University of Urbino Carlo Bo

Department of Biomolecular Sciences

Ph.D. Course in: Biomolecular and Health Sciences

XXXV cycle

From farm to fork: epidemiological study, genetic characterization and plasmid identification of antibiotic resistant *Salmonella* strains isolated along the food chain in Marche Region

ACADEMIC DISCIPLINE: MED/42

Coordinator: Prof. Marco Bruno Luigi Rocchi

Ph.D. student : Ilaria Russo

Supervisor: Prof. Giulia Amagliani

ACADEMIC YEAR 2021/2022

ASTRACT

Antimicrobial Resistance (AMR) is a serious public health problem; the use of antimicrobial agents for animals, plants and food production contributes to the development of antibiotic-resistant microorganisms transmissible to humans through food [1]. Genes involved in AMR can be localized on mobile genetic elements (e.i. transposons or plasmids): this allows them to be easily disseminated in the food chain and transferred to other bacteria. The epidemiology of resistance plasmids is a major issue for the description of AMR diffusion and may be investigated by the identification of plasmid families (Inc groups), assigned on the basis of their replication controls (replicons) [2]. Salmonellosis is the second most commonly reported gastrointestinal infection in humans and an important cause of food-borne outbreaks in the EU [3]. In 2020, high proportions of human *Salmonella* isolates were resistant to ampicillin (29.8%), sulfonamides (30.1%) and tetracyclines (31.2%) [3]. Moreover, multi-drug resistant (MDR) *Salmonella*, displaying also resistance to extended spectrum β- lactam antibiotics, critically important antibiotics for human health, were detected [4]. Among the different serovars of *Salmonella enterica*, *S.* Infantis is one of the five main causes of human salmonellosis in the European Union (EU) [3]. In Italy a MDR and ESBL producing *S*. Infantis clone, harboring a $bla_{\text{CTX-M-1}}$ pESI-like megaplasmid (~280–320 kb), has increasingly spread [5,6]. The transmission of this clone along the food chain could cause high-risk human illnesses because of MDR features of this clone, including resistance to third generation cephalosporins considered critically important antibiotics for human health.

The antibiotic resistance profiles of *Salmonella* spp. strains isolated in Marche Region from veterinary and food-related environments, food animals, foods and human clinical samples was investigated in order to possibly trace the spread of AMR and plasmid distribution in the food chain. Moreover, the presence of the pESI-like plasmid-carrying *S*. Infantis clone in AMR *S*. Infantis strains, isolated from various sources in Marche Region, was investigated.

A total of 101 AMR *Salmonella* strains, of serovars *S*. Derby (n. 20), *S*. Typhimurium (n. 18), MVST (n. 28) and *S*. Infantis (n. 35), were collected and analyzed for this study. Resistance to sulfisoxazole (86%) and tetracycline (81%) were the most common, followed by ampicillin (76%). FIIS was the most predominant replicon (17%), along with FII (11%) and FIB (11%), all belonging to IncF incompatibility group. Concerning the characterization of *tet* genes, *tet*B was the most frequently detected (27/86), followed by *tetA* (15/86), *tet*G (5/86) and *tet*M (1/86). 78% (79/101) of our strains

were phenotypically resistant to at least one β-lactam and were selected for ESBL gene detection by real-time PCR.

Most of the strains (n. 27 MVST, n. 6 S. Derby, n. 4 S. Typhimurium and n. 3 S. Infantis) were positive for *bla*$_{TEM}$ gene. Other strains (n. 23 S. Infantis and n. 1 S. Typhimurium) were positive for *bla*$_{CTX-A/B}$. Only one MVST strain was positive for *bla*$_{CMY}$.

In light of the importance that the third generation cephalosporins represent in human therapy, and the spread in the EU (included Italy) of *S*. Infantis MDR pESI-like clone, all *S*. Infantis strains of this study were sequenced and analyzed for their phylogenetical profile, for the purpose of detecting the presence of the plasmid.

Two different ESBL genetic profiles comparable to *S*. Infantis pESI-like clone were observed. 81% carried the *bla*$_{CTX-M-1}$ gene, prevalent in Europe, and 8% carried the *bla*$_{CTX-M-65}$, mainly detected in the USA. 11% carried *bla*$_{TEM-1}$ gene. IncFIB(K)_1_Kpn3 plasmids were detected in all *bla*$_{CTX}$ positive strains. Eleven clusters were observed after SNP analysis: by using a SNP threshold of <21, the 71% of strains cluster together. By reducing the SNP threshold down to 10, 6 subclusters were identified within this cluster, corresponding to strains isolated from different sources along the food chain. After S1-PFGE analysis, 92% of our strains showed the presence of IncFIB(K)_1_Kpn3 plasmids having size compatible with that of the pESI-like megaplasmid (224-310 kb). Close genetic relationship of *S*. Infantis

bla$_{CTX-M-1}$ harboring IncFIB(K)_1_Kpn3 megaplasmids from diverse sources confirmed the presence and spread of *S*. Infantis pESI-like *bla*$_{CTX-M-1}$ clone in Marche Region along the food chain. The implementation of a One Health approach, integrating surveillance of MDR strain spread from farm to fork, contributes to the monitoring of MDR zoonotic pathogens, such as *S*. Infantis.

References

1. Founou, L.L.; Founou, R.C.; Essack, S.Y. Antibiotic Resistance in the Food Chain: A Developing Country-Perspective. *Frontiers in Microbiology,* **2016**, *7*.

2. Carattoli, A. Resistance Plasmid Families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy* **2009**, *53*, 2227–2238.

3. Food, E.; Authority, S. The European Union One Health 2020 Zoonoses Report. *EFSA Journal* **2021**, *19*, doi:10.2903/j.efsa.2021.6971.

4. Food, E.; Authority, S. The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2019–2020. *EFSA Journal* **2022**, *20*, doi:10.2903/j.efsa.2022.7209.

5. Franco, A.; Leekitcharoenphon, P.; Feltrin, F.; Alba, P.; Cordaro, G.; Iurescia, M.; Tolli, R.; D'Incau, M.; Staffolani, M.; Di Giannatale, E.; et al. Emergence of a Clonal Lineage of Multidrug-Resistant ESBL-Producing Salmonella Infantis Transmitted from Broilers and Broiler Meat to Humans in Italy between 2011 and 2014. *PLoS ONE* **2015**, *10*, 1–15, doi:10.1371/journal.pone.0144802.

6. Alba, P.; Leekitcharoenphon, P.; Carfora, V.; Amoruso, R.; Cordaro, G.; Di Matteo, P.; Ianzano, A.; Iurescia, M.; Diaconu, E.L.; Pedersen, S.K.; et al. Molecular Epidemiology of Salmonella Infantis in Europe: Insights into the Success of the Bacterial Host and Its Parasitic Pesi-like Megaplasmid. *Microbial Genomics* **2020**, *6*, 1–12, doi:10.1099/mgen.0.000365.

CONTENT

INTRODUCTION

Salmonella: a foodborne pathogen

Salmonella was first observed by Karl Joseph Eberth in 1800 in a spleen of a human affected by typhoid fever [1]. In 1885, Theobald Smith and Daniel E. Salmon isolated some bacteria, similar to those described by their colleagues 85 years before, from swine gut affected by "swine fever", suggesting the possibility that similar bacteria can colonize both humans and animals [2]. These bacteria were named *Salmonella* in honor of Salmon, an American veterinary [3].

Since *Salmonella* was discovered, until today, this species is under continuous study because it is considered one of the most important pathogens cause of zoonosis worldwide [4].

Salmonella is classified as a Gram-negative bacterium, member of *Enterobacterales* family, recognized as one of the most diffuse foodborne pathogens and causes of enteric disease. The genus *Salmonella* is divided in two different species causing human illness: *S*. enterica and *S*. bongori [5] (Fig 1).

Salmonella enterica is classified in six subspecies divided in over than 2500 serovars, according to Kaufmann-White typing scheme [5,6], based on three antigenic determinants: flagellar (H), capsular (K) and somatic (O), that is a component of the lipopolysaccharide (LPS) of bacterial cell [6,7].

S. *enterica* subspecies *enterica,* is composed by more than 1500 serovars and is the most numerous subspecies within *S. enterica* [8]. It causes more than 99% of human and warm-blood animal infections [2] and it is divided in Typhoidal and Non-Typhoidal *Salmonella* on the bases on the diseases syndrome. Typhoidal *Salmonella* is composed by "specialist" serovars that have the capability to infect a small host range. For example, *S*. Typhi and *S*. Paratyphi can infect only humans and are cause of typhoid and paratyphoid fever [9].

Non-Typhoidal *Salmonella* (NTS), an important cause of foodborne infection acquired by the ingestion of food or beverages contaminated by several zoonotic serovars, is commonly considered to have the potential to interact with human and animal hosts. The most diffused serovars are *S*. Typhimurium and *S*. Enteritidis, cause of gastroenteritis and extraintestinal disease, nausea, vomiting, abdominal pain and fever [5,9]. Usually, symptoms appear 6-12h after bacteria ingestion and could last 10 days [9].

Salmonella is considered an actual health problem. Human salmonellosis, as typhoidal, paratyphoidal and non-typhoidal infection, is one of the most diffused diseases in both industrialized and developing countries [10].

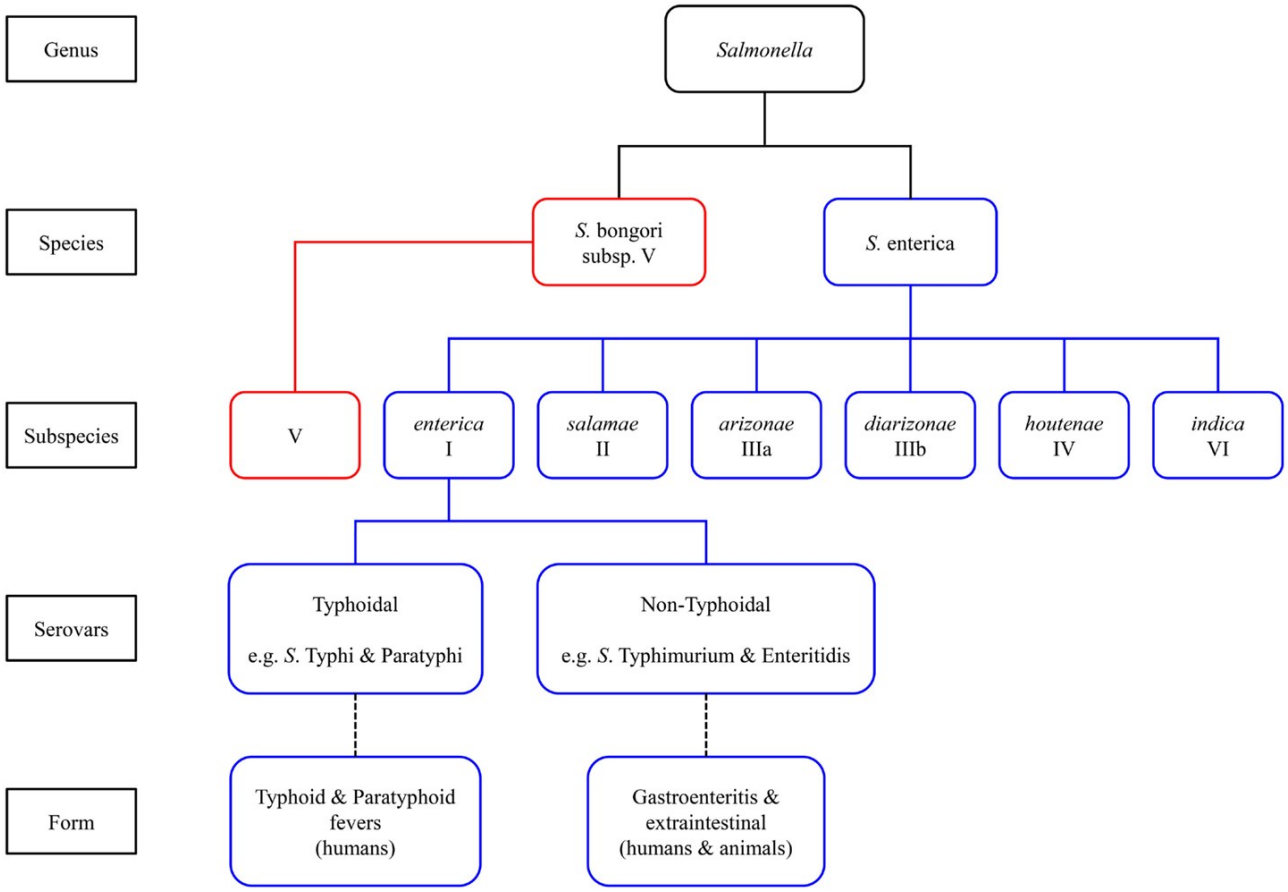


Fig 1. Classification of *Salmonella* species and subspecies [5].

Virulence factors

Almost all *Salmonella* serovars are considered pathogens due to their capability to invade, multiply and survive in the human and animal cells [2]. *Salmonella* pathogenesis is influenced by many virulence factors as flagella, adhesion systems, capsule, plasmids, and pathogenicity islands [6]. All of these factors allow the pathogen to adhere to and invade host cells bypassing the immune system defenses or other barriers like the gastric acidity, intestinal microbiome or gastrointestinal proteases [11]

Genes involved in virulence are often localized in specific chromosomal regions named pathogenicity islands [12].

Salmonella pathogenicity islands (SPIs) are composed by gene clusters located on chromosomal DNA regions involved in encoding virulence factors that increase the virulence of the pathogen [13]. Study about SPIs genetic structure showed that *Salmonella* acquired virulence genes through horizontal gene transfer (HGT) from other species. This hypothesis is based on the: (i) different GC content from the rest of bacterial chromosome; (ii) part of insertion sequences of bacteriophage

and transposons found near the boundary of SPIs; (iii) contiguous genes closely related with nonpathogenic bacteria flanking SPIs [12].

Twenty-one different types of SPI were described, some of them located on chromosome of *Salmonella* genus but other specific for some *Salmonella* serovars [14,15].

SPIs play an important role in *Salmonella* pathogenesis and virulence and are implicated in different virulence mechanism. The two most studied are SPI-1 and SPI-2. Both of them codify for a type III secretion system (T3SS) able to inject bacterial proteins named effectors through host membranes or in extracellular compartment to modify biochemical equilibrium and physiology of infected cells [16]. SPI-1 is a gene cluster composed by 39 genes encoding for T3SS-1, effector proteins and transcriptional regulators involved in the control of virulence genes implicated in invasion of host cells [17].

SPI-2 is important for the "parasitism" of the cell. It is involved in systemic infection allowing bacterial cells to overpass the immune response and permitting their survival in macrophages. Moreover, also SPI-2 codifies for T3SS-2, a system that was identified in inflammatory condition, highly important for *Salmonella* enterocolitis and systemic disease [16].

Presence of virulence plasmids, related to serovars, with a size from 50-100 kb was showed [6,18]. Virulence plasmids may carry the *Salmonella* plasmid virulence locus (*spv*), that includes genes involved in intracellular replication in the reticuloendothelial system as well as liver cells and spleen [6,18]. Genes belonging to the *spv* locus activate in condition with low presence of nutrients and low pH, typical of the intracellular environment [19].

Moreover, these genes are involved in systemic infection, promoting bacterial survival in the host cell [2].

The possibility to spread in the blood is due to the presence of *spv* that is strongly associated to non-typhoidal bacteremia [2].

Production of endotoxin and exotoxin has also considered an important factor that increases the virulence of *Salmonella* strains. In particular, exotoxins are divided in cytotoxin and enterotoxin involved in mammalian cell killing [6]. Kasturi et al revealed the correlation between virulence and enterotoxin production [20]. Virulent strains are able to produce more enterotoxin, compared to low virulent strains, facilitating the invasion of the intestinal membrane, but they also can be associated with extraintestinal infection. On the other hand, low virulent strains cause gastroenteritis confining their invasion only in the gut [6].

Fimbriae are the most common adhesion systems, allowing bacteria to adhere to host cells, food, and surfaces, and are involved in biofilm formation, cell invasion, macrophage interaction. The fimbrial system is composed by genes involved in encoding of structural, assembly and regulatory protein necessary for the filamentous used for the adhesion [21].

Flagella, the other structures located on cell surface, are involved in pathogenicity and motility of many *Salmonella* serovars. Flagella allow bacteria to swim in liquid and move in solid surface. For this reason they are also involved in biofilm formation [22,23]. These mobile structures are important virulence factors since mediate bacterial adhesion and invasion. Indeed, flagellated strains show greater ability to attach cells compared to the strains not equipped with flagella [24].

Pathogenicity

Salmonella is an ubiquitous pathogen: it can persist in the environment for long periods and has the ability to infect a wide range of hosts, except for a few serovars that are host-specific. *Salmonella* can be transmitted by vertical or horizontal transfer. Vertical transmission happens between parents to progeny in particular in poultry, while horizontal transmission occurs via feco-oral and is the most important way to infect humans and animals [25]. In this contest, it's also important to highlight the role of wild animals in *Salmonella* transmission. For example, birds, flies and rodents are considered reservoirs of *Salmonella* and are responsible of dissemination and transmission of the pathogen in livestock. Moreover, farmers are also exposed to and at high risk of salmonellosis through direct contact with infected animals [25].

Salmonellosis infection usually starts with the ingestion of contaminated food or water (Fig. 2). The first step for the pathogen is to overpass gastric acid barrier up to the gut to invade the epithelial cells of the intestinal lumen [2]. Interestingly, the pathogen displays acid-tolerance when is exposed to low pH like the gastric acid [26]. After passing the gastric barrier, thanks to the expression of several fimbriae, *Salmonella* adheres to epithelial intestinal cells [6].

When *Salmonella* is in contact with intestinal epithelial cells, SPIs encode for T3SS whereby bacterial effectors are moved from pathogen cell cytosol to host cell cytosol. The bacterial effectors have the task of modify the host cell membrane structure to allow the entrance of bacteria in the host cell resulting as phagocytosis process [27].

Once *Salmonella* is located in the host cells, it has to survive. Under normal conditions, the body recognizes the risk and starts to activate immune response system secreting lysosome and digestive

enzyme to damage the bacterial cell. Henceforth, *Salmonella* injects effector proteins that cause an alteration of cellular compartment structure and avoid the fusion with lysosomes, permitting bacterial cell survival and replication [2]. Moreover, the ability of *Salmonella* to survive within macrophages allows the pathogen to be carried in the reticuloendothelial system (RES) causing systemic infection [28].

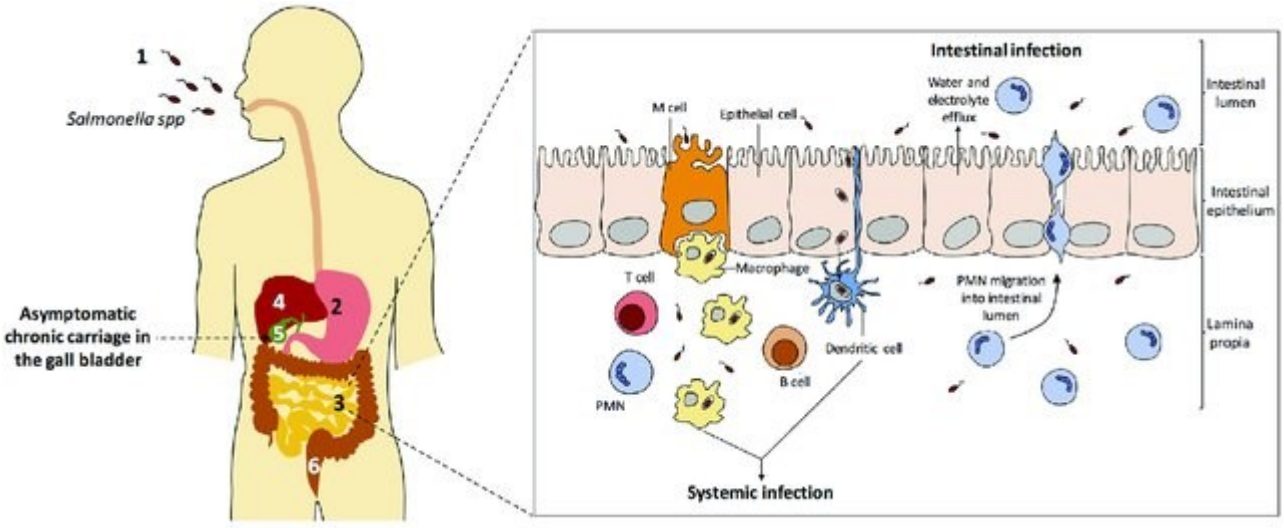


Fig. 2 Pathogenesis of *Salmonella* infection [29].

Clinical manifestation

The 4 clinical manifestations, following ingestion of typhoid and non-typhoid *Salmonella* (NST), are: enteric fever, gastroenteritis, bacteremia and extraintestinal infection.

Enteric fever

The term enteric fever includes two different clinical manifestation: typhoid fever and paratyphoid fever, with identical symptoms. *S.* Typhi is the cause of typhoid fever while *S*. Paratyphi A, B and C are the cause of paratyphoid fever [30]. Moreover *S*. Typhi and *S*. Parathyphi are host-specific since human is the only reservoir of the two serovars [2].

Enteric fever happens after the ingestion of contaminated food or water. It is characterized by an incubation period of one or more weeks, after which different symptoms, like headache, abdominal pain, diarrhea and high fever, start to appear [2]. The infection causes a fever with specific characteristic. The initial fever appears with low-grade (> 37.5°C to 38.2°C) and, in the following

days, slowly increase to high-grade fever (> 38.2°C to 41.5°C). The fever could persist for months if the patient is not treated [31].
Moreover, infected patients can develop infections that affect other organs, like liver or spleen, causing hepatomegaly, splenomegaly, myalgia, bradycardia and cutaneous rash [32].

Gastroenteritis

NTS, mainly found in animals, is the most important cause of gastroenteritis, and the severity of infection depends on the serotype and health state of human host [2].
When contaminated food is ingested, the pathogen attacks and invades the intestinal epithelium of the distal ileum [33,34].
The result of the infection appears as acute gastroenteritis within 4 to 72 h with fever, chills, nausea, vomiting, abdominal cramping, headache, and diarrhea as the main symptoms [33,34].
Usually, this condition doesn't need medicines and last, more or less, 10 days [2], but some patients may develop chronic sequelae such as reactive arthritis or irritable bowel syndrome [34,35].
On the other hand, in vulnerable patients (immunocompromised, very young or elderly persons) NTS infection can systematically spread to other body organs causing febrile illness [4,34,36]. Hence, NTS is a public health concern representing an economic burden for both developed and developing countries, due to costs associated with surveillance, prevention, and treatment of disease [10,33,37].

Bacteremia

After intestinal invasion, *Salmonella* can spread in the bloodstream causing bacteremia. It's a dangerous condition characterized by high fever and septic shock with a high mortality rate [2]. NTS carrying *spv* genes has an high capacity to cause bacteremia than serovars without *spv* [38]. SPVs show the presence of conserved operon composed by 3 genes involved in bypassing host immune system: *spvB* has a cytotoxic effect on macrophage cells enhancing intracellular bacterial proliferation [39]; *spvC* reduces inflammatory cytokines modulating the immune response in the first period of infection [40]; *spvD* downregulates the NF-kB pathway that plays important role in regulating the immune system response during the infection [41].

Due to the presence of *spv* gene, clinical manifestations of bacteremia are more common in NTS infection. Expression of this gene increases the virulence of NTS, extends apoptotic cell death and allows the persistence of bacteria in the host cell for a long period [38]. A small proportion of infected patient (5%) with NTS develops bacteremia, a condition in which the pathogen can spread beyond the gut causing extraintestinal infection with the compromission of other organs; indeed, one of the most commonly compromised organs is the lung. Urinary tract infection, meningitidis, endocarditis, cellulitis are other extraintestinal complication that could occur after *Salmonella* infection [2].

