in isolates from other regions and particularly high in isolates originating in Asia (22.7%), Africa (20.5%) and Europe (non-EU/EEA countries) (19.5%,) [19]. Susceptibility to all antimicrobials demonstrated to 25% of the isolates, 36% of the human, 25% of the animal and 12% of the wastewater. However, resistance levels can also differ substantially between *Salmonella* serovars, and therefore, an in-depth analysis among every serovar is essential.

According to our study, multiresistant isolates were 39% (n=88), in line to previous studies in Greece concerning poultry and poultry products [26,27]. Multi-drug resistance is high among isolates from humans in EU (28.9%) with the higher levels reported from Italy (63.1%) and Hungary (55.8%). The levels of resistance to ciprofloxacin and nalidixic acid are mostly moderate to very high within individual European countries, although some do not detect resistance or report low resistance to these compounds as we do in our study [19]. This considerable disparity in resistance to ciprofloxacin and nalidixic acid among *Salmonella* isolates, from different countries, may reflect the variability of serovars of *Salmonella* spp. included in the analyses of the different countries. Although antimicrobial use for animals is under veterinary prescription control in Greece, as in all European countries, farmers still sometimes use unprescribed antimicrobials for treatment or as growth promoters in cattle, poultry and swine. This practice leads into a possibility that bacterial resistance developing in the food animals' transfers to the human population thus posing a risk for public health by spreading of the resistance [28].

Cluster analysis did not reveal close genetic relationships between human and animal strains belonging to the same serovars. In most of the cases, distinct clusters were observed between human and non-human isolates, this can be clearly shown for *S*. Typhimurium, *S*. Blockley and *S.* Hadar. We suggest that this could be due to the isolates are epidemiologically unrelated.

Diversity among PFGE types varies for each particular serovar. It is well-known that *S.* Enteritidis is a clonal serovar. According to Pulsenet Europe database, which consists of 11,939 strains, 68 distinct and 509 unnamed profiles are present. Pulsenet PFGE-type SENTXB.0001 is predominant (56%), the top 5 PFGE-types are representing 86% of all the isolates (data not shown). *S.* Hadar is also not a diverse serovar in Europe as in Greece; Pulsenet profile SHADXB.0001 is ranking first (>50% of all isolates in Europe), the top five PFGE profiles represent 65% of all isolates [11]. On the contrary, *S.* Typhimurium is a very diverse serovar, according to Pulsenet Europe database consisting of 6,027 strains divided into 275 distinct profiles and 1,554 unnamed profiles. The most prevalent PFGE profile is STMXB.0061 representing 679 out of 6,027 isolates (11%) and the top five PFGE types are representing 1,887 isolates (31%) (Data not shown).

This study indicates that municipal wastewater would be of interest to further monitor the community prevalence of subclinical or non-reported *S.* Enterica infections. Wastewater samples may be good indicators of emerging antimicrobial resistance in the community as was shown by the detection of glycopeptide resistance in *Enterococcus faecium* in Germany [29]. The use of wastewater sampling could be incorporated into national surveillance programs to monitor *S.* Enterica, as well as other pathogens, to obtain further knowledge of serovar shifts and their resistance patterns.

Authors' Contributions

TP has designed and performed all experiments under supervision of AZ and AV. AZ, AV and GM provided the strains. GM, GV and VG have contributed in sample preparation and experimental procedure. EP participated in the manuscript preparation and advice during the work. All authors participated in draft and revision of the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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