Chronic carrier state

Salmonella can remain in the faeces for more than one year after the acute infection, causing the chronic carrier state that is more possible in elderly people, infants and immunocompromised patients [42]. Moreover, chronic carrier state is more observed in typhoidal *Salmonella* than in NTS and the primary reservoir of Typhoidal *Salmonella* is the human who spread the bacteria, contaminating food and water with infected faeces. On the other hand, in NTS, the chronic carrier state is less frequent because the primary reservoir is the animal instead human [43].

Epidemiological data

The World Health Organization (WHO) estimates that more or less than 2 billion people suffer from diarrhea each year and one third of this infections are transmitted by food [44]. In particular *Salmonella* is responsible of 180 million of diarrhea infection cases annually [44].

The Centers for Disease Control and Prevention (CDC) estimate *Salmonella* bacteria cause about 1.35 million illnesses, more than 26,500 hospitalizations, and 420 deaths in the United States every year [45].

Moreover, NTS, the main cause of gastroenteritis, is commonly distributed in Africa, while typhoidal *Salmonella* is more often found in Southeast Asia [46].

Salmonellosis is the second most commonly reported foodborne gastrointestinal infection in humans after campylobacteriosis and an important cause of foodborne outbreaks in the European Union (EU) [4].

In 2020, the number of confirmed cases of human salmonellosis was 52,702 with a hospitalization rate of 29.9% and a fatality rate of 0.19% [4].

Moreover, *Salmonella* caused 22.5% of foodborne outbreaks in 2020, mainly caused by *S*. Enteritidis. In particular, foods involved in *Salmonella* human infection were "eggs and egg products" an "pig meat and products thereof", highlighting that food animals are the most important reservoir for NTS infection in human and are related to many outbreaks [4,47].

In particular, a primary importance role, in the *Salmonella* infection, is attributed to poultry. Indeed, as the last European Food Safety Authority (EFSA) report showed, the highest proportion of positive samples was detected in meat and meat products from turkey (7.1%) and broiler (6.6%) [4].

Concerning food safety criteria, poultry meats is recognized as the food category with the highest proportion of *Salmonella* positive samples although the *Salmonella* national control programs in poultry have been applied for several years [48].

The role of ready-to-eat (RTE) foods is noteworthy , since RTE products are considered an important source of *Salmonella,* in *Salmonella* infection. For example, in 2020, 1.6% of 'meat and meat products from broilers', 0.8% of 'spices and herbs', 0.6% of 'meat and meat products from pigs', 0.5% of 'meat and meat products from turkeys' and 0.5% of 'other meat and meats products' were positive for *Salmonella* [4].

Comparing data of 2020 and the previous 4-years, in both RTE and no-ready-to-eat foods, an increase in *Salmonella* positive samples isolated from meat and meat products from broiler, turkey and pig was noticed [4].

In 2020, Czechia reported the highest notification rate (98.4 cases per 100,000 population) followed by Slovakia (62.1 cases per 100,000 population), while the lowest rates were reported by Bulgaria, Greece, Ireland, Italy, Portugal and Romania (≤ 4.4 cases per 100,000 population) [4].

Salmonellosis trend in 2016–2020 didn't show any statistically significant increase or decrease (Fig 3)[4]. Nevertheless, in 2020, the number of human cases were at the lowest compared to the other years, probably due to the COVID-19 pandemic [4]. The restrictive measures as hand washing, disinfection practices, and also the impossibility to trip resulted in the decreasing cases of salmonellosis.

The most affected age groups were 1–4 years (24.5%), 5–9 years (12.6%) and over 65 years old (17.8%).

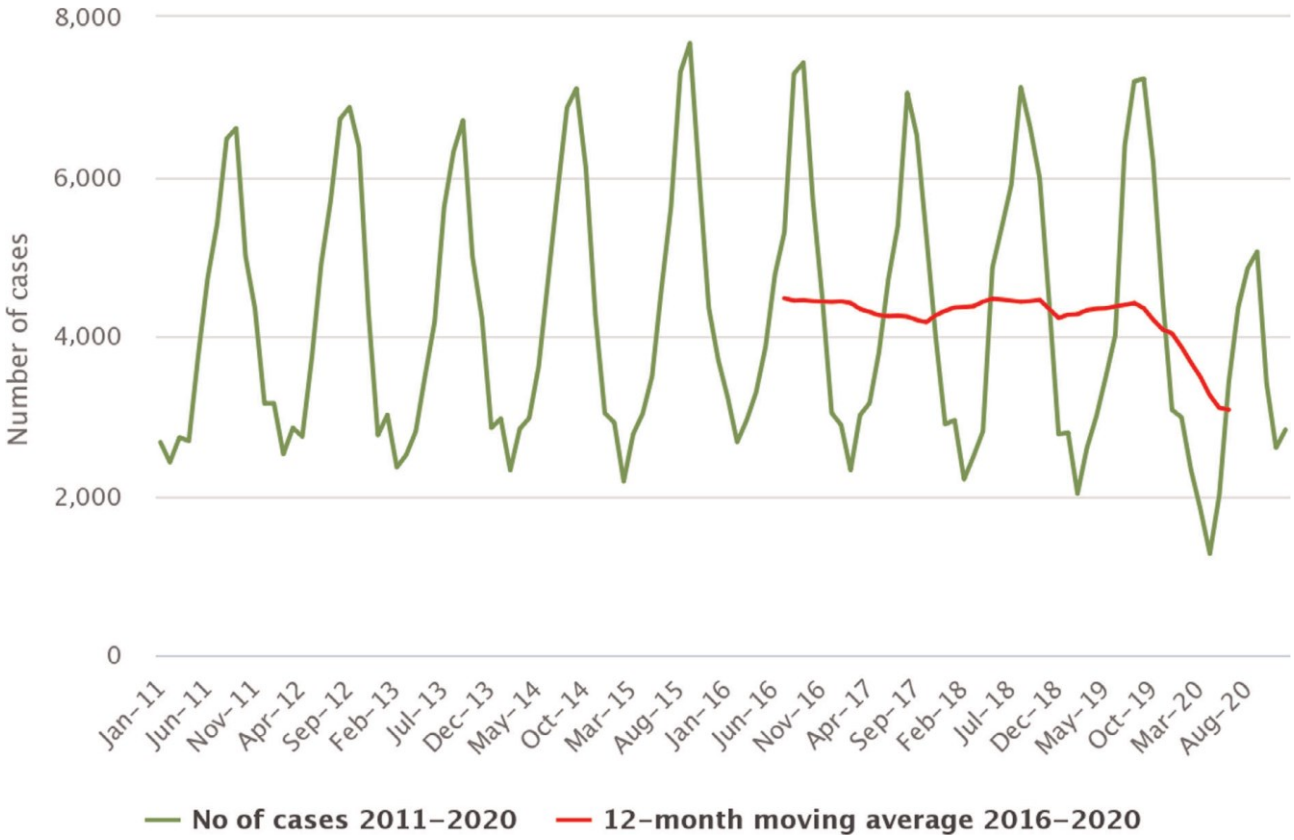


Fig. 3 Trend of salmonellosis human cases in the EU from 2016 to 2020 [4].

The top five *Salmonella* serovars involved in human infections overall were distributed as follows: *S*. Enteritidis (48.7%), *S*. Typhimurium (12.4%), monophasic variant of *S*. Typhimurium (MVST) (11.1%), *S*. Infantis (2.5%), that is the fourth serovar involved in domestically and travel associated infections, followed by *S*. Derby (1.2%) [4].

These serovars, responsible of human infection, are differently related to food sources. *S*. Enteritidis was related to laying hens and broiler sources; *S*. Typhimurium was detected, with a heterogeneous distribution, from poultry, pig and also bovine sources. MVST was related mainly to pig. *S*. Infantis was strictly related to broiler sources, whereas *S*. Derby was primarily linked with pigs [4].

In Italy in 2020, salmonellosis was confirmed as the most frequently reported zoonosis with 2.626 cases of human salmonellosis, a slight decrease compared to the previous year [4].

Salmonella was mainly isolated from food in particular in *Gallus gallus* (chicken, broiler, laying hen and breeding chicken) [4,49]. The most detected serovar is *S*. Infantis (26.4%) followed by MVST (10%) and *S*. Typhimurium (6.5%) [49].

Antibiotics used for salmonellosis treatment in human and veterinary medicine and consequences on antimicrobial resistance.

The WHO categorized antibiotics on the basis of their importance for human medicine. Antibiotics defined as *Critically Important* for human health (CIA) have to respond to certain characteristics: to be the only or one of the available therapies used to treat a serious infection in people, (ii) to treat human infections caused by bacteria transmitted from non-human sources to human or bacteria that may acquire antibiotic resistance genes (ARGs) from non-human sources [50].

The aim of this list is to limit the use of CIA for veterinary medicine but also to promote their prudent use in both human and veterinary medicine [50].

CIA are divided in highest priority and high priority; the CIA with highest priority are: cephalosporins (3^{rd}, 4^{th} and 5^{th} generation), glycopeptides, macrolides and ketolides, polymyxins, quinolones [50].

In the most of cases, salmonellosis occurs in a mild form and could be resolved without antibiotic use. When the infection become more serious or immunocompromised patients, infants and elderly people are involved, the antibiotic treatments are life-saving. Antibiotics used as a first-line therapy against serious form of salmonellosis are: fluoroquinolones and third-generation cephalosporins, classified as CIA and used for invasive infection and as first choice for treating children, due to less side-effect and pharmacodynamic properties [51–53].

Veterinary public health plays an important role in the fight against human disease. However, antibiotics used in veterinary medicine could affect the development of antimicrobial resistant strains. Moreover, the use of a large amount of antibiotics to treat diseases in animals used for food production results in the selective pressure for antimicrobial resistant microorganisms and their spread [54].

Antibiotics in animal treatment should be used only when other strategies are not efficient and not as first line treatment, to preserve the efficacy over the time of antibiotics and limit the transfer of antimicrobial resistant pathogens to human [54,55].

In 1977, FDA decided to restrict the use of penicillin and tetracycline from animal feed because categorized as important antibiotics for human health. This was the first step to fight against the antibiotic resistance (AMR), while the second step was to abolish the antibiotic use as growth promotors [54,56].

The misuse of antibiotics in the past as growth promoters as well as for prophylaxis and treatment of animal infections increased the selective pressure with a consequential emergence of resistant

bacteria that could spread to humans through direct contact and via food chain, or from environmental pollution [57].

Moreover, the wide use of antibiotics in human and veterinary medicine increases the development of antimicrobial resistant strains. Notably, the use of broad spectrum drugs, as β-lactams antibiotics, in veterinary and human medicine poses a risk to select strains producing β- lactamases and/or AmpC β-lactamases that can be transferred between humans and animals [58]. For example, ESBL *Salmonella* were isolated in animals, food producing animals, food, seafood, vegetables and also RTE foods, suggesting the spread along the food production chain [59].

These bacteria are implicated in failure of human therapies improving request of second line antibiotics to control the infections [60].

The indiscriminate use of antimicrobials in human and animal medicine causes the spread of antimicrobial resistant strains. Preventive actions are required to avoid the spread of AMR and also of antimicrobial resistant pathogens from farm to fork. WHO list for CIA is also a guide to conscious use of antibiotics in human and veterinary medicine to contain the increasingly widespread phenomenon of AMR.

Sales monitoring of human antibiotics and critically important antibiotics for human health used in veterinary medicine.

In the EU the use of antibiotics for growth promotion was banned since 1 January 2006 [56,61]. It's important to highlight that the use of antibiotics in veterinary medicine requires authorization by competent authority and the antibiotic sale is controlled. This allows to publish an annual report to summarize antibiotic sale in 31 European countries [62].

In the EU, the best-selling antibiotics, used in food-producing animal therapy, are penicillins (31.1%), tetracyclines (26.7%) and sulfonamides (9.9%) [62].

In 2020, the proportion of sales of CIA, like 3rd and 4th generation cephalosporins, fluoroquinolones, quinolones and polymyxins, varied substantially between the EU countries [62]. Overall, between 2011 and 2020 (Fig. 4), sales of 3rd and 4th generation cephalosporins, polymyxins, fluoroquinolones and other quinolones decreased over the years [62].

In particular in 2020, the total sales for 31 countries of 3rd and 4th generation cephalosporins, fluoroquinolones, other quinolones and polymyxins was 0.2, 2.3, 0.2, 2.5 mg/PCU (population correction unit) for each class, respectively [62].

In Italy a reduction of 51% in annual sales of antimicrobials was observed in 2020 compared with 2011. In 2020, the best-selling antibiotic classes were penicillins, tetracyclines and sulfonamides. Moreover, Italy is one of the countries with a low sale level of highest priority CIA, in particular 3rd and 4th generation cephalosporins, quinolones/fluoroquinolones and polymyxins used in veterinary medicine; while Romania, Poland, Portugal, Slovakia, Hungary and Germany showed a high sale proportion of CIA at highest priority used for food-producing animals (Fig. 4) [62].

Thanks to the Italian National Action Plan against Antimicrobial Resistance (2020), a 10% reduction compared to 2016 in the use of CIA and a sale reduction for colistin were observed [63].

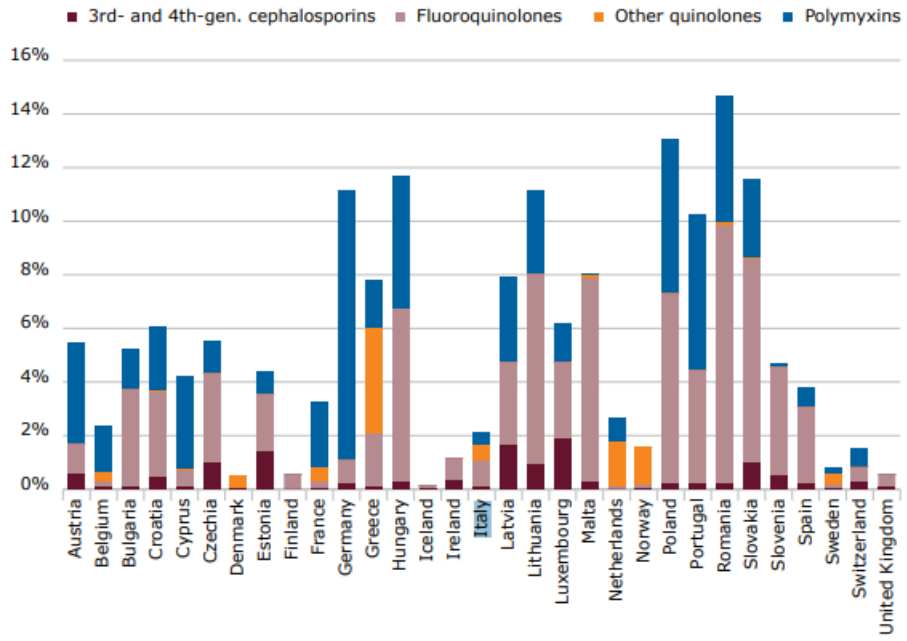


Fig. 4 Proportion of 3rd and 4th generation cephalosporins, fluoroquinolones, other quinolones and polymyxins sales for food-producing animals in 31 EU countries in 2019 [62].

From 2011 to 2020, in 25 EU countries, a decreasing trend in antibiotic sales was described (Fig. 5), in particular all antimicrobial classes, with the exception of amphenicols, showed a decrease in sales [62]. During this period, a decline in the sale of three highest-selling antimicrobial classes, tetracyclines, penicillins and sulfonamides, was noticed. The proportion of the reduction was 59.5%, 20.3% and 51%, respectively for the above mentioned antibiotics [62].

National campaigns for responsible and prudent use of antibiotics in animals, restriction of particular antibiotic classes in veterinary medicine, control measures for antibiotic sale, EU guidelines are actions that implement the antibiotic sales reduction for veterinary medicine. These measures are fundamental for the containment of the AMR phenomenon.

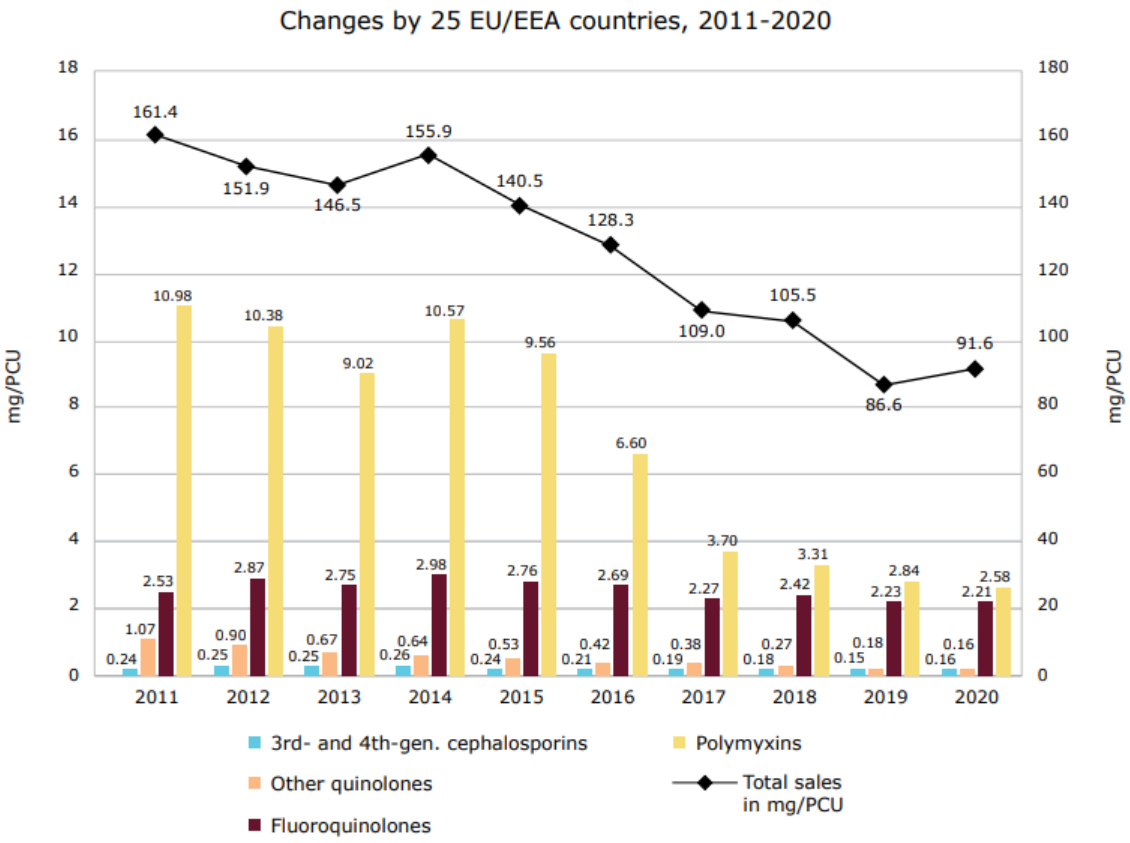


Fig. 5 Trend of antibiotic sales in veterinary medicine, in 25 EU countries, from 2011-2020 [62].

Determinants of AMR: Mobile genetic elements (plasmids, transposons and integrons) and horizontal gene transfer.

The emergence of resistant or multi-drug resistant (MDR) microorganisms, due to the acquisition of mobile genetic elements carrying ARGs, is becoming a Global Health problem [57].

Bacteria acquired with time the ability to adapt to environmental adverse conditions, such as the exposition to toxic substances like antibiotics. With the use of antibiotics, bacteria developed resistance mechanisms which can be: 1) DNA mutations that greatly facilitate the process of adaptation, 2) Acquisition of genetic material from other bacteria which expands the genome [64].

The acquisition of genes, from one cell to another, involved in AMR could occur by three methods: transformation, transduction and conjugation [64].

Transformation is the passage of free DNA fragments, originated from bacterial lysis, from a donor cell to a recipient cell. Transduction is the DNA transfer mediated by bacterial viruses called

bacteriophages. Conjugation, the most important transmission mechanism of ARGs, consists in the passage of plasmid DNA from one bacterial cell to another, through a “bridge” called pilus (Fig. 7) [65].

Moreover, mobile genetic elements are divided into 2 categories: elements that can move from one bacterial cell to another (conjugative plasmids and transposons) or elements that can move from one genetic location to another but in the same cell (transposons, gene cassettes and ISCR, Insertion Sequence Common Region-promoted gene mobilization).

The acquisition of genes from one bacterium to another, by HGT, takes place mainly throughout conjugative plasmids [64].

Plasmids

Plasmids are double-stranded, circular DNA molecules that replicate independently of the chromosome and could be transferred from one cell to another through HGT in particular by conjugation [66]. Plasmids show different sizes from a few to more than several hundred kilobase pairs (Kb) [67].

Conjugation is the most important HGT mechanism used to transfer DNA, in particular plasmid conjugation occurs between two different cells, a donor cell and a recipient cell, by direct contact. The donor cell produces a pilus that attaches the recipient cell like a bridge and allows the plasmid transfer (Fig.6) [68].

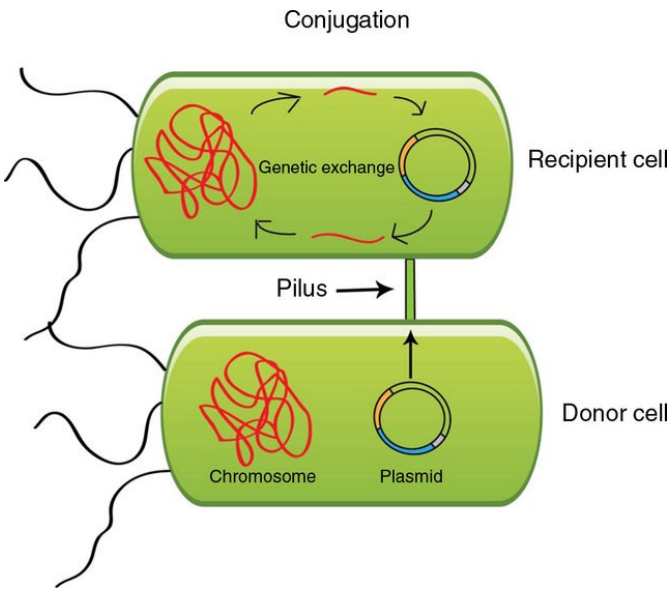


Fig. 6. Mechanism of conjugation between a donor cell and a recipient cell [69].

Hence, plasmids carry auxiliary genes involved in survival in particular environmental conditions, for example genes conferring resistance to antibiotics, heavy metals, or genes codifying enzymes able to improve the nutrition ability of the cells or virulence genes involved in invasion and adhesion of human/animal cells [64].

Moreover, these genes could be associated with mobile genetic elements (MGEs), like transposons, that move within the plasmid or spread from the plasmid to the chromosome [69]. Plasmids involved in AMR usually include genes conferring resistance to antibiotic classes used in clinical medicine or CIA used as first line therapy, for example such as extended-spectrum cephalosporins, carbapenems, colistin [70].

The variety of plasmids in nature required the ability to categorize these elements to study their distribution, the relationship with the host cell and also plasmid evolution during the time [71]. Indeed, the diffusion of resistance plasmids is one of the major concerns for the study of the AMR [72].

To have a better identification and classification of plasmids is necessary refer to elements that are stable and conserved, as those involved in their replication control. The replicon is a high conserved part of the plasmid where genes involved in activation and control of replication are located [69].

Carattoli et al (2005) proposed a plasmid classification scheme based on Incompatibility (Inc) groups throughout the identification of replication origin (replicon) [73]. The incompatibility is defined as the inability, for two plasmids, to be propagated in the same cell line [74]. Two plasmids are incompatible when their replicons belong to the same Inc group [73].

The identification of the Inc group is usually used to classify plasmids. This method allows to study the spread of plasmids involved in AMR and also trace the evolution and diffusion of emerging plasmids [73].

One bacterial cell could carry more than one plasmid, making difficult the identification of the whole plasmid content. The PCR-based replicon typing (PBRT) method, based on multiplexed-PCR detection, allows the identification of replicons of the most important plasmid families found in *Enterobacterales* [75,76].

The most recent version of PBRT allows a quick and easy plasmid characterization through replicon detection. The PBRT 2.0 is based on 8 multiplex PCR for the detection of 30 replicons that are representative of the major plasmid Incompatibility groups circulating among *Enterobacterales*, and are classified as follow:

HI1, HI2, I1, I2, X1, X2, X3, X4, L, M, N, FIA, FIB, FIC, FII, FIIS, FIIK, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M and FIB-M [72,73,77,78]

Plasmids characterization is important to study the association between plasmids and AMR features of bacterial strains. IncF plasmids have been associated with the wide spread of the extended-spectrum β-lactamases (ESBL) genes, but also with the diffusion of AmpC genes or quinolone and aminoglycosides resistance determinants. IncF plasmids are usually found in clinically relevant *Enterobacterales* [72]. Often, they can be found in a multi-replicon status since they carry more than one replicon. One classic example of multi-replicon plasmids relates to FII, FIA, FIB plasmids [72]. When the plasmid is in a multi-replicon status, the replicon not involved in the replication process can diverge and generate new variants able to overpass the problem of the incompatibility, upon the arrival of a new plasmid belonging to the same Inc group [72].

This phenomenon gives a great versatility and adaptability to the plasmid, explaining the large success and diffusion of IncF plasmids in clinically relevant enteric bacteria [72].

Transposons

Transposons (Tns) are DNA elements able to move from different places in the same DNA molecule or from one DNA molecule to another. The transposition of these mobile genetic elements is due to enzymes named transposases [79].

Tns are also involved in the AMR since they incorporate ARGs and, with a “jump-system”, allow the transposition of genes in different part of the same plasmid within one bacterial cell, in different plasmids belonging to two cells, or from one plasmid to a chromosome [64].

Beside transposons, Insertion sequences (IS) are also considered transposable elements [80]. Complex Tns are composed of a pair of IS elements and a central DNA, not able to transpose autonomously. In particular, IS elements are usually flanked to gene sequences that confer resistance to antibiotics [64]. An example of IS element is IS26, mainly found in composite Tns in Gram-negative bacteria [79]

IS elements have the ability to transpose as individual elements or as part of the complete transposable structure. The complex elements are not divisible; this is the example of Tn5 involved in aminoglycosides resistance or Tn10 involved in tetracycline resistance. They can be found in Gram-negative bacteria, in particular in *Enterobacterales* [64].

Tn3 codifies for resistance to β-lactam antibiotics, including ampicillin; Tn21 encodes for resistance to streptomycin, spectinomycin and sulphonamides and mercuric ions. These are examples of complex transposons, commonly found on *Enterobacterales* plasmids [64].
Composite Tns carrying drug ARGs and their spread among bacteria are the most critical challenge in infectious diseases treatment [79].

Integrons and gene cassettes

The integron is a gene capture system able to catch inactive mobile genes, called gene cassettes, and express them making them functional. Integrons are composed by: *intI* gene, that codifies for the enzyme named integrase, the *attI* recombination site, which is recognized by the integrase, Pc, a promoter essential for transcription and expression of gene cassettes present in the integron, and finally a small DNA sequence harbored genes (often ARGs) named gene cassette [64,81,82].
Through integrons, cassette genes are able to move and arrange genes, thus creating an integron with different genes. Integrons and gene cassettes are involved in the spread of AMR. Gene cassettes have the possibility to integrate in a single integron and to produce super-integrons harboring multiple genes also involved in AMR [83]. An example of AMR gene cassettes includes genes codifying resistance for imipenem and meropenem [64]
Integrons are classified in two different types: mobile integrons (MIs) and chromosomal integrons (CIs). CIs are not usually involved in antimicrobial resistance and can carry from zero to hundreds gene cassettes. On the other hand, MIs carry a limited number of gene cassettes and are often involved in the AMR spread [82].

Antibiotic resistance: a One Health and Global Health approach

The use of antibiotics in human and animal medicine is essential to treat infections. Since the antibiotics were discovered until now they are recognized as life-saving drugs. On the other hand, the use of antibiotics accelerates the natural development of resistance mechanisms activated by bacteria and the possible emergence of pathogens resistant to antibiotics considered critically important for human health [84]. Alexander Fleming was the first who mentioned the possible resistance to penicillin if the antibiotic was used in an incorrect way [85].

As said before, the AMR is a natural process for bacteria and occurs through gene mutations. In particular, antibiotics produce a selective pressure that cause adaptation phenomena, as gene mutations, that allow bacteria survival also in hostile conditions [85].

The AMR is one of the global health challenges of the 21th century. The AMR problem doesn't have to be associated with the only study of the complication in healthcare system, since different ecosystems contribute to the emergence, acquisition and spread of AMR. For this reason, a correct view of the AMR phenomenon should consider two important perspectives: One Health and Global Health [86]

These two concepts are connected and are based on the idea that human and animal health are strictly associated to the ecosystem of which they are part [86].

This holistic system offers a wide concept of the AMR problem since, for the first time, the ecosystem health, social aspect and public health are included in human and animal health [86,87]. One Health concept studies, in-depth, the association between human, animal and environment system at "local level", and highlights how the health of each system is influenced by the others.

Global health studies the AMR phenomenon in a global scale, focusing the attention on the global conditions that allow the AMR spread, in order to find a solution in the integrated approach involving political and socioeconomic actions in different countries all over the word [86]. For example, the globalization of food products, food import/export and travels, have also an important impact in food safety and AMR diffusion [88] (Fig. 7).

The AMR widespread viewed under global perspective, where more local ecosystems come in contact, finds its best conditions in phenomena like globalization, human travels, animal migration that increase the interconnection of different "local systems" in the word (Fig. 5) [86].

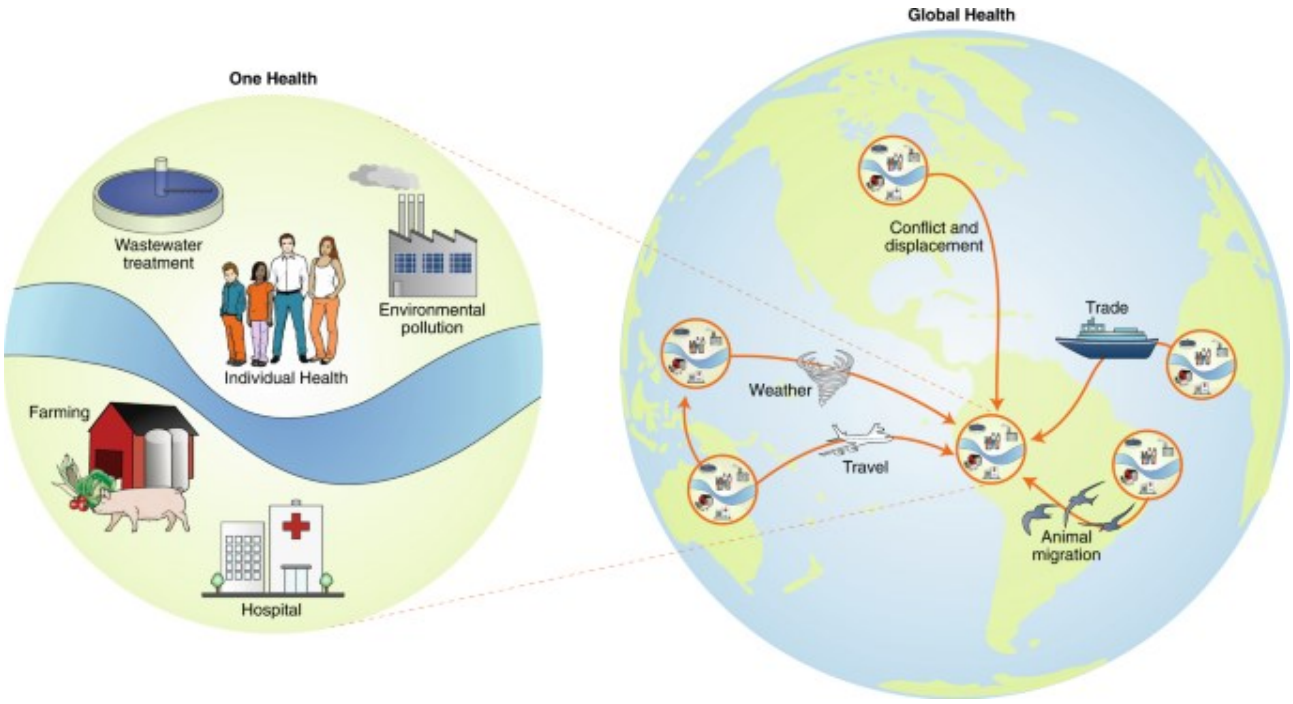


Fig. 7 AMR concern viewed under two perspectives: One Health and Global Health [86].

Under the One Health perspective, AMR problem is viewed as a public health problem also associated to the spread, among the environment-animal-human system, of AMR determinants due to MGEs acquired by HGT [89].

The exchange of AMR determinants could be explained as a Russian doll where all the elements involved are connected and influenced each other (Fig. 8). ARGs are usually associated with plasmid or other MGEs like integrons or transposons, able to spread from one bacterial cell to others. This bacterium could infect humans or animals or may be transmitted to hospital population through HGT. On the other hand, the acquisition of AMR determinants in a bacterial population could increase the possibility of AMR determinant spread also in environments where the antibiotics aren't used. For example, Salerno et al (2022) studied the presence of ARGs in an antibiotic free broiler farm. According to their results, although in the broiler farm the use of antibiotics was banned, ARGs were present in animal faeces and meat processing environment [90]. Another study, conducted in Italy, confirmed the presence of ARGs, in particular *tet* genes, in an antibiotic-free broiler farm. The broad diffusion of *tet* genes is a result of previous intensive use of tetracycline that established the persistence of resistant bacteria population in the farm environment [91]. This phenomenon suggests the possible diffusion of AMR bacteria by meat handlers, by water but also by wild animals, highlighting the easy spread of bacteria and AMR determinants in the environment [48,91].

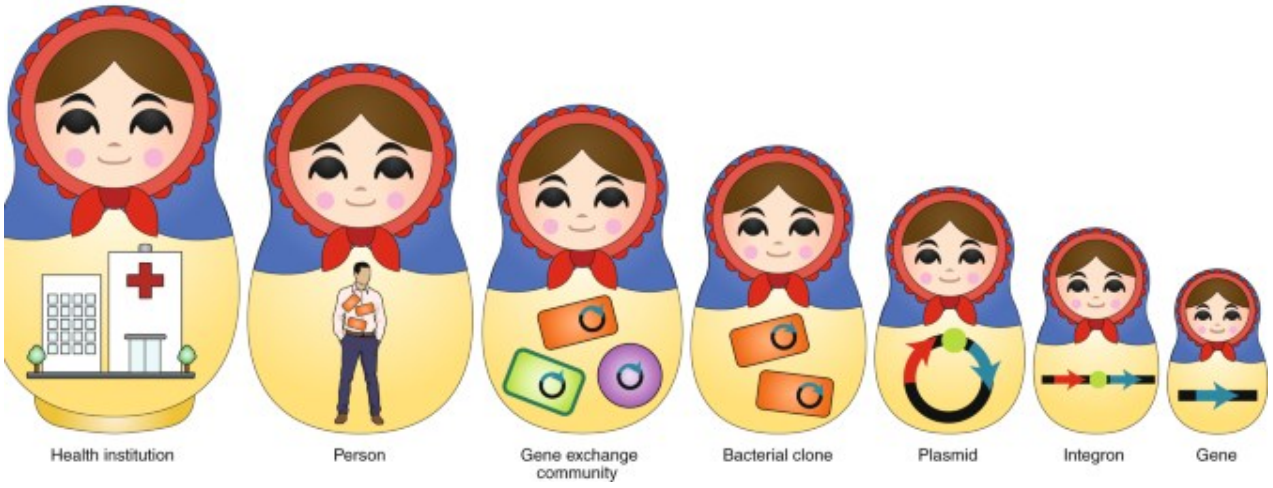


Fig. 8 AMR transmission is associated to a Russian doll model, since different elements involved in the spread of AMR, like integrons and other mobile elements, influence one other in a hierarchical system [86].

For this reason, in a Global Health view, the AMR phenomenon starts in a place and then spread all over in the word [92].

The emergence of AMR events in different places in the word is the results of a globalization process and unification of AMR problem due to the continuous spread of AMR determinants. To better understand the AMR spread it's important to focus the attention on the HGT that allows the ARGs spread in the environment-animal-human system.

Epidemiology of antibiotic resistant *Salmonella*

According to Commission Implementing Decision 2013/652/EU [93], monitoring of antibiotic resistant *Salmonella*, *Campylobacter jejuni* and indicator commensal *E. coli* is mandatory. In particular, in *Salmonella*, a specific monitoring in ESBL/AmpC and carbapenemases producing strains is required [93].

Moreover, *Salmonella* have to be monitored in the major food-producing animals as broilers, laying hens, fattening turkeys, fattening pigs, calves, their derived meat and also their carcass [93]. For this reason, every year, EFSA/ECDC publish a report on the results of the AMR monitoring in the most important zoonotic bacteria as *Salmonella.*

In 2019/2020, according to the last report of EFSA, resistance to ampicillin (29.8%), sulfonamides (30.1%) and tetracyclines (31.2%) was reported at high levels in animal and human isolates and, generally, MVST showed the highest resistance level for these 3 antibiotics [94].

Moreover, resistance to quinolones was observed at high levels among *Salmonella* isolates obtained from broilers, fattening turkeys and poultry carcasses and meat [94].

Poultry monitoring programs allow to observe high resistance levels in poultry for ciprofloxin/nalidixic acid. In particular, *S*. Infantis and *S*. Kentucky showed resistance to these two compounds in poultry [94,95].

Moreover, these resistances are usually associated to sulfamethoxazole and tetracycline resistance prevalent in *S*. Infantis clones isolated in Europe [94,96].

Monitoring of resistance to highest priority CIA is also essential to control the spread of *Salmonella* resistant to these important antibiotics. Resistance to third-generation cephalosporins, cefotaxime and ceftazidime, in *Salmonella,* was detected at low levels in human isolates. In particular, ESBL or AmpC strains were detected at low level in human and animal samples [94]. Cefotaxime and ceftazidime resistance was mainly found in broilers in the EU. ESBL producers were isolated in particular in poultry and associated to specific serovars like *S*. Infantis, *S*. Kentucky, *S*. Bareilly and *S.* Bredeney

Resistance to ciprofloxacin (14.1%) was reported at high levels in *S*. Infantis and *S*. Kentucky.

Carbapenem resistance is not diffused in *Salmonella*, in 2019/2020: none carbapenemases-producing *Salmonella* isolates were detected from animals, while one isolate was detected in 2019 and two isolates in 2020 from humans [94].

In Italy the ESBL phenotype was detected in 24.8%. from broilers and 26.5% from turkeys; the serovar isolated in poultry was, in all samples, *S*. Infantis.

MDR *Salmonella* was detected at high levels from human cases and the most involved serovars are *S*. Kentucky (76.6%) and MVST (74.2%). Moreover, MDR was observed at very high levels in broilers, calves, pigs [94].

From farm to fork: spread of AMR

Food chain is considered an ecosystem composed by different ecological niches interacting each other, where large amounts of antibiotics are used and numerous microorganisms co-exist [97,98]. In these ecological niches two different pathways are involved in AMR spread: (i) microorganisms with innate resistance due to genetic errors in resistant genes can transfer resistance to the progeny through vertical gene transfer; (ii) genetic exchange through HGT where mobile genetic elements are involved [98].

Antibiotics are used in food animal production not only to treat infections but also as metaphylactic, where the identification of infection in one animal results in the treatment for whole flock, or as prophylactics approach, where sub-therapeutic doses are used to avoid adverse effects due to antibiotic therapy [98,99].

The emergence of antimicrobial resistance in the food chain is considered a cross-sectoral problem due to: (i) antibiotic misuse and overuse in agricultural and livestock production, (ii) the spread of ARGs through bacteria intentionally added during processing (e.g., starter cultures and probiotics), (iii) environmental contamination before or after food processing, (iv) the cross-contamination with antibiotic resistant bacteria colonizing other foods during industrial processing and handling by the consumer [98,100].

Human can be exposed to antibiotic resistant bacteria through direct or indirect contact. Direct contact occurs between human and infected animal or biological contaminated substances like urine, faeces, blood, saliva [98,101].

Indirect contact occurs with the ingestion of contaminated food products [98].

Food products mainly described as source of antibiotic resistant bacteria are RTE meat, cooked meat and bulk milk but also from various animals such as cattle, poultry, swine, goat and sheep [98,102]

Focusing the attention on the different ecological niches that constitute the food chain, it is possible to study how they are involved in the spread of antibiotic resistant pathogens, as *Salmonella*, and AMR determinants.

Food animal microflora is considered as a reservoir of AMR determinants. The misuse of antibiotics has two different effects in animal gut microflora: (i) competitive effects due to the suppression of the normal flora allowing the pathogen to colonize the site; (ii) selective effect due to the selection of resistant species that could transmit their AMR determinants to food pathogens through HGT [103].

Moreover, the extensive use of antibiotics in human and animals causes an accumulation of these compounds in the environment that become as a reservoir of AMR determinants and antibiotic resistant bacteria [57].

Environment has a significant role in the AMR spread, it is a niche of microorganisms present outside the host (in water, soil, air) which can interact with foodborne pathogens. These environmental microorganisms are an important source of ARGs and are named environmental resistome. The term “Resistome” describe microorganism living in the environment or commensal microbes which

play an important role in the AMR spread since they transfer AMR determinants to foodborne pathogens involved in human infection [104].

For example, antibiotic resistant *S*. Typhimurium were found from swine and poultry housed in antibiotic-free production systems, to emphasize the import role of environment in the spread of antibiotic resistant determinants [105–107].

Water also is a reservoir of AMR determinants [104]. Indeed, the bacteria recovered in aquaculture showed a similar resistance pattern to bacteria isolated in agriculture [108]. Irrigation practice can occur with surface water that include treated and untreated wastewater that can originate also from hospital and municipal wastewater and could be contaminated by antibiotic resistant bacteria or AMR determinants [48]. The use of contaminated water for irrigation could spread the resistant bacteria in fresh products as vegetables and fruits [104].

Veterinarians, farmers or food handlers are highly exposed to infection with antibiotic resistant bacteria [48]. On the other hand, they could also be a vector of antibiotic resistant microorganisms. Evidences that humans could be a primary source of AMR for farm animals are presented in literature. Guillon et al reported that highly ciprofloxacin resistant *S.* Kentucky was introduced by a worker, after a trip in Africa, in a farm [48,109].

As described, until now the use of antibiotics in the food chain is considered an important cause of AMR spread; this problem is worrying when foodborne pathogens, like *Salmonella,* are involved since the infection may result in high mortality. So, it's important to reduce the use of antibiotics in food process but also, it's important to study the MGEs involved in AMR spread through different species during the food production process, too.

Moreover, to limit ARGs transfer during food processing, physical parameters (such as temperature and exposition time) have to be observed and good hygienic practices applied at all stages of the food chain, from farm to fork [100].

Xin Wu et al demonstrated that high level of antibiotic resistant *Salmonella* contamination occurs mostly during the food processing but with the observation of good hygiene practices the *Salmonella* occurrence is reduced [110].

For this reason, it' important to decrease the use of antibiotics in food production animals but also it's necessary to follow the HACCP system to decrease the spread of antibiotic resistant pathogens during the food production practices.

Salmonella Infantis pESI-like megaplsmid

S. Infantis is one of the five main causes of human salmonellosis in the EU. In 2020, according to the last report of EFSA [94], MDR *S*. Infantis in broilers and turkeys reached 73% and 22%, respectively. Moreover, an increase in the number of MDR *S*. Infantis has been also noticed in human clinical isolates (45.3%), in 2020, in contrast with 2019 (35.7%) [94].

The issue of MDR in food production systems became more relevant when CIAs for human health are involved in the resistance. In the last decade, MDR and ESBL *S*. Infantis has been frequently reported from food-producing animals and humans in Italy [94,111]. In fact, Italy possesses high number of isolates from both, broiler and turkeys, exhibiting a MDR ESBL phenotype and some of them reported also AmpC profile [94] although the use of 3rd and 4th generation cephalosporin is not permitted in poultry and off-label use is prohibited according to EU legislation [51,94,112].

For this reason, the high proportion of MDR ESBL/AmpC-producers in *S*. Infantis can be attributed to a clonal expansion and spread as described by Franco et al [51].

Indeed, in Italy a MDR and ESBL producing *S*. Infantis clone harboring an IncI/IncP chimeric megaplasmid (~280–320 kb), carrying virulence, fitness and MDR genes, in particular the ESBL encoding gene *bla*$_{\text{CTX-M-1,}}$ increasingly spread during the last years [113]. This megaplasmid is named pESI-like since it was similar to that, named pESI, described in Israel [114].

The ancestral pESI isolated in Israel was reported conferring tetracycline, sulfamethoxazole and trimethoprim resistance to *S*. Infantis extended spectrum cephalosporin sensible. Moreover, the pESI plasmid promotes pathogenicity mechanisms and it's able to increase intestinal inflammation in experimental mouse infections. The Israel pESI also harbored genes able to increase bacterial tolerance to environmental mercury (mer operon) and oxidative stress. It is defined a chimeric plasmid evolved by recombination between, at least, IncI1 and IncP ancestral plasmid groups [51,114]. The *S*. Infantis clone harboring the pESI-like plasmid was first identified in Italy, in 2014, by Franco et al from food-producing animals and humans [51].

The pESI-like megaplasmid acquired more resistance genes. In particular it is characterized by a specific gene panel that includes third-generation cephalosporin (*bla*$_{\text{CTX-M}}$), tetracyclines (*tet*A), trimethoprim (*dfr*A), sulphonamides (*sul*1), aminoglycosides (*aad*A). Moreover, other advantageous genes enhancing virulence traits of *S*. Infantis clone were associated to pESI-like and include yersiniabactin biosynthetic protein gene *irp*2, fimbria gene *ipf*, toxin/antitoxin (T/AT) system and genes conferring resistance to quaternary ammonium compounds [113]. Of particular concern is the *bla*$_{\text{CTX-M-65}}$ gene presented in a large part of pESI-like plasmids in the U.S. [115].

Due to ARGs, genes involved in virulence, colonization and fitness, previously described, once acquired, pESI-like plasmid became established in the local population because of the selective advantages it confers to the host bacteria [113,116],

In the U.S., in 2018, an outbreak of an ESBL producing *S*. Infantis which harbored a pESI-like megaplasmid that carried $bla_{\text{CTX-M-65}}$, associated with raw chicken, was reported. The majority of the strains harboring $bla_{\text{CTX-M-65}}$ were additionally resistant to ciprofloxacin, nalidixic acid, chloramphenicol, gentamicin, sulfamethoxazole, tetracycline and trimethoprim [94].

The ESBL-producing *Enterobacterales* are considered "Critical Priority Pathogens" by the WHO since they show resistance to third-generation cephalosporin considered as CIA for human health and first choice treatment for children and invasive infection [51,117,118].

Due to these characteristics and the ability to spread and establish well in the host cell, this plasmid can spread along the food chain and arrive to humans in the way "from farm to fork" [113].

AIM OF THE STUDY

The aim of this study was to investigate the antibiotic resistance profiles of *Salmonella* spp. strains isolated in Marche Region from veterinary and food-related environments, food animals, foods, and human clinical samples, in order to possibly trace the spread of AMR in the food chain, according to the One Health approach.

Distribution and prevalence of major plasmid replicons in the same strains have been determined to assess the presence and spread of plasmids carrying AMR determinants in the food chain. Moreover, the presence of the pESI-like plasmid-carrying *S*. Infantis clone in AMR *S*. Infantis strains, isolated from various sources in Marche Region, was investigated for the purpose of a better understanding of their genetic characteristics, AMR profile and confirm their spread along the food chain using a phylogenetic approach.

PAPERS

Included in the thesis

This thesis includes conference proceeding, conference poster, original research paper and paper in preparation, divided in section and presented in chronological order. Moreover, the only Italian conference proceeding was translated in English immediately below the Italian original version.

Conference proceeding paper

I. **Ilaria Russo**, Daniela Bencardino, Francesca Andreoni, Giuditta Fiorella Schiavano, Maira Napoleoni, Giorgio Brandi, Giulia Amagliani. Application of the PCR-based replicon typing (PBRT) method to trace antimicrobial resistance plasmids in the food chain; *Journal of Preventive Medicine and Hygiene*, 2021 Dec; 62(1 Suppl 2): E1–E60 https://doi.org/10.15167/2421-4248/jpmh2021.62.1s2

II. **Russo I.**, Bencardino D., Andreoni F., Schiavano G.F., Brandi G., Napoleoni M., Staffolani M., Amagliani G. Studio epidemiologico e caratterizzazione molecolare di ceppi di *Salmonella* antibiotico-resistenti isolati nella filiera alimentare della regione Marche; ATTI- 54° Congresso Nazionale SItI, 2021. "LA SANITA' PUBBLICA NEL POST COVID".

III. **I. Russo**, J. Fischer, A. Groger, A. Irrgang, G.F. Schiavano, F. Andreoni, M. Napoleoni, G. Brandi, G. Amagliani. From farm to fork: Spread of multidrug resistant Salmonella Infantis pESI-like blaCTX-M-1 clone in Marche Region; *Journal of Preventive Medicine and Hygiene,* 2022 Jun; 63 (2 Suppl 1): E1-E443 https://doi.org/10.15167/2421-4248/jpmh2022.63.2S1

Poster

I. **Russo I**., Bencardino D., Andreoni F., Schiavano G.F., Brandi G., Napoleoni M., Staffolani M., Amagliani G. Studio epidemiologico e caratterizzazione molecolare di ceppi di *Salmonella* antibiotico-resistenti isolati nella filiera alimentare della regione Marche; ATTI- 54° Congresso Nazionale SItI 2021. "LA SANITA' PUBBLICA NEL POST COVID". 3-6 novembre 2021, Lecce.

Original research paper

I. **Russo, I**., Bencardino, D., Napoleoni, M., Andreoni, F., Schiavano, G. F., Baldelli, G., Brandi, G., Amagliani, G. Prevalence, Antibiotic-Resistance, and Replicon-Typing of Salmonella Strains among Serovars Mainly Isolated from Food Chain in Marche Region, Italy. *Antibiotics*, **2022**, *11*(6), 725.

https://doi.org/10.3390/antibiotics11060725

Paper in preparation

I. **I. Russo**, J. Fischer, L. Uelze, A. Irrgang, G.F. Schiavano, F. Andreoni, M. Napoleoni, G. Brandi, G. Amagliani. From farm to fork: Spread of multidrug resistant *Salmonella* Infantis pESI-like blaCTX-M-1 clone in Marche Region.

Not included in the thesis

Conference proceeding paper

I. **Schiavano G.F**., Baldelli G., Amagliani G., Russo I., Brandi G. INATTIVAZIONE DI L. MONOCYTOGENES SU SUPERFICI DI VARIA NATURA MEDIANTE LAMPADA UV-C. I. ATTI- 54° Congresso Nazionale SItI, 2021. “LA SANITA’ PUBBLICA NEL POST COVID”.

CONFERFERENCE PROCEEDING I

In this study, the epidemiological data about the circulation of AMR *Salmonella* strains in Marche region was investigated. Moreover, the molecular characterization of the main resistance plasmids Incompatibility group carried by *Salmonella* strains isolated at different levels in the food chain and of human origin were detected, in order to trace their potential spread.

Application of the PCR-based replicon typing (PBRT) method to trace antimicrobial resistance plasmids in the food chain

Ilaria Russo[1], Daniela Bencardino[1], Francesca Andreoni[1], Giuditta Fiorella Schiavano[2], Maira Napoleoni[3], Giorgio Brandi[1], Giulia Amagliani[1]

[1]Department of Biomolecular Sciences, University of Urbino, Urbino (PU), Italy
[2]Department of Humanities, University of Urbino, Urbino (PU), Italy
[3]Istituto Zooprofilattico Sperimentale Umbria e Marche (IZSUM), Tolentino (MC), Italy

INTRODUCTION

Antimicrobial resistance (AMR) is the capacity of microorganisms to resist medicines used to treat infections and represents a major global threat of increasing concern. Antimicrobial use in agriculture and farming contributes to the emergence of AMR, and food systems play an important role in its development and spread. Food can become contaminated with AMR bacteria carrying antimicrobial resistance genes (ARGs) at every stages of the food chain, serving as a vehicle of foodborne exposure to resistant bacteria.

They may also serve as a source of ARGs that can be acquired by other microorganisms in the gastrointestinal tract, including human pathogens, mainly through mobile genetic elements (i.e. plasmids) by horizontal gene transfer (HGT). Plasmids are the main vectors of ARGs in *Enterobacteriaceae* and plasmid typing is essential for the analysis of evolution and spread of AMR in the food chain. This can be accomplished through the application of the PCR-based replicon typing (PBRT) method, that allows fast and easy identification of resistance plasmids through replicons detection[1].

In 2018, salmonellosis was the second bacterial foodborne zoonosis in the EU[2]; the isolation from food of *Salmonella* strains resistant to tetracycline, ampicillin, nalidixic acid and trimethoprim/sulfamethoxazole has been recently reported, at the European level[3] and in Italy[4].

The aims of the study were: the epidemiological investigation about the circulation of AMR *Salmonella* strains in Marche region; the molecular characterization of the main resistance plasmids carried by *Salmonella* strains isolated at different levels in the food chain and of human origin, in order to trace their potential spread.

MATERIALS AND METHODS

Epidemiological data about *Salmonella* prevalence in food, animals, food-related environments and humans were retrieved from official reports[2, 5, 6, 7, 8], and scientific literature[4]. Information about AMR in *Salmonella* was also obtained[3, 6, 7, 8].

AMR and multidrug resistant (MDR) *Salmonella* strains were provided by the Regional Reference Center for Pathogenic Enterobacteria (CRREP) of IZSUM. Total bacterial DNA was amplified with the PBRT 2.0 Kit (Diatheva, Fano, Italy), able to identify, through 8 multiplex PCRs targeting 30 replicons, the presence of the main *Enterobacteriaceae* resistance plasmids. The amplicons recognized by the PBRT kit were analyzed by capillary electrophoresis with an AATI Fragment Analyzer (Agilent, Santa Clara, CA, USA).

RESULTS

Epidemiological data highlight that *Salmonella* spp. showed an increasing trend in the last three years in human and veterinary samples in Italy. *S*. Infantis, the Monophasic Variant of *Salmonella* Typhimurium (MVST), *S*. Bredeney *S*. Derby and *S*. Typhimurium are the most prevalent serovars in food and veterinary samples, and data of Marche Region are in accordance with national information. Poultry is the animal species most often contaminated with *Salmonella*. At the EU level, *S*. Bredeney is replaced by *S*. Enteritidis among the “top five serovars”. The same serovars are also the most diffused in human infections.

Salmonella strains of Marche Region showed resistance mainly to tetracycline and ampicillin followed by nalidixic acid, trimethoprim/sulfamethoxazole and cefotaxime. Intermediate resistance to ciprofloxacin was found in veterinary and human strains.

Concerning resistance plasmid distribution, 26/52 strains tested positive for at least one replicon (69% 1 replicon, 23% 2, and 8% 3 replicons). In our study, and in accordance with previous data about *Salmonella*, FIIS and FII (IncF incompatibility group) are the most frequently detected replicons. IncF plasmids confer resistance to all major classes of antimicrobials, including β-lactams, aminoglycosides, tetracyclines, chloramphenicol, and quinolones[9].

However, replicon distribution was different among serovars. Strains of the serovar *S*. Typhimurium were those in which replicons were detected most frequently, while the most heterogeneity was detected in S. Derby (4 different replicons distributed in distinct strains).

Replicons of different types were found mainly in food and human strains. FIB, FII, X1 were found in animal, food and human strains. FIIS, I1α and FII were found in food and human strains. X4 was found in all sample sources.

CONCLUSIONS

Surveillance of AMR and antimicrobial use in primary food production environments is crucial to obtain data necessary for risk assessment and risk management.

The PBRT method is a useful tool to trace the spread of the main resistance plasmids along the food chain. However, a larger number of strains from different sources, including humans, will be needed to provide reliable data and statistical association between AMR phenotype and replicon typing profile, especially in case of MDR.

Results of this study highlight the epidemiological importance of tracing AMR plasmids along the food chain.

Spread of resistance plasmids in the food chain through HGT is highly probable.

REFERENCES

[1] Carattoli A., Bertini A., Villa L., Falbo V., Hopkins K.L., Threlfall E.J. 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods. 63:219-228.

[2] EFSA and ECDC. The European Union One Health 2018 Zoonosis Report. EFSA J. 2019, 17.

[3] EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control), 2020. The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018. EFSA Journal 2020;18 (3):6007, 166 pp. https://doi.org/10.2903/j.efsa.2020.6007.

[4] Peruzy M.F., Capuano F., Proroga Y.T.R., Cristiano D., Carullo M.R., Murru N. 2020. Antimicrobial susceptibility testing for *Salmonella* serovars isolated from food samples: five-year monitoring (2015-2019). Antibiotics 9(7):365.

[5] National surveillance centre for *Salmonella* (Enter-Vet) https://www.izsvenezie.it/temi/malattie-patogeni/salmonella/enter-vet/

[6] Regional Reference Center for Pathogenic Enterobacteria (CRREP) of Marche region. Napoleoni M., Medici L., Staffolani M., Fisichella S. 2018. Dati relativi agli isolamenti di batteri enteropatogeni

effettuati da casi clinici umani, da animali, da alimenti e da ambiente nella Regione Marche – anno 2017. Sanità Pubblica Veterinaria. 110:1-22.

[7] Regional Reference Center for Pathogenic Enterobacteria (CRREP) of Marche region. Napoleoni M., Medici L., Staffolani M., Fisichella S. 2019. Dati relativi agli isolamenti di batteri enteropatogeni effettuati da casi clinici umani, da animali, da alimenti e da ambiente nell'anno 2018 nella Regione Marche. Sanità Pubblica Veterinaria. 114:1-26.

[8] Regional Reference Center for Pathogenic Enterobacteria (CRREP) of Marche region. Napoleoni M., Staffolani M., Pazzaglia I, Fisichella S. 2020. Dati relativi agli isolamenti di batteri enteropatogeni effettuati da casi clinici umani, da animali, da alimenti e da ambiente nell'anno 2019 nella Regione Marche. Sanità Pubblica Veterinaria. 118:1-35.

[9] Yang QE, Sun J, Li L, Deng H, Liu BT, Fang LX, Liao XP, Liu YH. IncF plasmid diversity in multi-drug resistant Escherichia coli strains from animals in China. Front Microbiol. 2015 Sep 22;6:964. doi: 10.3389/fmicb.2015.00964. PMID: 26441898; PMCID: PMC4585273.

CONFERFERENCE PROCEEDING II

The focus of this paper is to study gene determinants involved in AMR, in particular the gene variants involved in tetracycline resistance (*tet*) were detected. Moreover, the diffusion of AMR in the food chain by tracing the distribution of the main plasmid families in *Salmonella* strains isolated in the Marche Region was studied in dept, in particular the association between phenotypic AMR and the presence of plasmids was statistically evaluated.

Studio epidemiologico e caratterizzazione molecolare di ceppi di *Salmonella* antibiotico-resistenti isolati nella filiera alimentare della regione Marche

Russo I.[1], Bencardino D.[1], Andreoni F.[1], Schiavano G.F.[2], Brandi G.[1], Napoleoni M.[3], Staffolani M.[3], Amagliani G.[1]

[1]Università degli Studi di Urbino Carlo Bo, Dipartimento di Scienze Biomolecolari
[2]Università degli Studi di Urbino Carlo Bo, Dipartimento di Studi Umanistici
[3]Centro di Riferimento Regionale Patogeni Enterici, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", sezione di Tolentino (MC)

INTRODUZIONE

L'uso di antimicrobici nella catena alimentare contribuisce alla selezione di microrganismi resistenti potenzialmente trasmissibili all'uomo. La diffusione dell'antimicrobico-resistenza (AMR) lungo la filiera si verifica mediante trasferimento orizzontale di elementi genetici mobili. Pertanto, è possibile tracciare la diffusione dell'AMR identificando la presenza di plasmidi classificati in base ai gruppi di incompatibilità (Inc).

La salmonellosi è al secondo posto in Europa tra le zoonosi più frequenti; la resistenza alla tetraciclina è una delle più diffuse in *Salmonella* spp. ed è correlata con la presenza dei geni *tet*.

Questo lavoro approfondisce la diffusione di AMR nella filiera alimentare tracciando la distribuzione delle principali famiglie plasmidiche in *Salmonelle* isolate nella regione Marche e caratterizzando le varianti geniche di resistenza alla tetraciclina (*tet*).

MATERIALI E METODI

Lo studio è stato eseguito su un pannello di isolati AMR di *Salmonella* (n. 96) collezionati dal Centro di Riferimento Regionale per gli Enteropatogeni (CRREP presso ISZUM; anni 2015-2021) da alimenti (n. 33), ambienti veterinari e di produzione alimentare (n. 9), animali (n. 16) e uomo (38), tra cui diversi erano produttori di beta lattamasi a spettro esteso (ESBL). La presenza delle principali famiglie plasmidiche è stata determinata mediante PCR-based replicon typing (PBRT). L'associazione tra AMR fenotipica e presenza di plasmidi è stata valutata statisticamente (Fisher's test).

I geni *tet* sono stati caratterizzati tramite PCR secondo il protocollo del Laboratorio EU di riferimento per l'AMR, che ha fornito anche i controlli positivi.

RISULTATI

Le principali resistenze riscontrate erano verso sulfisossazolo (85%), tetraciclina (80%) e ampicillina (78%), in linea con dati nazionali ed europei. I ceppi erano principalmente multiresistenti (91%; resistenti a 3-7 classi di antibiotici). Il gruppo Inc più frequente (40%) era IncF, riscontrato in ceppi di origine alimentare, animale e umana. Tale gruppo è risultato significativamente associato alla resistenza verso cloramfenicolo e streptomicina. La variante genica prevalente era *tet*B (35%), seguita da *tet*A (10%), *tet*G (4%) e *tet*M (1%), mentre *tet*C e *tet*D non sono stati rilevati.

CONCLUSIONI

I risultati mostrano un'elevata prevalenza di ceppi AMR, suggerendo anche la diffusione di una principale famiglia plasmidica lungo la filiera alimentare regionale. Inoltre, la sorveglianza epidemiologica dell'AMR mediante tipizzazione molecolare può rappresentare un efficace strumento di prevenzione, in linea con un approccio One Health.

Keywords: antimicrobico-resistenza (AMR); *Salmonella* spp.; tetraciclina; plasmidi

Epidemiological study and molecular characterization of antibiotic-resistance *Salmonella* strains isolated along the food chain in Marche Region.

Russo I.[1], Bencardino D.[1], Andreoni F.[1], Schiavano G.F.[2], Brandi G.[1], Napoleoni M.[3], Staffolani M.[3], Amagliani G.[1]

[1]Università degli Studi di Urbino Carlo Bo, Dipartimento di Scienze Biomolecolari
[2]Università degli Studi di Urbino Carlo Bo, Dipartimento di Studi Umanistici
[3]Centro di Riferimento Regionale Patogeni Enterici, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", sezione di Tolentino (MC)

INTRODUCTION

The use of antimicrobials in the food chain contributes to the selection of resistant microorganisms potentially transmissible to humans. The spread of antimicrobial resistance (AMR) along the food chain occurs through horizontal transfer of mobile genetic elements. Therefore, it is possible to trace the spread of AMR by identifying the presence of plasmids classified according to incompatibility groups (Inc).

Salmonellosis the second zoonosis in Europe; resistance to tetracycline is one of the most common in *Salmonella* spp. and is correlated with the presence of the *tet* genes.

The aim of this work is to study the diffusion of AMR in the food chain by tracing the distribution of the main plasmid families in *Salmonella* strains isolated in the Marche Region and characterize the gene variants involved in tetracycline resistance (*tet*).

MATERIALS AND METHODS

The study was performed on a panel of AMR *Salmonella* isolates (n.96) collected by the Regional Reference Center for Enteropathogens (CRREP at ISZUM; years 2015-2021) from food (n.33), veterinary and food production environments (9), animals (16) and humans (38), many of that were producers of extended-spectrum β-lactamase (ESBL). The presence of the plasmid families was determined by PCR-based replicon typing (PBRT). The association between phenotypic AMR and the presence of plasmids was statistically evaluated (Fisher's test).

The *tet* genes were characterized by PCR according to the protocol of the EU Reference Laboratory for AMR that also provided the positive controls.

RESULTS

Our strains were mainly resistant to sulphysoxazole (85%), tetracycline (80%) and ampicillin (78%), in line with national and European data. The strains were mainly multidrug-resistant (91%; resistant to 3-7 classes of antibiotics). The most frequent Inc group (40%) was IncF, found in food, animal and human strains. This group was significantly associated with chloramphenicol and streptomycin resistance. The prevalent gene variant was *tet*B (35%), followed by *tet*A (10%), *tet*G (4%) and *tet*M (1%), while *tet*C and *tet*D were not detected.

CONCLUSIONS

The results show a high prevalence of AMR strains, also suggesting the spread of a main plasmid family along the regional food chain. In addition, epidemiological surveillance of AMR by molecular typing can represent an effective prevention tool, in line with a One Health approach

SUPPLEMENTARY MATERIAL

Serovar	HI2	I1α	N	I2	FIB	I1γ	A/C	FIIS	X1	FIB KN	FII	X4	Prevalent Incompatibility group	N° of untypable strains
S. Derby	2	5		1		1							IncI	11
S. Typhimurium			1	1				13		1			IncF	3
VMST	1	1			10		1	4	1		11		IncF	15
S. Infantis									3		2	4	IncX	24

Tab. 1. Plasmid incompatibility group assigned by PBRT test.

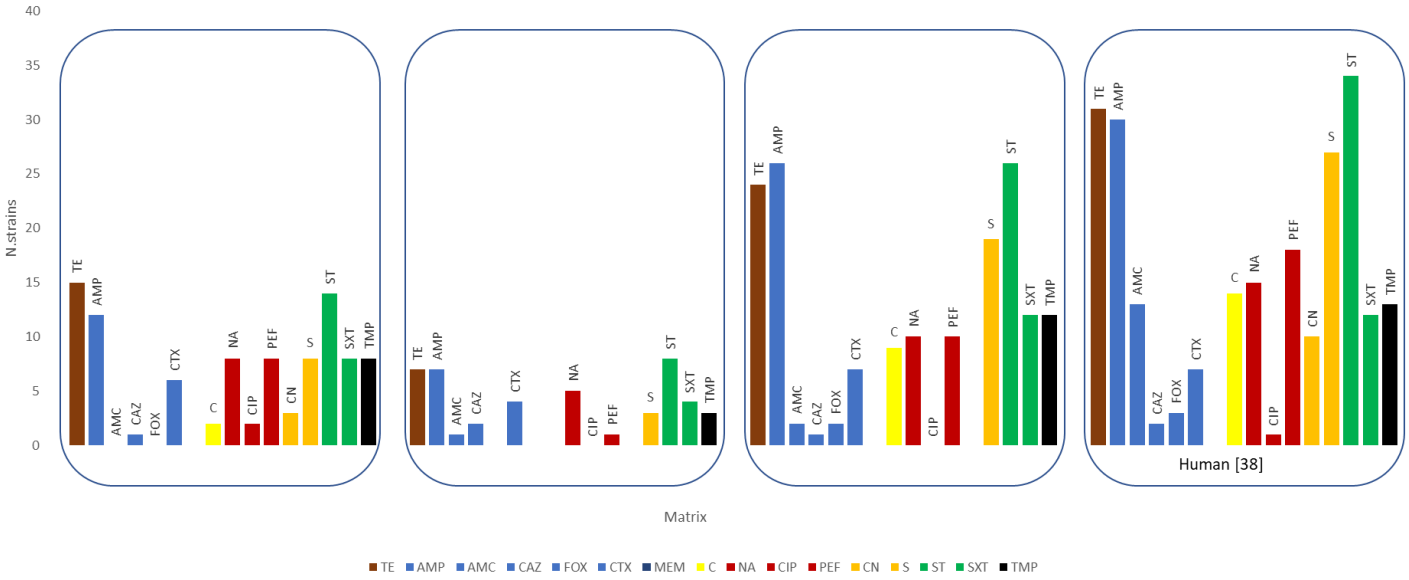


Fig. 1. AMR in *Salmonella* strains according to source of isolation

TE, Tetracyclin; AMP, Ampicillin; AMC, Amoxicillin+ Clavulanic Acid; CAZ, Ceftazidime; FOX, Cefoxitin; CTX, Cefotaxim; MEM, Meropenem; C, Chloramphenicol; NA, Nalidixic Acid; CIP, Ciprofloxacin; PEF, Pefloxacin; CN, Gentamicin; S, Streptomycin; ST, Sulphysoxazole; SXT, Trimetoprim-Sulfamethoxazole; TMP, Trimetoprim.

CONFERFERENCE PROCEEDING III

This study is focused on a specific *Salmonella* serovar: *S*. Infantis.

The aim was to reveal the presence of the pESI-like plasmid carrying *S*. Infantis clone in AMR *S*. Infantis strains from various sources in Marche Region, study their genetic characteristics, antibiotic resistance profile and confirm their spread along the food chain using a phylogenetic approach.

From farm to fork: spread of multidrug resistant *Salmonella* Infantis pESI-like $bla_{CTX\text{-}M\text{-}1}$ clone in Marche Region

Ilaria Russo[1], Jennie Fischer[2], Angelina Groger[2], Alexandra Irrgang[2], Giuditta Fiorella Schiavano[3], Francesca Andreoni[1,4], Maira Napoleoni[5], Giorgio Brandi[1], Giulia Amagliani[1]

[1] Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy
[2] BfR, German Federal Institute for Risk Assessment, Berlin, Germany
[3] Department of Humanities, University of Urbino Carlo Bo, Urbino, Italy
[4] Clinical Pathology, Urbino Hospital, Asur Marche, Urbino, Italy
[5] Regional Reference Center for Enteric Pathogens Marche, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy

INTRODUCTION

Salmonella Infantis is one of the five main causes of human salmonellosis in the European Union (EU). In Italy a multidrug resistant (MDR), extended-spectrum β-lactamases (ESBL) producing *S*. Infantis clone harboring a $bla_{CTX\text{-}M\text{-}1}$ pESI-like megaplasmid (~280–320 kb) has increasingly spread. The transmission of this clone along the food chain could cause high-risk human illnesses because of MDR including resistance to third generation cephalosporins. The aim of our study was to reveal the presence of the pESI-like plasmid carrying *S*. Infantis clone in AMR *S*. Infantis strains from various sources in Marche Region, study their genetic characteristics, antibiotic resistance profile and confirm their spread along the food chain using a phylogenetic approach.

MATERIALS AND METHODS

36 *S*. Infantis strains, isolated from food (n. 11), veterinary and food processing environments (n. 5), animals (n. 8) and humans (n. 12), were selected from 102 *Salmonella* strains collected in Marche Region. PCR screening revealed 72% ESBL *S*. Infantis strains that were subsequently submitted to Illumina short read based whole genome sequencing (WGS) and *bla* variants were determined using the NCBI resistance gene database (ncbi-AMRFinderPlus version 3.6.15) via BakCharak-Pipeline. Phylogenetic analyses were carried out in order to investigate SNP and cgMLST profiles using ChewieSnake and SnippySnake automated pipelines. Plasmid detection was carried out by multiplex

PCR Inc-rep-typing and WGS analysis (PlasmidFinder). Plasmid size was assessed with S1-PFGE profiling.

RESULTS

Two different ESBL genetic profiles comparable to *S*. Infantis pESI-like clone were observed. 81% carried the $bla_{\text{CTX-M-1}}$ gene, prevalent in Europe and 8% carried the $bla_{\text{CTX-M-65}}$, mainly detected in USA. 11% carried $bla_{\text{TEM-1}}$ gene. IncFIB plasmids were detected in all bla_{CTX} positive strains. Nine clusters were observed after SNP analysis. 69% belonged to the same cluster using a SNP threshold of <20. Within this Cluster, we detected 3 subclusters, when reducing the SNP threshold down to 10, with strains isolated from different sources along the food chain. After S1-PFGE analysis, 92% of our strains showed presence of IncFIB plasmids with the same size of pESI-like megaplasmid (224-310 kb).

CONCLUSION

Close genetic relationship of CTX-M-1 producing *S*. Infantis isolates harboring IncFIB megaplasmids from diverse sources confirm the presence and spread of *S*. Infantis pESI-like $bla_{\text{CTX-M-1}}$ clone in Marche Region along the food chain. The implementation of a One Health approach, integrating surveillance of MDR strains spread from farm to fork, contributes to the monitoring of MDR zoonotic pathogens, such as *S*. Infantis.

POSTER

The following poster was presented during 54° Congress of the Italian Society of Hygiene (SItI) held in Lecce on November 2021.

The poster summarize data obtained to the epidemiological study and molecular characterization of *Salmonella* strains isolated in Marche Region along the food chain.

Studio epidemiologico e caratterizzazione molecolare di ceppi di Salmonella antibiotico-resistenti isolati nella filiera alimentare della regione Marche.

Russo I.[1], Bencardino D.[1], Andreoni F.[1], Schiavano G.F.[2], Brandi G.[1]
Napoleoni M.[3], Staffolani M.[3], Amagliani G.[1]

[1]Università degli Studi di Urbino Carlo Bo, Dipartimento di Scienze Biomolecolari
[2]Università degli Studi di Urbino Carlo Bo, Dipartimento di Studi Umanistici
[3]Centro di Riferimento Regionale Patogeni Enterici, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", sezione di Tolentino (MC)

INTRODUZIONE

La diffusione dell'antimicrobico-resistenza (AMR) si verifica mediante trasferimento orizzontale di elementi genetici mobili come i plasmidi. La loro classificazione sulla base dei gruppi di incompatibilità (Inc) consente di tracciare la diffusione dell'AMR nella filiera. Nei ceppi di *Salmonella* spp., la resistenza alla tetraciclina è una delle più diffuse ed è correlata con la presenza dei geni *tet*. In questo lavoro è stata valutata la distribuzione delle principali famiglie plasmidiche in *Salmonelle* isolate nella regione Marche e la successiva caratterizzazione delle varianti geniche di resistenza alla tetraciclina (*tet*).

MATERIALI E METODI

Lo studio è stato eseguito su 96 ceppi di *Salmonella* AMR collezionati, dal 2015 al 2021, dal Centro di Riferimento Regionale Patogeni Enterici (ISZUM) da: alimenti (n. 33), ambienti veterinari e di produzione alimentare (n. 9), animali (n. 16) e uomo (38). Le famiglie plasmidiche sono state caratterizzate mediante PCR-based replicon typing (PBRT) e l'associazione con l' AMR fenotipica è stata valutata statisticamente (Fisher's test). I geni *tet*A, *tet*B, *tet*C, *tet*D, *tet*G, *tet*M sono stati amplificati tramite PCR secondo il protocollo del Laboratorio EU di riferimento per l'AMR, che ha fornito anche i controlli positivi.

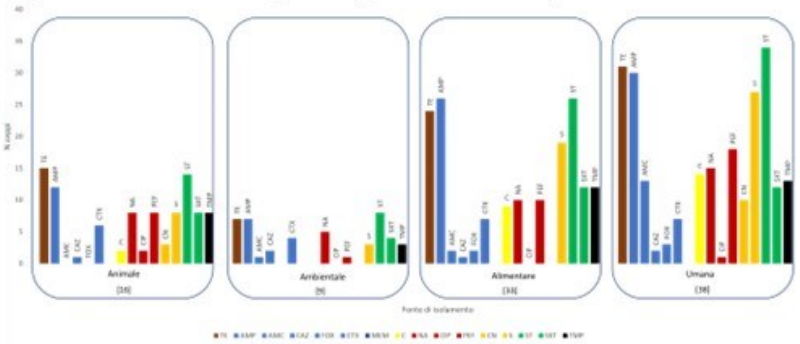
RISULTATI

Diversi ceppi erano produttori di β-lattamasi a spettro esteso (ESBL) e le principali resistenze erano verso sulfisossazolo (85%), tetraciclina (80%) e ampicillina (78%), in linea con dati nazionali ed europei. Il 91% dei ceppi era multiresistente e IncF era il gruppo Inc più frequente (40%), riscontrato in ceppi di origine alimentare, animale e umana. Tale gruppo è risultato significativamente associato alla resistenza verso cloramfenicolo e streptomicina. La variante genica prevalente era *tet*B (35%), seguita da *tet*A (10%), *tet*G (4%) e *tet*M (1%), mentre *tet*C e *tet*D non sono stati rilevati.

CONCLUSIONI

I risultati mostrano un'elevata prevalenza di ceppi AMR, suggerendo anche la diffusione di una principale famiglia plasmidica lungo la filiera alimentare regionale. La sorveglianza epidemiologica dell'AMR mediante tipizzazione molecolare può rappresentare un efficace strumento di prevenzione, in linea con un approccio One Health.

Fig.1 Distribuzione resistenze fenotipiche nei ceppi di *Salmonella* suddivisi per fonte di isolamento



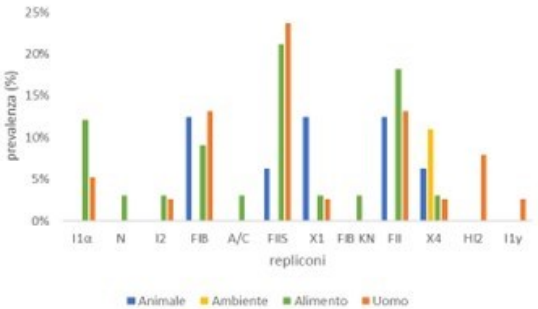


Fig.2 Frequenza dei ceppi di *Salmonella* contenenti i diversi repliconi secondo la matrice di isolamento.

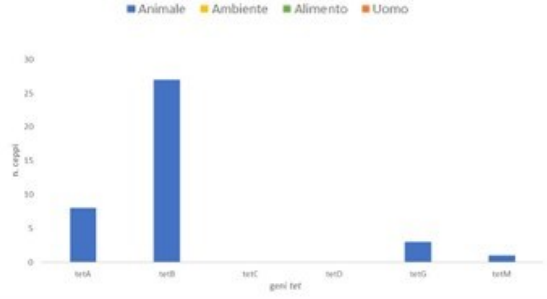


Fig.3 Distribuzione varianti geni *tet* nei ceppi di *Salmonella*

ORIGINAL RESEARCH PAPER

In this study, the number of selected *Salmonella* strains was increased to 102. We selected a group of strains isolated in the Marche Region including those serotypes mainly detected in Salmonella strains. Within this collection, we evaluated their distribution in animal, food, environment, and human matrix, their antimicrobial resistance, and plasmid profiles in order to obtain a general view of isolates circulating in our region. Our aim was to investigate the presence and the potential dissemination of AMR Salmonella isolates via the food chain.

antibiotics

MDPI

Article

Prevalence, Antibiotic-Resistance, and Replicon-Typing of *Salmonella* Strains among Serovars Mainly Isolated from Food Chain in Marche Region, Italy

Ilaria Russo [1], Daniela Bencardino [1], Maira Napoleoni [2], Francesca Andreoni [1], Giuditta Fiorella Schiavano [3], Giulia Baldelli [1], Giorgio Brandi [1] and Giulia Amagliani [1,*]

[1] Department of Biomolecular Sciences, University of Urbino Carlo Bo, 61029 Urbino, Italy; i.russo6@campus.uniurb.it (I.R.); daniela.bencardino@uniurb.it (D.B.); francesca.andreoni@uniurb.it (F.A.); giulia.baldelli@uniurb.it (G.B.); giorgio.brandi@uniurb.it (G.B.)
[2] Istituto Zooprofilattico Sperimentale dell'Umbria e Delle Marche "Togo Rosati", 06126 Perugia, Italy; m.napoleoni@izsum.it
[3] Department of Humanities, University of Urbino Carlo Bo, 61029 Urbino, Italy; giuditta.schiavano@uniurb.it
* Correspondence: giulia.amagliani@uniurb.it; Tel.: +39-0722-303540

Abstract: Nontyphoidal salmonellosis (NTS) is the second most commonly reported gastrointestinal infection in humans and an important cause of food-borne outbreaks in Europe. The use of antimicrobial agents for animals, plants, and food production contributes to the development of antibiotic-resistant *Salmonella* strains that are transmissible to humans through food. The aim of this study was to investigate the presence and the potential dissemination of multidrug-resistant (MDR) *Salmonella* strains isolated in the Marche Region (Central Italy) via the food chain. Strains were isolated from different sources: food, human, food animal/livestock, and the food-processing environment. Among them, we selected MDR strains to perform their further characterization in terms of resistance to tetracycline agent, carriage of *tet* genes, and plasmid profiles. Tetracycline resistance genes were detected by PCR and plasmid replicons by PCR-based replicon typing (PBRT). A total of 102 MDR *Salmonella* strains were selected among the most prevalent serovars: *S*. Infantis (n = 36/102), *S*. Derby (n = 20/102), *S*. Typhimurium (n = 18/102), and a monophasic variant of *S*. Typhimurium (MVST, n = 28/102). Resistance to sulfisoxazole (86%) and tetracycline (81%) were the most common, followed by ampicillin (76%). FIIS was the most predominant replicon (17%), followed by FII (11%) and FIB (11%) belonging to the IncF incompatibility group. Concerning the characterization of *tet* genes, *tet*B was the most frequently detected (27/89), followed by *tet*A (10/89), *tet*G (5/89), and *tet*M (1/89). This study showed the potential risk associated with the MDR *Salmonella* strains circulating along the food chain. Hence, epidemiological surveillance supported by molecular typing could be a very useful tool to prevent transmission of resistant *Salmonella* from food to humans, in line with the One Health approach.

Keywords: *Salmonella* serovars; antibiotic resistance; *tet* genes; multidrug-resistant (MDR); extensively drug-resistant (XDR); antimicrobial resistance genes (ARGs); plasmid profile; PCR-based replicon typing (PBRT); food chain

Citation: Russo, I.; Bencardino, D.; Napoleoni, M.; Andreoni, F.; Schiavano, G.F.; Baldelli, G.; Brandi, G.; Amagliani, G. Prevalence, Antibiotic-Resistance, and Replicon-Typing of *Salmonella* Strains among Serovars Mainly Isolated from Food Chain in Marche Region, Italy. *Antibiotics* **2022**, *11*, 725. https://doi.org/10.3390/antibiotics11060725

Academic Editor: Kálmán Imre

Received: 9 May 2022
Accepted: 25 May 2022
Published: 28 May 2022

1. Introduction

Nontyphoidal salmonellosis (NTS) is a common infection mainly caused by the ingestion of food or beverages contaminated by several zoonotic serovars with the potential to interact with human and animal hosts [1,2]. The most prevalent serovars responsible for human illnesses acquired in the European Union during 2019 were, in decreasing order, *S*. Infantis, *S*. Enteritidis, the monophasic variant of *S*. Typhimurium (MVST), *S*. Typhimurium, and *S*. Derby [3].

Food-producing livestock and domestic pets are most often sources of NTS food-borne outbreaks [1]. Indeed, NTS is linked to the consumption of *Salmonella*-contaminated food mostly from poultry, pork, and egg products. However, in the last few decades, the sporadic occurrence of these microorganisms was also detected in fruit and vegetable produce [3,4]. Furthermore, poor hand washing and contact with infected pets are some of the contamination routes. Thus, as a consequence of the growth in consumption of products of animal origin and the integration of companion animals in households, there is an increased potential for exposure to *Salmonella* via the food chain [4].

When contaminated food is ingested, the pathogen attacks and invades the intestinal epithelium of the distal ileum triggering sickness that appears as acute gastroenteritis within 4 to 72 h. Fever, chills, nausea, vomiting, abdominal cramping, headache, and diarrhea are the main symptoms [1,4]. Usually, in healthy individuals, NTS is a self-limited disease resolving in a few days without medical intervention, but some patients may develop chronic sequelae such as reactive arthritis or irritable bowel syndrome [4,5]. On the other hand, in vulnerable patients (immunocompromised, very young or elderly persons) NTS infection can systematically spread to other body organs causing febrile illness [2–4]. Hence, NTS is a public health concern representing an economic burden for both developed and developing countries, due to costs associated with surveillance, prevention, and treatment of disease [1,6,7].

The rise in the occurrence of antimicrobial-resistant (AMR) strains in the food chain increases the risk associated with this zoonotic infectious disease minimizing treatment options and increasing human mortality [8,9]. The emergence of antimicrobial resistance in the food chain is considered a cross-sectoral problem due to: (i) the misuse and overuse of antibiotics in agricultural and livestock production, (ii) the dissemination of antimicrobial resistance genes (ARGs) among bacteria intentionally added during processing (e.g., starter cultures and probiotics), (iii) the post-contamination by the environment, after food processing, (iv) the cross-contamination with AMR bacteria colonizing other foods during industrial processing and handling by the consumer [10,11].

The selective effects of antimicrobial use accelerate the natural development of resistance mechanisms activated by bacteria, and the acquisition of ARGs can occur at any stage of the food chain facilitated by mobile genetic structures such as plasmids, integrons, and transposons, in addition to vertical transfer [11,12]. Indeed, the presence of resistance determinants in foodstuffs increases the gene pool by which pathogens can achieve and transfer ARGs to other bacteria, thus representing an indirect risk to public health [11].

Horizontal gene transfer has been repeatedly described in *Salmonella*, especially for tetracycline resistance genes (*tet*) because some of the most frequently detected, such as *tet*A and *tet*G, are located on mobile genetic elements [13]. As observed in the latest report of the European Food Safety Authority (EFSA), Italy showed a high level of resistance toward tetracyclines, confirming the alarming effects caused by the abuse of these broad-spectrum agents in both human and veterinary medicine [3,13]. In light of recent scientific issues on antimicrobial resistance, the EFSA updated technical specifications for the implementation of molecular typing methods during routine monitoring. EFSA proposal aims to reach harmonised surveillance of antimicrobial resistance in food-producing animals and derived food to ensure continuity in following up the dynamic evolution of AMR food-borne pathogens [14]. Mobile genetic elements play a pivotal role in the dissemination of antimicrobial resistance along the food chain and their study is crucial to better understand their epidemiology, apply control measures and reduce the presence of the pathogen in food [11].

In this study, we selected a group of strains isolated in the Marche Region including those serotypes mainly detected in *Salmonella* strains. Within this collection, we evaluated their distribution in each one of the niches considered (animal, food, environment, and human), their antimicrobial resistance, and plasmid profiles in order to obtain a general view of isolates circulating in our region. Our aim was to investigate the presence and the potential dissemination of AMR *Salmonella* isolates via the food chain.

2. Results

2.1. Salmonella Strains

A total of 102 AMR Salmonella strains, of serovars S. Derby (n. 20), S. Typhimurium (n. 18), MVST (n. 28), and S. Infantis (n. 36) were collected and analysed for this study. Strains of animal origin (n. 16) were from pigs (n. 5) and poultry (broiler, n. 8; pigeon, turkey, and laying hen, n. 1 each); food samples (n. 35) included meat products (pork meat, n. 20; bovine, n. 4; chicken, n. 7), and mollusks (n. 4). Environmental samples (n. 9) were swabs from food processing rooms (n. 2), slaughter rooms (n. 4), and poultry farms (n. 3) (Table 1). Human clinical *Salmonella* strains (n. 42) were isolated from faeces (n. 37), blood (n. 2) urine (n. 2), and other clinical samples (n. 1) (Table 1).

Table 1. Source and serotypes of *Salmonella* strains.

Source	Serovars	Strain n.
Animals	*S*. Infantis	8
	S. Derby	1
	S. Typhimurium	1
	MVST	6
Environment	*S*. Infantis	5
	S. Derby	1
	S. Typhimurium	0
	MVST	3
Foods	*S*. Infantis	11
	S. Derby	8
	S. Typhimurium	7
	MVST	9
Humans	*S*. Infantis	12
	S. Derby	10
	S. Typhimurium	10
	MVST	10
Total		102

2.2. Antibiotic Susceptibility Profiles

All *Salmonella* strains analysed in this study were resistant to one or more antibiotic classes, except for one unique environmental *Salmonella* isolate, that was intermediate resistant to streptomycin and ciprofloxacin and sensitive to all other antibiotics tested. Nevertheless, this last strain was included in our collection in order to obtain a reasonable number of environmental strains, in light of the limited size of this niche. The supplementary Figure S1 showed the distribution of antibiotic resistance detected among strains isolated from the different niches. Independently of isolation origin and serovar, a high rate of resistance was recorded to sulfisoxazole (88 out of 102 strains, 86%) and tetracycline (83/102, 81%), followed by ampicillin (78/102, 76%), streptomycin (61/102, 60%) and nalidixic acid (38/102, 37%). Finally, 26 strains (25%) were resistant to cefotaxime and confirmed as extended-spectrum β-lactamase (ESBL)-producing *Salmonella*. Moreover, a high proportion of *Salmonella* isolates (42%; 43/102) showed intermediate sensitivity to ciprofloxacin, a 2nd generation fluoroquinolone, whereas meropenem resistant strains were not detected (Figure 1A).

As shown in Figure 1B, the majority of strains (47/102, 46%) were resistant to three or more antibiotic classes, hence they were classified as multidrug-resistant (MDR) [15], whereas the 44% of strains (45/102) were classified extensively drug-resistant (XDR) because they were resistant to at least one agent in all but two or fewer antimicrobial categories (i.e., bacterial isolates remain susceptible to only one or two categories) [15]. The remaining 9 strains showed resistance to up two antibiotics of two categories.

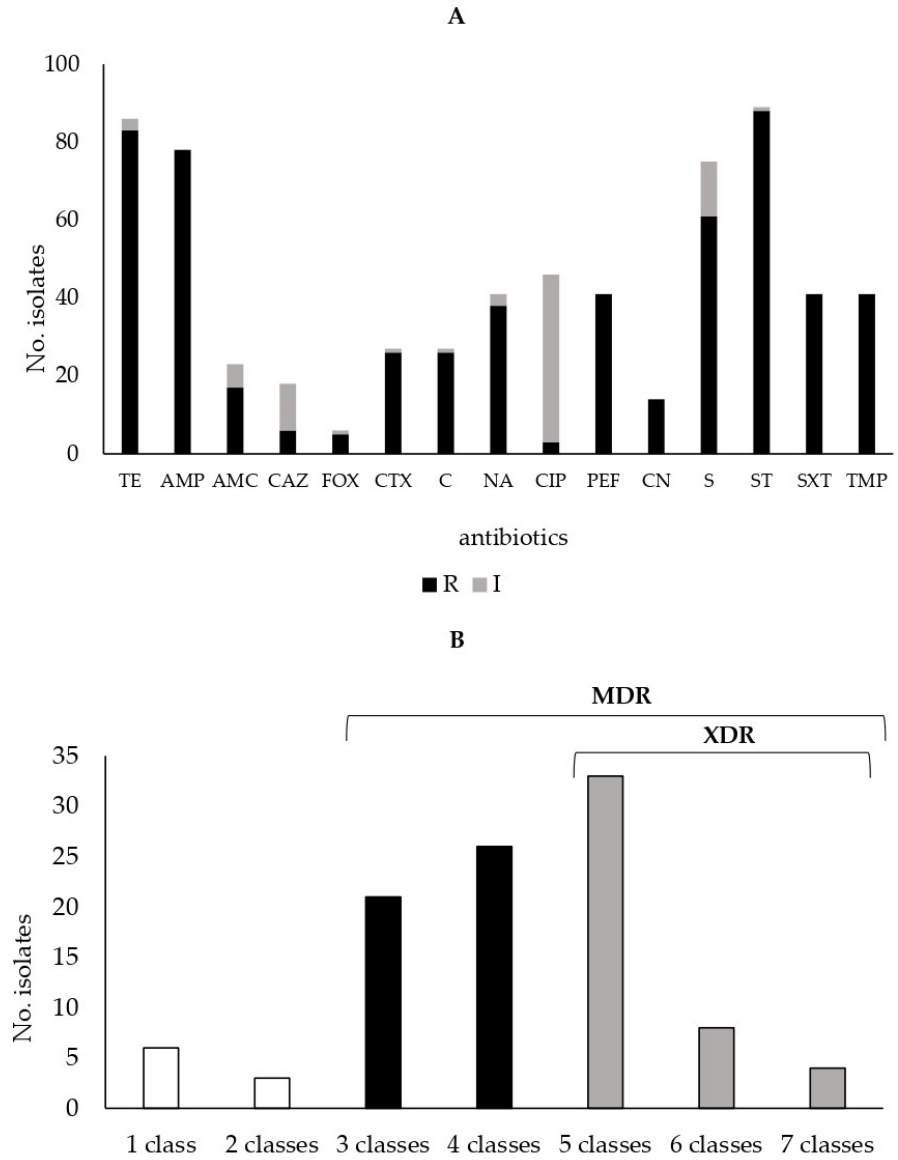


Figure 1. Resistance rate of *Salmonella* strains analysed in this study. (**A**) Strains were resistant to one or more antibiotic classes, and the major resistance was detected against sulfisoxazole and tetracycline. R: resistant (black), and I: intermediate (grey) resistant strains. AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; AMC, amoxicillin+clavulanic acid; FOX, cefoxitin; TE, tetracycline; C, chloramphenicol; CIP, ciprofloxacin; NA, nalidixic acid; PEF, pefloxacin; CN, gentamicin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; ST, sulfisoxazole; TMP, trimethoprim. (**B**) Distribution of Multi-and Extensively Drug-Resistant *Salmonella* strains. The major part of strains were resistant to three or more antibiotic classes (multidrug-resistant; MDR) whereas 44% were resistant to at least one agent in all but two or fewer antimicrobial categories (extensively drug-resistant; XDR) (**B**).

The resistant phenotype was not equally distributed among serovars. In *S*. Infantis, strains resistant to tetracycline, sulfamides, streptomycin, nalidixic acid, pefloxacin, ampicillin, and cefotaxime were the most frequently observed, while reduced sensitivity to ciprofloxacin was noticed among a relevant number of strains which were resistant to other antibiotics. Streptomycin, sulfisoxazole, and tetracycline were the most observed resistance phenotypes in *S*. Derby. *S*. Typhimurium and MVST showed similar antibiotic resistance profiles: ampicillin, sulfisoxazole, streptomycin, and tetracycline were the most diffused resistance phenotypes in both serovars, and *S*. Typhimurium also showed a certain proportion of strains resistant to chloramphenicol (Figure 2).

2.3. Tetracycline Resistance Genes

Among the 83 resistant strains and the three strains with intermediate sensitivity to tetracycline, a *tet* gene was determined in 43 strains, while the remaining 43 strains tested negative for each target. The gene most frequently detected was *tet*B (27/86), followed by *tet*A (10/86) and *tet*G (5/86), while *tet*M was found in a single strain isolated from humans and was in combination with *tet*B. On the other hand, *tet*C and *tet*D were not detected (Table 2).

Table 2. *tet* genes detected in tetracycline-resistant and intermediate strains, and serovars.

tet Genes	Resistant Strains	Intermediate Strains
*tet*A	10 2 *S*. Derby, 7 *S*. Infantis, 1 *S*. Typhimurium	0
*tet*B	27 1 *S*. Derby, 24 MVST, 2 *S*. Typhimurium	0
*tet*C	0	0
*tet*D	0	0
*tet*G	3 *S*. Typhimurium	2 *S*. Typhimurium
*tet*M	1 MVST	0

To note, almost all determinants were heterogeneously distributed both in terms of serovars and niches. However, *tet*A was not found in MVST that was instead characterised by the carriage of *tet*B for the 97% of tetracycline-resistant strains. Further, *tet*G gene was detected only in *S*. Typhimurium strains, and all were isolated from food (Table 2).

2.4. Replicon Typing

Among the 102 strains analysed by PBRT only 12 out 30 replicons were detected (HI2, I1α, N, I2, FIB, I1γ, A/C, FIIS, X1, FIB KN, FII, X4), as described in Figure 3A.

Overall, 58 strains were untypeable because they were negative for all replicons whereas 28 strains were positive for 1 replicon, 11 for 2 replicons, and 5 for 3 replicons. FIIS was the most predominant replicon (17%), followed by FII (11%), and FIB (11%). However, we observed specific replicon distribution among strains belonging to the serovars considered in this study. Indeed, the IncX group was detected in *S*. Infantis and MVST, IncF in *S*. Typhimurium, *S*. Infantis, and MVST whereas IncI and IncH were detected only in *S*. Derby.

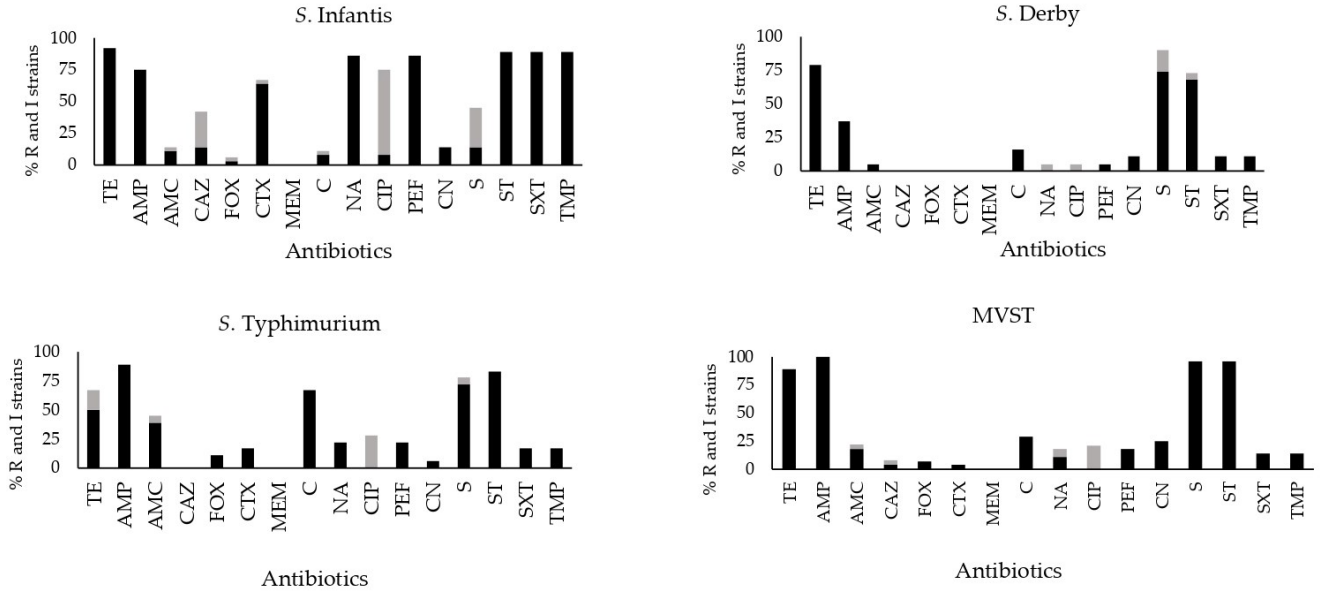


Figure 2. AMR profile of *Salmonella* strains investigated in this study, according to serovar. R: resistant (black), and I: intermediate (grey) strains. MVST: monophasic variant of *S.* Typhimurium. AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; AMC, amoxicillin+clavulanic acid; FOX, cefoxitin; MEM, meropenem; TE, tetracycline; C, chloramphenicol; CIP, ciprofloxacin; NA, nalidixic acid; PEF, pefloxacin; CN, gentamicin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; ST, sulfisoxazole; TMP, trimethoprim.

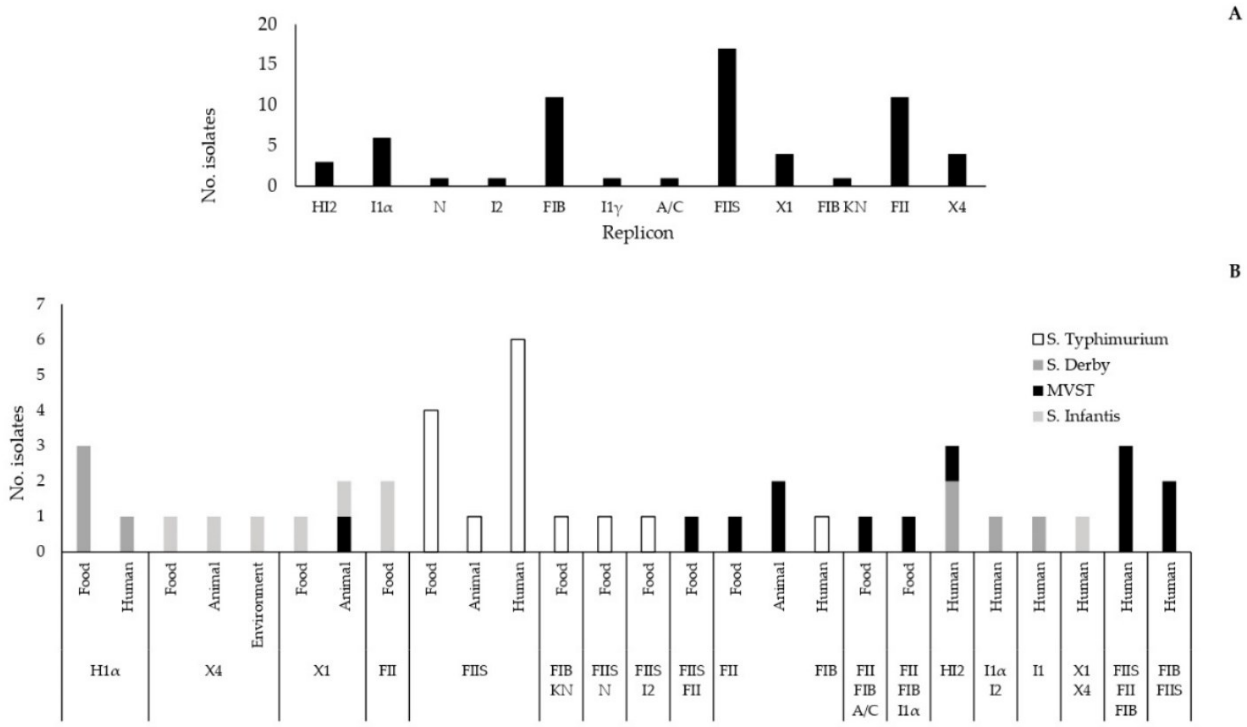


Figure 3. Replicons detected by PBRT and distribution of replicon patterns among *Salmonella* serovars isolated from different sources. (**A**) Replicons detected by PBRT. (**B**) Replicon patterns and distribution among the serovars of *Salmonella* strains isolated from different sources. MVST: monophasic variant of *S*. Typhimurium.

Furthermore, replicons IncI and IncH were detected in strains isolated from human and food samples, IncF group was the most detected in strains isolated from animal, food, and human samples whereas, among strains isolated from the environment, only the IncX group was detected (Figure 3B).

Results of the PBRT analysis allowed us to define 18 PBRT profiles (i.e., combinations of replicons). The prevalent profile included strains with the single replicon FIIS (n = 11/102), followed by I1α (n = 4/102), and FIB,FII (n = 4/102). All PBRT profiles detected in the present study comprised strains of the same serovar, except for HI2 detected in both *S.* Derby and MVST, and X1 detected in MVST and *S.* Infantis (Figure 3B). Multireplicon status (two or more replicons) was recorded in 16 strains, with a maximum of three replicons in five MVST strains. Multireplicon profiles were the following: FIB,A/C,FII (n = 1), I1α,FIB,FII (n = 1), and FIB,FIIS,FII (n = 3). Four out of these five strains having the multireplicon profile were resistant to tetracycline, three of which carried *tet*B gene or *tet*B combined with *tet*M.

In this study, the possible association between the Inc group and resistance phenotypes was also investigated. Results of statistical analysis (Hypothesis test–difference between two frequencies in large samples) showed significant association between IncF presence and resistance to sulfisoxazole ($p < 0.00001$), chloramphenicol ($p < 0.0001$), streptomycin ($p < 0.01$), ampicillin ($p < 0.01$) and tetracycline ($p < 0.05$).

3. Discussion

In this study, the characterisation of *Salmonella* strains belonging to the main serovars isolated from the food chain in the Marche Region (Central Italy) is reported. The decision to select *S.* Infantis, *S.* Derby, *S.* Typhimurium, and MVST as the target of the investigation was derived from taking into account the epidemiology of main serovars circulating at the regional level in both veterinary and human samples [16–21]. The dominance of these four serovars was consistent with that observed at the national and European levels, as described by the last reports of Entervet and EFSA/ECDC [3,22].

With the exception of *S.* Typhimurium which presents a global distribution [23], geographical differences emerged comparing the serovars predominant in Europe with those in the USA, with the prevalence of *S.* Enteritidis and *S.* Newport for the latter [23,24].

All serovars considered in the present work are commonly found along the food chain and they are able to cause infections in humans [23]. *S.* Typhimurium and its monophasic variant (MVST) are associated with pig and pork meat [3]. In food-processing environments, these pathogens can spread throughout processing lines and, thus, reach utensils, surfaces, and hands of employees [25]. Indeed, the prevalence of *S.* Typhimurium and its monophasic variant is similar in both farms and slaughterhouses, and strains isolated from swine and humans show important correlations in terms of molecular profiles [23,26]. *S.* Derby is continuously detected in pig, slaughter, and pork meat [3], and several studies demonstrated correlations between strains isolated from pigs and those of human origin [27,28]. *S.* Infantis is reported as the most frequent poultry-adapted *S.* enterica serovar with an increasing occurrence in broiler flocks, derived meat, and breeding hens [29,30].

From a health perspective, these findings are remarkable because the spread along the food chain of serovars with the ability to cause infections in humans represents a great concern. Indeed, *Salmonella* is the second pathogen responsible for food-transmitted diseases and is an important cause of foodborne outbreaks in the EU [3].

Over recent years, the risk associated with the increasing incidence of these serovars in humans and animals has been complicated by the spread of MDR clones in several European countries, including Italy [31,32]. The overuse of antimicrobial agents in therapy and prophylaxis of human and animal infections, as well as growth-promoting agents in food animal production, favored the emergence of *Salmonella* strains resistant to several classes of antibiotics, including those used as the primary choice for clinical treatment [33]. In light of this, we presented new and updated data concerning not only the occurrence of the most prevalent serovars detected at the regional level but also their molecular

features in terms of antibiotic resistance and plasmid profiles. We found a heterogeneous distribution of phenotypic resistance among the serovars investigated, confirming also the typical multi-resistance pattern frequently determined in *Salmonella* strains circulating in Europe [34].

Tetracyclines, which are broad-spectrum drugs widely used in human and veterinary medicine, represent one of the main agents toward which *Salmonella* developed a high level of resistance [35]. As reported by the latest ESVAC (European Surveillance of Veterinary Antimicrobial Consumption) report, of the overall antimicrobials used in the 31 countries in 2020, the largest amounts were for penicillins (31.1%), tetracyclines (26.7%), and sulfonamides (9.9%) [36]. In our study the highest resistance rate was determined toward sulfisoxazole, followed by tetracycline, both classified as highly important antibiotics for human medicine [37]. Thus, the responsible use of them is strongly recommended in order to keep the associated risk as low as possible [38].

This study demonstrated wide dissemination of tetracycline resistance in *Salmonella* strains (80%) along with the considered food chain settings. This was in accordance with the last report released by EFSA where a rate of tetracycline resistance higher than the European average was described for Italy [34]. Among all tetracycline-resistant strains of our collection, 49% harboured one or more *tet* genes (*tet*A, *tet*B, *tet*C, *tet*D, *tet*G, *tet*M), matching with other studies carried out both in Italy and in Europe [39–41]. The fact that only 49% of strains resistant to tetracycline were positive for at least one *tet* gene is not surprising because this resistance could be due to different genes or other mechanisms such as mutations within the ribosomal binding site, activity of efflux pumps or enzymatic inactivation of tetracycline drugs [42]. In agreement with other authors, the genes most frequently detected in the present work were *tet*A and *tet*B belonging to Group-I and associated with an efflux pump mechanism [43–46]. On the contrary, all strains of our collection were negative for *tet*C and *tet*D. This result is not uncommon and could be due to the low ability of those genes to confer resistance to tetracycline [46], leading to their infrequent detection. The presence of these genes in strains isolated from animals increases the risk associated with circulating *Salmonella* strains in livestock for many reasons. Firstly, animals can transfer these strains to humans when farmers come into close contact with them. Secondly, animals can release strains through faecal material contributing to the spread of ARGs within livestock, and cross-contamination events, due to low hand hygiene compliance which can occur during processing.

Remarkably, the localization of many ARGs on mobile genetic elements (plasmids, integrons, and transposons) makes them easily transferred to both other bacteria and the environment [47]. This is the case of *tet*A and *tet*B which are the most frequently detected genes in our collection, confirming their increased ability to spread.

Hence, screening programmes supported by molecular typing methods are very useful for the epidemiological tracing of serovar distribution in different sources along the food chain, and also for the prompt identification of potential risks for the health of farmers, food handlers, and consumers. For this reason, all strains were further characterised by PBRT in order to understand the distribution of plasmid-related antimicrobial resistance determinants based upon replicon types.

It is known that a variety of plasmid families are frequently found in *Enterobacterales* promoting the rapid and consistent dissemination of ARGs [48]. The most common Inc replicon types found in our collection were IncFIIS, followed by IncFII and IncFIB. The dominance of IncF plasmids in AMR *Enterobacterales* isolated from humans and animals has been widely assessed [48,49]. It was also demonstrated that IncF plasmids carry multireplicon patterns where one replicon is strongly conserved while the others are free to diverge. So, the selective pressure imposed leads plasmids to duplication and dissemination [49]. Furthermore, the low copy number IncF plasmids contain the ADP-ribosylating toxin, SpvB causing the systemic virulence typical of some serovars such as *S.* Derby and *S.* Typhimurium [50]. For all of these reasons, the implementation of molecular investigation in surveillance programmes achieves more and more relevance.

Besides the described dominance of IncF, previous studies carried out in Italy recorded the occurrence of different plasmid replicons. Dionisi and colleagues [32] characterised *S*. Infantis isolated from different sources, between 2005 and 2011, identifying IncHI1 as the dominant replicon. Instead, Franco and colleagues [31] investigated *S*. Infantis isolates from different broiler chicken flocks detecting IncP replicon in all of them. Interestingly, Di Cesare and colleagues [51] reported a large variety of Inc-plasmids among isolates from animals and foodstuffs, highlighting the diversity of the *Salmonella* serovars. To note, IncHI2 and IncFIIS were prevalent and detected only in specific serovars suggesting that their distribution could be serovar-dependent [51]. This issue was found in accordance with that observed in our study where IncH and IncI were found to be associated only with *S*. Derby while IncX was detected only in MVST and *S*. Infantis strains originated from food and human samples, suggesting their potential transmission along the food chain.

A large part of strains was untypeable by PBRT, and what could be a limitation for the study actually highlights the need of monitoring circulating strains continuously, in order to follow the dynamic evolution of *Salmonella*. This foodborne pathogen, as well as other bacteria, has a tendency to modify its molecular elements to obtain advantageous adaptability resulting in the escape of current assays.

It is clear that a better knowledge of molecular aspects in terms of resistance determinants and plasmid content can be helpful to understand how resistant bacteria spread within food settings.

4. Materials and Methods

4.1. Study Design and Selection of Strains

This study considered *Salmonella* strains isolated from different samples collected in the Marche Region, Italy, between 2015 and 2021, in the framework of the official controls provided by the Regulation (EC) No 2073/2005 on microbiological food safety criteria [52] the National Poultry monitoring plan [53,54] and during self-monitoring controls both in food [52] and veterinary sectors [54].

Salmonella strains were collected from microbiological analysis performed as part of these controls and serotyped at the Regional Reference Centre for Enteric Pathogens of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche, section of Tolentino (Italy).

Only AMR strains of the main circulating serovars were selected including *S*. Derby (n = 20/102; 20%), *S*. Infantis (n = 36/102; 35%), *S*. Typhimurium (n = 18/102; 18%), and MVST (n = 28/102; 27%). The dominance of these serovars was derived from epidemiological information about the main serovars circulating in both veterinary and human samples [3,21]. Among them, we selected MDR strains to perform their further characterisation in terms of resistance to tetracycline agent, carriage of *tet* genes, and plasmid profiles.

Isolation sources included different points of the food chain, from farm and veterinary environments to food and food processing plants. Animal samples were obtained mainly from pigs and poultry (faeces, dust, and boot swabs collected on farms, viscera collected during necropsy) while food samples included meat and fish products. Environmental samples were obtained by collecting swabs from food processing rooms, slaughter rooms, and poultry farms. Finally, human *Salmonella* strains were isolated from people with gastrointestinal symptoms, some of them hospitalized. These strains were sent to the Regional Reference Centre for Enteric Pathogens from the Regional hospitals' analysis laboratories participating in Enter-Net surveillance for Marche Region. Strain origin and serotypes were reported in Table 1.

4.2. Serotyping Analysis and Antibiotic Susceptibility Testing

All *Salmonella* isolates from either veterinary, food or human samples were serotyped according to ISO/TR 6579-3:2014 [55].

Antimicrobial susceptibility of the *Salmonella* strains was determined by the disk diffusion method, according to the Clinical and Laboratory Standards Institute guidelines (CLSI, 2021) toward the following antibiotic agents: ampicillin (AMP, 10 µg), cefotaxime (CTX, 30 µg), ceftazidime (CAZ, 30 µg), amoxicillin+clavulanic acid (AMC, 30 µg), cefoxitin (FOX, 30 µg), meropenem (MEM, 10 µg), tetracycline (TE, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), nalidixic acid (NA, 30 µg), pefloxacin (PEF, 5 µg), gentamicin (CN, 10 µg), streptomycin (S, 10 µg), trimethoprim-sulfamethoxazole (SXT, 23.75/1.25 µg), sulfisoxazole (ST, 300 µg), trimethoprim (TMP, 5 µg). *Escherichia coli* ATCC 25922 was used as a control strain.

ESBL-producing *Salmonella* strains were confirmed by the double-disk synergy test performed by positioning a disk of amoxicillin+clavulanic acid 30 µg between a disk of cefotaxime 30 µg and a disk of ceftazidime 30 µg [56]. The CLSI interpretive criteria for disk diffusion susceptibility testing of *Salmonella* were used [57].

4.3. Bacterial DNA Extraction and Plasmid Typing

Bacterial DNA was obtained by the boiling lysis method, incubating the isolated colonies in distilled water for 10 min at 100 °C. The samples were then centrifuged at 15,000× *g* for 5 min and the supernatants were used for the following reactions.

All strains were typed by PCR-based replicon typing (PBRT) using the PBRT kit 2.0 (Diatheva, Fano, Italy) in order to identify plasmid replicons. This PBRT assay consists of eight multiplex PCRs and allows the detection of 30 replicons of the main plasmids in *Enterobacterales*.

All PCR reactions were carried out in accordance with the manufacturer's instructions, including positive controls. The amplicons were detected through capillary electrophoresis on the AATI Fragment Analyzer (Agilent, Santa Clara, CA, USA) using the dsDNA 906 Reagent kit (Advanced Analytical, Ankeny, IA, USA). This amplicon analysis allows the combination of two multiplex PCRs in the same lane, resolving up to eight peaks, as previously published [58]. The positive peaks were successively analysed using the tool "PBRT plugin" [58] developed in cooperation with the Advanced Analytical Company that allows automatic peak calling and the recording of positive replicons.

4.4. PCR screening of Tetracycline Resistance Genes

Salmonella strains with tetracycline-resistant (n. 83) and intermediate (n. 3) phenotypes were investigated to detect the presence of *tet*A, *tet*B, *tet*C, *tet*D, *tet*G, and *tet*M genes by PCR. All PCR reactions were carried out following protocols indicated by the European Reference Laboratory for Antimicrobial Resistance (EURL-AR, Technical University of Denmark, National Food Institute) [59] (Table 3).

Table 3. Primer and amplicon features of PCR assays used for *tet* gene characterization.

Target Gene	Primer Sequence	Amplicon Size (bp)	Tm (°C)	Ref.
tetA	5′-GTAATTCTGAGCACTGTCGC-3′ 5′-CTGCCTGGACAACATTGCTT-3′	956	57	[60]
tetB	5′-CTCAGTATTCCAAGCCTTTG-3′ 5′-ACTCCCCTGAGCTTGAGGGG-3′	414	52	[60]
tetC	5′-GGTTGAAGGCTCTCAAGGGC-3′ 5′-CCTCTTGCGGGATATCGTCC-3′	505	65	[60]
tetD	5′-CATCCATCCGGAAGTGATAGC-3′ 5′-GGATATCTCACCGCATCTGC-3′	436	57	[61]
tetG	5′-GCAGCGAAAGCGTATTTGCG-3′ 5′-TCCGAAAGCTGTCCAAGCAT-3′	662	62	[62]
tetM	5′-GTTAAATAGTGTTCTTGGAG-3′ 5′-CTAAGATATGGCTCTAACAA-3′	657	45	[62]

Positive control strains for the above-mentioned *tet* genes were also provided by the EURL-AR (Table 4).

The PCR products were separated by electrophoresis on a 1.5% (*w/v*) agarose/*midori* green advance color gel (Resnova, Rome, Italy) and finally recorded using UV transillumination. A 100 bp DNA ladder (GeneRuler 100 bp Plus, ThermoFisher Scientific, Waltham, US) was included in all agarose gels as a molecular weight standard.

4.5. Statistical Analysis

The association between the presence of the Inc plasmid group and the resistance phenotype was analyzed by the Hypothesis test–difference between two frequencies in large samples. A p-value < 0.05 was considered significant.

Table 4. Positive control strains used for *tet* gene PCR-based characterisation provided by the European Reference Laboratory for Antimicrobial Resistance (EURL-AR, Technical University of Denmark, National Food Institute).

Species	Strain Name	*tet* Gene
E. coli	*tet*A, NCTC 50078	*tet*A
E. coli	*tet*B, CSH50::Tn10	*tet*B
E. coli	*tet*C DO7 pBR 322, Tet	*tet*C
E. coli	*tet*D C600 psl 106, Tet *tet*Dx2	*tet*D
S. Typhimurium	P502212 DT104 sul1 and *tet*G	*tet*G
Staph. intermedius	2567, chromosomal *tet*M	*tet*M

5. Conclusions

In conclusion, this study shows that a heterogeneous *Salmonella* serovars population colonizes the food chain with the ability to reach consumers and cause serious illnesses. Isolates analysed here were characterised by a high rate of resistance to a wide panel of antibiotics, and particular attention was focused on tetracyclines considering the extensive use of this agent in veterinary medicine. The presence of MDR strains carrying genetic determinants on mobile genetic elements is a matter of concern for the spread of resistance in food settings.

In this context, the monitoring of different serovars along the food chain and a better knowledge of them from a molecular point of view are essential to managing risks associated with consumer health.

Greater coordination across all sectors of the food chain is needed to fight antibiotic resistance, and the implementation of molecular typing methods within routinary screening programmes can become an important tool of the One Health preventive approach.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antibiotics11060725/s1, Figure S1: AMR profiles of *Salmonella* strains investigated in this study according to the isolation source. AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; AMC, amoxicillin+clavulanic acid; FOX, cefoxitin; MEM, meropenem; TE, tetracycline; C, chloramphenicol; CIP, ciprofloxacin; NA, nalidixic acid; PEF, pefloxacin; CN, gentamicin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; ST, sulfisoxazole; TMP, trimethoprim.

Author Contributions: Conceptualization, F.A., G.F.S., D.B., G.A. and G.B. (Giorgio Brandi); methodology, I.R., D.B., F.A. and M.N.; formal analysis, D.B., F.A., G.F.S. and G.A.; investigation, I.R., D.B., M.N., G.B. (Giulia Baldelli); data curation, G.A., G.B. (Giorgio Brandi), G.F.S.; writing—original draft preparation, D.B., and I.R.; writing—review and editing, I.R., D.B., F.A., G.B. (Giorgio Brandi), G.F.S. and G.A.; supervision, G.B. (Giorgio Brandi). All authors have read and agreed to the published version of the manuscript.

Funding: This work was partially funded with internal funds from the Department of Biomolecular Sciences, University of Urbino Carlo Bo (Funds dedicated to Research Projects about Food Safety, Delibera del Consiglio del DISB n. 30/2020, 5 February 2020).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are present within the article.

Acknowledgments: The authors are grateful to the EURL-AR, Technical University of Denmark, National Food Institute, Lyngby, Denmark, for providing bacterial strains used as positive controls in PCR targeting *tet* genes. The authors would also like to thank M. Carletti for her help with the statistical analysis.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Gal-Mor, O. Persistent Infection and Long-Term Carriage of Typhoidal and Nontyphoidal *Salmonellae*. *Clin. Microbiol. Rev.* **2018**, *32*, e00088-18. [CrossRef] [PubMed]
2. Haselbeck, A.H.; Panzner, U.; Im, J.; Baker, S.; Meyer, C.G.; Marks, F. Current perspectives on invasive nontyphoidal *Salmonella* disease. *Curr. Opin. Infect. Dis.* **2017**, *30*, 498–503. [CrossRef] [PubMed]
3. European Food Safety Authority; European Centre for Disease Prevention and Control. The European Union One Health 2019 Zoonoses Report. *EFSA J.* **2021**, *19*, e06406.
4. Ehuwa, O.; Jaiswal, A.K.; Jaiswal, S. *Salmonella*, Food Safety and Food Handling Practices. *Foods* **2021**, *10*, 907. [CrossRef] [PubMed]
5. Keithlin, J.; Sargeant, J.M.; Thomas, M.K.; Fazil, A. Systematic review and meta-analysis of the proportion of non-typhoidal *Salmonella* cases that develop chronic sequelae. *Epidemiol. Infect.* **2015**, *143*, 1333–1351. [CrossRef]
6. Ao, T.T.; Feasey, N.A.; Gordon, M.A.; Keddy, K.H.; Angulo, F.J.; Crump, J.A. Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerg. Infect. Dis.* **2015**, *21*, 941–949. [CrossRef]
7. Majowicz, S.E.; Musto, J.; Scallan, E.; Angulo, F.J.; Kirk, M.; O'Brien, S.J.; International Collaboration on Enteric Disease "Burden of Illness" Studies. The global burden of nontyphoidal *Salmonella* gastroenteritis. *Clin. Infect. Dis.* **2010**, *50*, 882–889. [CrossRef]
8. V.T. Nair, D.; Venkitanarayanan, K.; Kollanoor Johny, A. Antibiotic-Resistant *Salmonella* in the Food Supply and the Potential Role of Antibiotic Alternatives for Control. *Foods* **2018**, *7*, 167. [CrossRef]
9. Wang, X.; Biswas, S.; Paudyal, N.; Pan, H.; Li, X.; Fang, W.; Yue, M. Antibiotic Resistance in *Salmonella* Typhimurium Isolates Recovered from the Food Chain Through National Antimicrobial Resistance Monitoring System Between 1996 and 2016. *Front. Microbiol.* **2019**, *10*, 985. [CrossRef]
10. Founou, L.L.; Founou, R.C.; Essack, S.Y. Antibiotic Resistance in the Food Chain: A Developing Country-Perspective. *Front. Microbiol.* **2016**, *7*, 1881. [CrossRef]
11. Verraes, C.; Van Boxstael, S.; Van Meervenne, E.; Van Coillie, E.; Butaye, P.; Catry, B.; de Schaetzen, M.-A.; Van Huffel, X.; Imberechts, H.; Dierick, K.; et al. Antimicrobial resistance in the food chain: A review. *Int. J. Environ. Res. Public Health* **2013**, *10*, 2643–2669. [CrossRef] [PubMed]
12. Bennani, H.; Mateus, A.; Mays, N.; Eastmure, E.; Stärk, K.D.C.; Häsler, B. Overview of Evidence of Antimicrobial Use and Antimicrobial Resistance in the Food Chain. *Antibiotics* **2020**, *9*, 49. [CrossRef] [PubMed]
13. Pezzella, C.; Ricci, A.; DiGiannatale, E.; Luzzi, I.; Carattoli, A. Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella* enterica isolates from animals in Italy. *Antimicrob. Agents Chemother.* **2004**, *48*, 903–908. [CrossRef] [PubMed]
14. European Food Safety Authority (EFSA); Aerts, M.; Battisti, A.; Hendriksen, R.; Kempf, I.; Teale, C.; Tenhagen, B.; Veldman, K.; Wasyl, D.; Guerra, B.; et al. A Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. *EFSA J.* **2019**, *17*, e05709.
15. Magiorakos, A.P.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; Monnet, D.L. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: An international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* **2012**, *18*, 268–281. [CrossRef]
16. Staffolani, M.; Medici, L.; Napoleoni, M.; Fisichella, S. Data on isolates of enteric bacteria from human clinical cases, animals, food and environment samples, in the year 2015 in Marche Region (Italy). *Sanità Pubblica Vet.* **2016**, 95. Available online: http://www.spvet.it/archivio/numero-95/626.html (accessed on 9 May 2022).
17. Staffolani, M.; Medici, L.; Napoleoni, M.; Fisichella, S. Data on isolates of enteric bacteria from human clinical cases, animals, food and environment samples, in the year 2016 in Marche Region (Italy). *Sanità Pubblica Vet.* **2017**, 101. Available online: http://www.spvet.it/archivio/numero-101/660.html (accessed on 9 May 2022).

18. Napoleoni, M.; Medici, L.; Staffolani, M.; Fisichella, S. Data on isolates of enteric bacteria from human clinical cases, animals, food and environment samples, in the year 2017 in Marche Region (Italy). *Sanità Pubblica Vet.* **2018**, 110. Available online: http://www.spvet.it/archivio/numero-110/688.html (accessed on 9 May 2022).
19. Napoleoni, M.; Medici, L.; Staffolani, M.; Fisichella, S. Data on isolates of enteric bacteria from human clinical cases, animals, food and environment samples in the year 2018 in Marche Region (Italy). *Sanità Pubblica Vet.* **2019**, 114. Available online: http://spvet.it/archivio/numero-114/697.html (accessed on 9 May 2022).
20. Napoleoni, M.; Staffolani, M.; Pazzaglia, I.; Fisichella, S. Data on isolates of enteric bacteria from human clinical cases, animals, food and environment samples in the year 2019 in Marche Region (Italy). *Sanità Pubblica Vet.* **2020**, 118. Available online: http://spvet.it/archivio/numero-118/705.html (accessed on 9 May 2022).
21. Napoleoni, M.; Silenzi, V.; Staffolani, M.; Guidi, F.; Blasi, G.; Fisichella, S.; Rocchegiani, E. Data on isolates of enteric bacteria from human clinical cases, animals, food and environment samples in the year 2020 in Marche Region (Italy). *Sanità Pubblica Vet.* **2021**, 125. Available online: http://www.spvet.it/archivio/numero-125/718.html (accessed on 9 May 2022).
22. Leati, M.; Cibin, V.; Mancin, M.; Pestelli, P.; Barco, L. *Enter-Vet Report Dati 2019*; Centro di Referenza Nazionale per le Salmonellosi; Istituto Zooprofilattico Sperimentale delle Venezie: Legnaro (PD), Italy, 2022; pp. 1–38.
23. Ferrari, R.G.; Rosario, D.K.A.; Cunha-Neto, A.; Mano, S.B.; Figueiredo, E.E.S.; Conte-Junior, C.A. Worldwide Epidemiology of *Salmonella* Serovars in Animal-Based Foods: A Meta-analysis. *Appl. Environ. Microbiol.* **2019**, *85*, e00591-19. [CrossRef] [PubMed]
24. Crim, S.M.; Iwamoto, M.; Huang, J.Y.; Griffin, P.M.; Gilliss, D.; Cronquist, A.B.; Cartter, M.; Tobin-D'Angelo, M.; Blythe, D.; Smith, K.; et al. Incidence and Trends of Infection with Pathogens Transmitted Commonly Through Food—Foodborne Diseases Active Surveillance Network, 10 U.S. Sites, 2006–2013. *MMWR Morb. Mortal. Wkly. Rep.* **2020**, *63*, 328–332.
25. Gomes-Neves, E.; Antunes, P.; Tavares, A.; Themudo, P.; Cardoso, M.F.; Gärtner, F.; Costa, J.M.; Peixe, L. *Salmonella* cross-contamination in swine abattoirs in Portugal: Carcasses, meat and meat handlers. *Int. J. Food Microbiol.* **2012**, *157*, 82–87. [CrossRef] [PubMed]
26. Ferrari, R.G.; Panzenhagen, P.H.N.; Conte-Junior, C.A. Phenotypic and Genotypic Eligible Methods for *Salmonella* Typhimurium Source Tracking. *Front. Microbiol.* **2017**, *8*, 2587. [CrossRef] [PubMed]
27. Wu, C.; Yan, M.; Liu, L.; Lai, J.; Chan, E.W.; Chen, S. Comparative characterization of nontyphoidal *Salmonella* isolated from humans and food animals in China, 2003–2011. *Heliyon* **2018**, *4*, e00613. [CrossRef] [PubMed]
28. Kerouanton, A.; Rose, V.; Weill, F.X.; Granier, S.A.; Denis, M. Genetic diversity and antimicrobial resistance profiles of *Salmonella* enterica serotype derby isolated from pigs, pork, and humans in France. *Foodborne Pathog. Dis.* **2013**, *10*, 977–984. [CrossRef]
29. Alba, P.; Leekitcharoenphon, P.; Carfora, V.; Amoruso, R.; Cordaro, G.; Di Matteo, P.; Engage-Eurl-Ar Network Study Group. Molecular epidemiology of *Salmonella* Infantis in Europe: Insights into the success of the bacterial host and its parasitic pESI-like megaplasmid. *Microb. Genom.* **2020**, *6*, e000365. [CrossRef]
30. Mughini-Gras, L.; van Hoek, A.H.; Cuperus, T.; Dam-Deisz, C.; van Overbeek, W.; van den Beld, M.; Wit, B.; Rapallini, M.; Wullings, B.; Franz, E.; et al. Prevalence, risk factors and genetic traits of *Salmonella* Infantis in Dutch broiler flocks. *Vet Microbiol.* **2021**, *258*, 109120. [CrossRef]
31. Franco, A.; Leekitcharoenphon, P.; Feltrin, F.; Alba, P.; Cordaro, G.; Iurescia, M.; Battisti, A. Emergence of a Clonal Lineage of Multidrug-Resistant ESBL-Producing *Salmonella* Infantis Transmitted from Broilers and Broiler Meat to Humans in Italy between 2011 and 2014. *PLoS ONE* **2015**, *10*, e0144802. [CrossRef]
32. Dionisi, A.M.; Lucarelli, C.; Benedetti, I.; Owczarek, S.; Luzzi, I. Molecular characterisation of multidrug-resistant *Salmonella* enterica serotype Infantis from humans, animals and the environment in Italy. *Int. J. Antimicrob. Agents* **2011**, *38*, 384–389. [CrossRef] [PubMed]
33. Elnekave, E.; Hong, S.L.; Lim, S.; Johnson, T.J.; Perez, A.; Alvarez, J. Comparing serotyping with whole-genome sequencing for subtyping of non-typhoidal *Salmonella* enterica: A large-scale analysis of 37 serotypes with a public health impact in the USA. *Microb. Genom.* **2020**, *6*, mgen000425. [CrossRef] [PubMed]
34. European Food Safety Authority; European Centre for Disease Prevention and Control. The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2018/2019. *EFSA J.* **2021**, *19*, e06490.
35. Frech, G.; Schwarz, S. Molecular analysis of tetracycline resistance in Salmonella enterica subsp. enterica serovars Typhimurium, enteritidis, Dublin, Choleraesuis, Hadar and Saintpaul: Construction and application of specific gene probes. *J. Appl. Microbiol.* **2000**, *89*, 633–641. [CrossRef]
36. European Medicines Agency; European Surveillance of Veterinary Antimicrobial Consumption. *Sales of Veterinary Antimicrobial Agents in 31 European Countries in 2019 and 2020*; Eleventh ESVAC Report EMA/58183/2021; 2021; Available online: https://www.ema.europa.eu/en/documents/report/sales-veterinary-antimicrobial-agents-31-european-countries-2019-2020-trends-2010-2020-eleventh_en.pdf (accessed on 9 May 2022).
37. WHO. Critically Important Antimicrobials for Human Medicine, 6th Revision. 2019. Available online: https://www.who.int/foodsafety/publications/antimicrobials-sixth/en/https://www.who.int/publications/i/item/9789241515528 (accessed on 9 May 2022).
38. European Union (EU). Commission Notice—Guidelines for the prudent use of antimicrobials in veterinary medicine (2015/C 299/04). *Off. J. Eur. Union* **2015**, *58*, 7–26. Available online: https://ec.europa.eu/health/system/files/2016-11/2015_prudent_use_guidelines_en_0.pdf (accessed on 9 May 2022).

39. Gargano, V.; Sciortino, S.; Gambino, D.; Costa, A.; Agozzino, V.; Reale, S.; Vicari, D. Antibiotic Susceptibility Profile and Tetracycline Resistance Genes Detection in *Salmonella* spp. Strains Isolated from Animals and Food. *Antibiotics* **2021**, *10*, 809. [CrossRef]
40. Bacci, C.; Lanzoni, E.; Vismarra, A.; Alpigiani, I.; Nuvoloni, R.; Bonardi, S.; Brindani, F. Antibiotic resistance and resistance genes in *Salmonella enterica* isolated from pork meat and pig carcasses in Northern Italy. *Large Animal. Rev.* **2014**, *20*, 201–207.
41. García-Soto, S.; Abdel-Glil, M.Y.; Tomaso, H.; Linde, J.; Methner, U. Emergence of Multidrug-Resistant *Salmonella enterica* Subspecies *enterica* Serovar Infantis of Multilocus Sequence Type 2283 in German Broiler Farms. *Front. Microbiol.* **2020**, *11*, 1741. [CrossRef]
42. Grossman, T.H. Tetracycline Antibiotics and Resistance. *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a025387. [CrossRef]
43. Lauteri, C.; Maggio, F.; Serio, A.; Festino, A.R.; Paparella, A.; Vergara, A. Overcoming Multidrug Resistance in *Salmonella* spp. Isolates Obtained From the Swine Food Chain by Using Essential Oils: An in vitro Study. *Front. Microbiol.* **2022**, *12*, 808286. [CrossRef]
44. Peruzy, M.F.; Capuano, F.; Proroga, Y.T.R.; Cristiano, D.; Carullo, M.R.; Murru, N. Antimicrobial Susceptibility Testing for *Salmonella* Serovars Isolated from Food Samples: Five-Year Monitoring (2015–2019). *Antibiotics* **2020**, *9*, 365. [CrossRef]
45. Pavelquesi, S.L.S.; de Oliveira Ferreira, A.C.A.; Rodrigues, A.R.; de Souza Silva, C.M.; Orsi, D.C.; da Silva, I.C.R. Presence of Tetracycline and Sulfonamide Resistance Genes in *Salmonella* spp.: Literature Review. *Antibiotics* **2021**, *10*, 1314. [CrossRef] [PubMed]
46. Camarda, A.; Pugliese, N.; Pupillo, A.; Oliva, M.; Circella, E.; Dionisi, A.M.; Ricci, A.; Legretto, M.; Caroli, A.; Pazzani, C. Resistance genes, phage types and pulsed field gel electrophoresis pulsotypes in *Salmonella* enterica strains from laying hen farms in southern Italy. *Int. J. Environ. Res. Public Health* **2013**, *10*, 3347–3362. [CrossRef] [PubMed]
47. Tawyabur, M.; Islam, M.; Sobur, M.; Hossain, M.; Mahmud, M.; Paul, S.; Rahman, M. Isolation and Characterization of Multidrug-Resistant *Escherichia coli* and *Salmonella* spp. from Healthy and Diseased Turkeys. *Antibiotics* **2020**, *9*, 770. [CrossRef] [PubMed]
48. Carattoli, A. Plasmids in Gram negatives: Molecular typing of resistance plasmids. *Int. J. Med. Microbiol.* **2011**, *301*, 654–658. [CrossRef] [PubMed]
49. Carattoli, A. Resistance plasmid families in Enterobacteriaceae. *Antimicrob. Agents Chemother.* **2009**, *53*, 2227–2238. [CrossRef]
50. Oluwadare, M.; Lee, M.D.; Grim, C.J.; Lipp, E.K.; Cheng, Y.; Maurer, J.J. The Role of the *Salmonella* spvB IncF Plasmid and Its Resident Entry Exclusion Gene traS on Plasmid Exclusion. *Front. Microbiol.* **2020**, *11*, 949. [CrossRef]
51. Di Cesare, A.; Losasso, C.; Barco, L.; Eckert, E.M.; Conficoni, D.; Sarasini, G.; Corno, G.; Ricci, A. Diverse distribution of Toxin-Antitoxin II systems in *Salmonella enterica* serovars. *Sci. Rep.* **2016**, *6*, 28759. [CrossRef]
52. European Union. Commission Regulation (EC) No 2073/2005 of 15 November 2005 on Microbiological Criteria for Foodstuffs. *Off. J. Eur. Union* **2005**, *50*, 1–26. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX:32005R2073 (accessed on 9 May 2022).
53. *ISO 6579-1:2017/A1:2020*; Microbiology of the Food Chain—Horizontal Method for the Detection, Enumeration and Serotyping of *Salmonella*—Part 1: Detection of *Salmonella* spp.—Amendment 1 Broader Range of Incubation Temperatures, Amendment to the Status of Annex D, and Correction of the Composition. International Organization for Standardization: Geneva, Switzerland, 2020.
54. Italian Ministry of Health. National Control Plan for Salmonellosis in Poultry 2019–2021 (Piano Nazionale di Controllo delle Salmonellosi negli Avicoli 2019–2021). Directorate General for Animal Health and Veterinary Medicines of the Italian Ministry of Health. 2018. Available online: https://www.salute.gov.it/imgs/C_17_pubblicazioni_2849_allegato.pdf (accessed on 9 May 2022).
55. *ISO/TR 6579-3:2014*; Microbiology of the Food Chain—Horizontal Method for the Detection, Enumeration and Serotyping of *Salmonella*—Part 3: Guidelines for Serotyping of *Salmonella* spp. International Organization for Standardization: Geneva, Switzerland, 2014.
56. Jarlier, V.; Nicolas, M.H.; Fournier, G.; Philippon, A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in enterobacteriaceae: Hospital prevalence and susceptibility patterns. *Clin. Infect. Dis.* **1988**, *10*, 867–878. [CrossRef]
57. CLSI. *Performance Standards for Antimicrobial Susceptibility Testing*, 32nd ed.; CLSI Supplement M100; Clinical and Laboratory Standards Institute: Wayne, PA, USA, 2022.
58. Barbadoro, P.; Bencardino, D.; Carloni, E.; Omiccioli, E.; Ponzio, E.; Micheletti, R.; Andreoni, F. Carriage of carbapenem-resistant enterobacterales in adult patients admitted to a university hospital in italy. *Antibiotics* **2021**, *10*, 61. [CrossRef]
59. European Reference Laboratory for Antimicrobial Resistance (EURL-AR, Technical University of Denmark, National Food Institute). List of Primers for Detection of Antimicrobial Resistance Genes. Available online: https://www.eurl-ar.eu/CustomerData/Files/Folders/25-resourcer/459_primerliste-til-web-07-11-2018.pdf (accessed on 9 May 2022).
60. Sengeløv, G.; Agersø, Y.; Halling-Sørensen, B.; Baloda, S.B.; Andersen, J.S.; Jensen, L.B. Bacterial antibiotic resistance levels in Danish farmland as a result of treatment with pig manure slurry. *Environ. Int.* **2003**, *28*, 587–595. [CrossRef]
61. Miranda, C.D.; Kehrenberg, C.; Ulep, C.; Schwarz, S.; Roberts, M.C. Diversity of tetracycline resistance genes in bacteria from Chilean salmon farms. *Antimicrob. Agents Chemother.* **2003**, *47*, 883–888. [CrossRef] [PubMed]
62. Agersø, Y.; Sandvang, D. Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. isolated from pigsties and manured soil. *Appl. Environ. Microbiol.* **2005**, *71*, 7941–7947. [CrossRef] [PubMed]

SUPPLEMENTARY MATERIALS

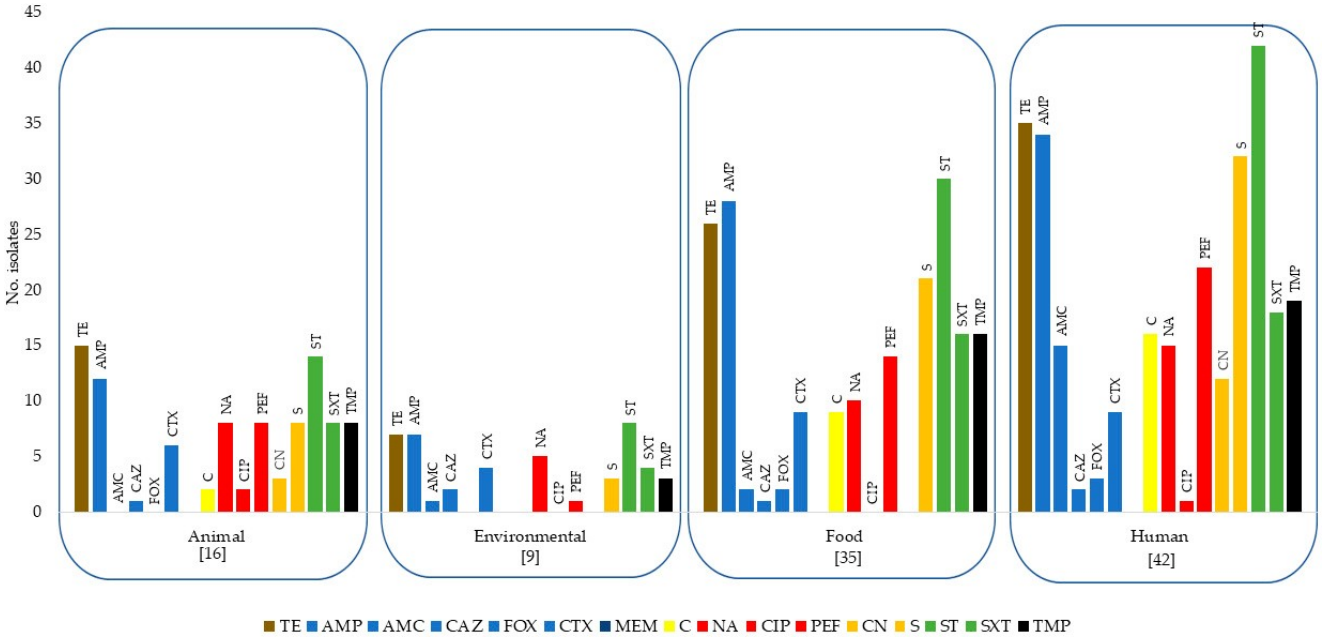


Figure S1. AMR profiles of *Salmonella* strains investigated in this study according to the isolation source. AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; AMC, amoxicillin+clavulanic acid; FOX, cefoxitin; MEM, meropenem; TE, tetracycline; C, chloramphenicol; CIP, ciprofloxacin; NA, nalidixic acid; PEF, pefloxacin; CN, gentamicin; S, streptomycin; SXT, trimethoprim-sulfamethoxazole; ST, sulfisoxazole; TMP, trimethoprim.

PAPER IN PREPARATION

The following paper in preparation is based on results obtained after an in dept molecular analysis carried out at the German Federal Institute for Risk Assessment
(Bundesinstitut für Risikobewertung, BfR), Berlin, under the supervision of dr Jennie Fischer, during a 7-months period abroad of my PhD course.
After a preliminary screening based on the AMR profile of investigates strains, 35 *S*. Infantis were selected among *Salmonella* strains isolated during the routine surveillance activity of the Regional Reference Center for Enteric Pathogens of the Instituto Zooprofilatico Sperimentale dell´Umbria e delle Marche, section of Tolentino.
The aim of this study was to reveal the presence of the *S*. Infantis pESI-like in antibiotic resistant *S*. Infantis strains isolated from the food chain, in particular, from food environment, animals, food and humans in Marche Region, and to study the pESI-like genetic characteristics, AMR profile and confirm their spread along the food chain using a phylogenetic approach

From farm to fork: Spread of multidrug resistant *Salmonella* Infantis pESI-like *bla*$_{CTX-M-1}$ clone in Marche Region

Ilaria Russo[1], Jennie Fischer[2], Laura Uelze[2], Alexandra Irrgan[2], Giuditta Fiorella Schiavano[3], Francesca Andreoni[1,4], Maira Napoleoni[5], Giorgio Brandi[1], Giulia Amagliani[1]

[1] Department of Biomolecular Sciences, University of Urbino Carlo Bo, Urbino, Italy
[2] BfR, German Federal Institute for Risk Assessment, Berlin, Germany
[3] Department of Humanities, University of Urbino Carlo Bo, Urbino, Italy
[4] Clinical Pathology, Urbino Hospital, Asur Marche, Urbino, Italy
[5] Regional Reference Center for Enteric Pathogens Marche, Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche "Togo Rosati", Perugia, Italy

1. Introduction

Salmonella Infantis is one of the five main causes of human salmonellosis in the European Union (EU). In 2020, according to the last report of EFSA [1], multi-drug resistant (MDR) *S*. Infantis in broilers and turkeys reached 73% and 22%, respectively. Moreover, an increase in the number of MDR *S*. Infantis has been also noticed in human clinical isolates (45.3%), in 2020, in contrast with 2019 (35.7%) [1].

The issue of MDR in food production systems become even more relevant when Critically important antibiotics (CIAs) for human health are involved in the resistance. In the last decade, MDR and extended spectrum β-lactamase (ESBL) AmpC-producing *S*. Infantis has been frequently reported from food-producing animals and humans in Italy [1,2].

In fact, Italy possesses high number of isolates from both, broiler and turkeys, exhibiting a MDR ESBL phenotype and some of them reported also AmpC profile [1], although the use of 3rdand 4th generation cephalosporin is not permitted in poultry and off-label use is prohibited according to EU legislation [1,3,4].

For this reason, the high proportion of MDR ESBL/AmpC-producers in *S*. Infantis can be attributed to a clonal expansion and spread as described by Franco et al [3].

Indeed, in Italy a MDR and ESBL producing *S*. Infantis clone harboring an IncI/IncP chimeric megaplasmid (~280–320 kb), carrying virulence, fitness and MDR genes, in particular the ESBL encoding gene *bla*$_{CTX-M-1,}$ increasingly spread during the last years [5]. This megaplasmid is named pESI-like since it was similar to that, named pESI, described in Israel [6]. The *S*. Infantis clone

harboring the pESI-like plasmid was first identified in Italy, in 2014, by Franco et al from food-producing animals and humans [3].

The ancestral pESI isolated in Israel was reported conferring tetracycline, sulfamethoxazole and trimethoprim resistance to *S*. Infantis extended spectrum cephalosporin sensible. Moreover, the pESI plasmid promotes pathogenicity mechanisms and it's able to increase intestinal inflammation in experimental mouse infections. The Israel pESI also harbored genes able to increase bacterial tolerance to environmental mercury (mer operon) and oxidative stress. It is defined a chimeric plasmid evolved by recombination between, at least, IncI1 and IncP ancestral plasmid groups [3,6].

The pESI-like megaplasmid acquired more resistance genes. In particular it is characterized by a specific gene panel that includes 3rd generation cephalosporin (*bla*$_{CTX-M}$), tetracyclines (*tet*A), trimethoprim (*dfr*A), sulphonamides (*sul*1), aminoglycosides (*aad*A). Moreover, other advantageous genes enhancing virulence traits of *S*. Infantis clone were associated to pESI-like and include yersiniabactin biosynthetic protein gene *irp*2, fimbria gene *ipf*, toxin/antitoxin (T/AT) system and genes conferring resistance to quaternary ammonium compounds [5].

Due to antibiotic resistance genes (ARGs), genes involved in virulence, colonization and fitness, previously described, once acquired, pESI-like plasmid became established in the local population because of the selective advantages it confers to the host bacteria [5,7].

In the United States (U.S.), in 2018, an outbreak of an ESBL producing *S*. Infantis which harbors a pESI-like megaplasmid that carries *bla*$_{CTX-M-65}$, associated with raw chicken, was reported. The majority of the strains harboring *bla*$_{CTX-M-65}$ were additionally resistant to ciprofloxacin, nalidixic acid, chloramphenicol, gentamicin, sulfamethoxazole, tetracycline and trimethoprim [1,8]. Of particular concern is the *bla*$_{CTX-M-65}$ gene presented in a large part of pESI plasmids in U.S. [9].

The ESBL-producing *Enterobacterales* are considered "Critical Priority Pathogens" by the World Health Organization (WHO) since they show resistance to 3rd generation cephalosporin considered as CIA for human health and first choice treatment for children and invasive infection [3,10,11].

Due to these characteristics and the ability to spread and establish well in the host cell, this plasmid can spread along the food chain and arrive to humans in the way "from farm to fork" [5].

The aim of our study was to reveal the presence of the *S*. Infantis pESI-like in antibiotic resistant *S*. Infantis strains isolated from the food chain, in particular, from environment, animal, food and human in Marche Region, Central Italy and to study the pESI-like genetic characteristics, AMR profile and confirm their spread along the food chain using a phylogenetic approach.

2. Materials and Methods

2.1. S. Infantis strains selection

For this study 35 *S*. Infantis strains were selected among *Salmonella* strains isolated during the routine surveillance activity of the Regional Reference Center for Enteric Pathogens of the Instituto Zooprofilatico Sperimentale dell´Umbria e delle Marche, section of Tolentino.

The strains were isolated along the food chain, from 2016 to 2021 from food (7 chicken meat, 1 pig meat, 1 mollusk and 2 mixed meat); environment (3 livestock, 1 slaughterhouse and 1 from food processing laboratory); animals (8 from broiler) and humans (10 from faeces and 1 other human clinical samples), and were selected for this study for in depth molecular characterization using real-time PCR, S1 nuclease pulsed-field gel electrophoresis (S1-PFGE) and whole genome sequencing (WGS).

2.2. Real-time PCR screening for the presence of genes conferring resistance against 3rd generation cephalosporins and carbapenems.

2.2.1. DNA extraction via thermal lysis

Bacterial isolates were grown in Trypticase soy agar (TSA) plates overnight. A single colony was resuspended in 300 µl of TE and heated to 100°C for 10 minutes. Afterwards the suspension was pelletized at 14.000 rpm for 2 minutes and 50 µl of supernatant was added to 100 µl of TE buffer (10mM Tris, 0.1 mM EDTA, pH8) to dilute the final concentration of DNA. The DNA was stored at -20°C and used for PCR detection of carbapenemases and ESBL encoding genes.

2.2.2. Multiplex real-time PCR analysis

The extracted DNA was used as template in two real-time PCR assays, one for the detections of genes encoding ESBL and AmpC enzymes and one for carbapenemases enzymes.

The detection of ESBL and Amp-C encoding genes were investigated *via* multiplex real-time PCR for the identification of $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and CIT-Type AmpC genes using the protocol published by Roschanski et al (2014) [12].

Multiplex real-time PCR was performed in 20 µL including, PCR mastermix (Platinum Multiplex PCR Master Mix, Thermo Fisher, Waltham, MA, USA), primers and probes as described in Table 1 and 1 µL of DNA. Sequences of primers and probes are listed in Table 2 according to Roschanki et al (2014) [12]. The PCRs were performed with an initial denaturation step of 95°C for 2 minutes followed by 30 cycles of denaturation at 95°C for 5 sec, annealing and elongation at 60°C for 60sec.

Components	Concentration	Volume (µL)
Platinum MultiplexMasterMix (ThermoFisher Scientific)		10
TEM_fwd	10pmol(=1:10)	0,5
TEM_rev	10pmol(=1:10)	0,5
TEM_probe	5pmol(=1:10)	0,2
CMY_fwd	10pmol(=1:10)	0,4
CMY_rev	10pmol(=1:10)	0,4
CMY_probe	10pmol(=1:10)	0,1
SHV_fwd	10pmol(=1:10)	0,5
SHV_rev	10pmol(=1:10)	0,5
SHV_probe	10pmol(=1:10)	0,2
CTX-A_fwd	10pmol(=1:10)	0,5
CTX-A_rev	10pmol(=1:10)	0,5
CTX-A_probe	10pmol(=1:10)	0,2
CTX-B_fwd	10pmol(=1:10)	0,5
CTX-B_rev	10pmol(=1:10)	0,5
CTX-B_probe	10pmol(=1:10)	0,3

Table 1. Multiplex real-time PCR protocol conditions used for ESBL/AmpC gene identification

Gene target	Primer and Probe	Gene sequence (5'- 3')
bla$_{TEM}$	TEM_fwd	GCATCTTACGGATGGCATGA
	TEM_rev	GTCCTCCGATCGTTGTCAGAA
	TEM_probe	**6-Fam**-CAGTGCTGCCATAACCATGAGTGA-**BHQ-1**
bla$_{CMY}$	CMY_fwd	GGCAAACAGTGGCAGGGTAT
	CMY_rev	AATGCGGCTTTATCCCTAACG
	CMY_probe	**ROX**-CCTACCGCTGCAGATCCCCGATG-**BHQ-2**
bla$_{SHV}$	SHV_fwd	TCCCATGATGAGCACCTTTAAA
	SHV_rev	TCCTGCTGGCGATAGTGGAT
	SHV_probe	**Cy5**-TGCCGGTGACGAACAGCTGGAG-**BBQ-650**
bla$_{CTX-M}$	CTX-A_fwd	CGGGC**R**ATGGCGCA**R**AC
	CTX-A_rev	TGC**R**CCGGT**S**GTATTGCC
	CTX-A_probe	**Yakima Yellow**-CCARCGGGCGCAGYTGGTGAC-**BHQ1**
	CTX-B_fwd	ACCGAGCC**S**ACGCTCAA
	CTX-B_rev	CCGCTGCCGGTTTTATC
	CTX-B_probe	**Yakima Yellow**- CCCGCGYGATACCACCACGC-**BHQ1**

Table 2. Primers and probes used for ESBL and CIT type AmpC detection in this study [12].

For the detection of genes encoding most frequently detected carbapenemases genes *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{NDM}$, *bla*$_{OXA-48}$, and *bla*$_{GES}$, we conducted a multiplex real-time PCR.

The used primer and probe sequences for the detection of the tested carbapenemases genes were taken from previously published protocols [13,14] and later adapted by Roschanski et al (2017) as described in Table 3 [15]

Multiplex real-time was performed in 25 µL including, PCR mastermix (Platinum Multiplex PCR Master Mix, Thermo Fisher), primers and probes as described in Table 4 and 1 µL of DNA .

The PCRs were performed with an initial denaturation step of 95°C for 15 minutes followed by 30 cycles of denaturation at 95°C for 15 sec, annealing and elongation at 60°C for 60sec.

Gene target	Primer and Probe	Gene sequence (5'- 3')
bla$_{OXA-48}$	OXA-48_fwd	GCGTGGTTAAGGATGAACAC
	OXA-48_rev	CATCAAGTTCAACCCAACCG
	OXA-48_probe	**Rox**-AGCCATGCTGACCGAAGCCAATG-**BHQ2**
bla$_{VIM}$	VIM_fwd	GAGATTCCCACGCAYTCTCTAGA
	VIM_rev	AATGCGCAGCACCAGGATAG
	VIM_probe	**Yakima Yellow**-ACGCAGTGCGCTTCGGTCCAGT-**BHQ1**
bla$_{NDM}$	NDM_fwd	CATTAGCCGCTGCATTGATG
	NDM_rev	GTCGCCAGTTTCCATTTGCT
	NDM_probe	**6-Fam**-CATGCCCGGTGAAATCCGCC-**BHQ1**
bla$_{KPC}$	KPC_fwd	TGCAGAGCCCAGTGTCAGTTT
	KPC_rev	CGCTCTATCGGCGATACCA
	KPC_probe	**Cy5**-TTCCGTCACGGCGCGCG-**BBQ650**
bla$_{GES}$	GES_fwd	CGGTTTCTAGCATCGGGACACAT
	GES_rev	CCGCCATAGAGGACTTTAGCMACAG
	GES_probe	**Atto425**-CGACCTCAGAGATACAACTACGCCTATTGC-**DDQ1**

Table 3. Primers and probes used for carbapenemases gene via multiplex real-time PCR [15]

Components	Concentration	Volume (µL)
Platinum MultiplexMasterMix (ThermoFisher Scientific)		12,5
OXA-48_fwd	10pmol(=1:10)	1
OXA-48_rev	10pmol(=1:10)	1
OXA-48_probe	10pmol(=1:10)	0,2
VIM_fwd	10pmol(=1:10)	1
VIM_rev	10pmol(=1:10)	1
VIM_probe	10pmol(=1:10)	0,2
NDM_fwd	10pmol(=1:10)	1
NDM_rev	10pmol(=1:10)	1
NDM_probe	10pmol(=1:10)	0,1
KPC_fwd	10pmol(=1:10)	1
KPC_rev	10pmol(=1:10)	1
KPC_probe	10pmol(=1:10)	0,3
GES_fwd	10pmol(=1:10)	1
GES_rev	10pmol(=1:10)	1
GES_probe	10pmol(=1:10)	0,2

Table 4. Multiplex real-time conditions used for the detection of carbapenemases genes tested in our study.

2.3. Whole genome sequencing (WGS)

The 35 *S*. Infantis strains were cultivated in TSA. A single colony was inoculated in Luria-Bertani (LB) liquid medium and incubated at 37°C under overnight shaking conditions.

The PureLink® Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) was used for Genomic DNA extraction.

Sequencing libraries were prepared with the Nextera™DNA Flex Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

Paired-end sequencing was performed in in 2x149 cycles on the Illumina NextSeq™ 500 benchtop sequencer (Illumina, San Diego, CA, USA) using the NextSeq™ 500/550 Mid Output Kit v2 or v2.5 (300-cycle) (Illumina, San Diego, CA, USA).

AQUAMIS pipeline v1.3.8 (Assembly-based QUality Assessment for Microbial Isolate Sequencing) was used for the assembly and trimming of raw reads [16] (https://gitlab.com/bfr_bioinformatics/AQUAMIS)

Based on the draft assemblies produced by AQUAMIS, the Bakcharak pipeline v2.1.0 was used to conduct a characterization of the *Salmonella* genome (https://gitlab.com/bfr_bioinformatics/bakcharak)

In Bakcharak implemented tools were used to carry out the analysis: ncbi-amrfinderplus v3.9.3 to detect antimicrobial resistance genes [17]; PlasmidFinder database for plasmid incompatibility group detection [18]; ABRicate v1.0.1 for antimicrobial resistance and virulence factor screening (https://github.com/tseemann/abricate); Prokka 1.14.6 for gene annotation; AMR finder 2021-03-01.1; Platon was used to determine whether an AMR gene-harboring contig was classified as part of a plasmid or the chromosome; mlst 2.19.0 for ST typing. *Salmonella* serovar is determined with software SISTR v1.1.1 [19].

All contained software versions are fully tracked with the conda software management tool.

2.4. Phylogenetic analysis

The cgMLST analysis was carried out as pre-analysis of isolates genetic diversity and included all *S*. Infantis strains, positive or negative for $bla_{\text{CTX-M}}$ gene or presence of plasmids.

The cgMLST analysis was conducted with the chewieSnake pipeline v3.1.1 [20], which implements chewBBACA v2.0.12 [21] and is based on 3000 loci of the *Salmonella* Enterobase scheme (http://enterobase.warwick.ac.uk/species/senterica/download_data; accessed on 8 September 2021). Allele profiles are generated and allele differences are calculated pairwise. An allelic distance

matrix is generated and a minimum spanning tree was calculated using GrapeTree (https://enterobase.warwick.ac.uk/ms_tree?tree_id=23518).

The threshold with a maximum cut-off of 20 allelic differences was used to include strains in the same cluster.

Moreover, cgMLST results were used for reference selection for SNP analyses with a focus on a certain genetic cluster and for the selection of SNP reference used for the analysis.

SNP calling was conducted with the snippySnake pipeline v1.0 that implements snippy v4.6.0 for variant calling [22] A maximum-likelihood based phylogenetic tree was inferred with IQ-TREE v2.0.3 from the core SNPs. Phylogenetic trees were visualized with iTOL v5.

Single-linkage clustering was performed on the pairwise SNP difference between all isolates using a distance threshold of <21 SNPs [23].

22-SA00401-0 was used as internal reference for SNP analysis

2.5. PFGE-S1 plasmids size characterization

To confirm presence and molecular sizes of plasmids, S1 digested DNA was used for PFGE analysis. Analysis were conducted with the CHEF-DR III system (Bio-Rad Laboratories GmbH, Munich, Germany) using a 1% agarose gel (Biozyme LE GP agarose; Biozym Scientific GmbH, Hessisch Oldendorf, Germany) under following running conditions: 1s-25s, 17 h, 6 V/cm, 120 V according to the PulseNet standardized laboratory protocol [24]. Plasmids sizes were evaluated using the molecular size marker *Salmonella enterica* serotype Braenderup H9812 digested with XbaI enzyme.

2.6. pESI-like prediction

WGS sequence data of the 35 *S*. Infantis strains were aligned against reference sequences of 13 target genes proposed by McMillan et al [8] (Tab.5), indicative of pESI-like plasmid presence, using Geneious Prime® 2020.2.2.

According to Mc Millan et al, isolates carrying genes with >95% identity to the target sequences were considered positive for the presence of the gene [8].

Moreover, for genes with a coverage <95% to the target sequences, short read sequences of *S*. Infantis strains were additionally aligned *in silico* using Geneious Prime® 2020.2.2, using PCR primers proposed by Franco et al to verify the presence of the gene [25].

Gene target	Gene Function
*ard*A	Restriction modification enzyme
*tra*I	Relaxase (IncI1)
*sog*S	Primase (IncI1)
trbA	Conjugal transfer protein (IncI1)
pESI rep. origin	Plasmid replication
bla_{CTX-M}	Extended-Spectrum Betalactamase
IncP	Interons associated with IncP
hypothetical backbone protein	Hypothetical protein associated with pESI
K88	Fimbriae
Ybt	*irp*2 (Ybt operon); yersiniabactim biosynthesis
merA	Mercuric reductase (*mer* operon)
ipf	Fimbriae
pilL	Pilus biosynthesis (IncI1)

Table 5. Gene targets used to verify the presence of pESI-like plasmid in *S*. Infantis strains as proposed by McMillan et al [8,25]

3. Results

3.1. Presence of 3rd generation cephalosporin and carbapenemases encoding genes in MDR S. Infantis isolates.

The AMR profile previously investigated [26], showed a total of 74% (26/35) of *S*. Infantis resistant to at least one beta-lactam. Among them, 88% (23/26) were resistant to 3rd generation cephalosporins.

Real-time PCR analysis showed 88% of isolates positive for $bla_{CTX-A/B}$ gene family, 11% for bla_{TEM} gene family and one strain was negative for all genes tested in this study.

None of our strains were tested positive for the carbapenemase encoding genes of analysis.

3.2. Whole genome sequencing of S. Infantis strains

3.2.1. Gene content

The WGS of the 35 *S*. Infantis strains revealed two different *bla*$_{CTX-M}$ variants: 60% (21/35) of strains carried *bla*$_{CTX-M-1}$ and 6% (2/35) *bla*$_{CTX-M-65}$, both involved in 3rd generation cephalosporin resistance. The 8% (3/35) carried *bla*$_{TEM-1}$ and 26% (9/35) were negative for all ESBL/AmpC genes.

Moreover, the most detected genes involved in AMR were in particular: *tet*A, *dfr*A1, *dfr*A14 variants were detected in 97% (34/35) of strains, followed by *sul*1 detected in 94% (33/35) strains; *aph*(3') in 40% (14/35) strains. Additional genes were detected: *aad*A1, *ant(2")*, *mdsA* and *mdsB*. Notably, *aac*(3)-IVa, *aph*(4), *floR* and *fosA3* genes were detected only in *bla*$_{CTX-M-65}$ strains. Mutation in the *gyr*A gene with D87G variant were detected in all strains and usually associated with fluoroquinolone resistance. All ARGs detected are listed in details in supplementary Table S1.

118 virulence genes were detected in isolates harboring plasmid, such as genes encoding fimbriae, *feaD*, *feaE*; yersiniabactin operon genes *fyuA*, *irp*1, *irp*2, *ybt*A, *ybt*E, *ybt*P, *ybt*Q, *ybt*S, *ybt*T, *ybt*U, *ybt*X; mercury operon involved in mercury resistance such as *mer*A gene. In the strains without the plasmid 105 virulence genes were noticed (Table S2).

3.2.2. Plasmid content

The investigation of plasmid replicon profiles revealed 97% (34/35) of *S*. Infantis isolates showing the presence of IncFIB(K)_1_Kpn3, which was present also in strains lacking *bla*$_{CTX-M-1}$. In 8 strains, IncFIB(K)_1_Kpn3 was detected together with others Incompatibility (Inc) group. The second most common Inc group, detected in 4 isolated with IncFIB(K)_1_Kpn3, was IncX4, followed by IncX1 detected in 3 isolates and X3, FII and FIC detected in 1 isolates. Two strains showed the presence of Col(MG828)_1 replicons.

3.2.3. pESI-like gene pattern

All 35 *S*. Infantis strains were screened for gene target sequences used to determine the most common genes carried by pESI-like plasmid and used to predict the presence of the plasmid. As shown in table 6, strains harboring IncFIB(K)_1_Kpn3 plasmids showed the presence of all genes used to determine the presence of pESI-like or a partial presence of gene pESI-like pattern.

Genes *ard*A, I1 relaxase, *sog*S, *trb*A, K88, Yersinia bactime operon, *ipf* and *pilL* were found in all IncFIB(K)_1_Kpn3 positive strains. The replication origin *rep*B is associated with the IncFIB(K)_1_Kpn3 plasmid. IncP was found in 94% (33/35) of strains, pESI backbone in 91% (32/35), *mer*A in 94% (33/35), *pilL* in 97% (34/35).

The pESI backbone was not detected in 3 strains, in particular in the two pESI-like $bla_{CTX\text{-}M\text{-}65}$ harboring isolates and in the only strain lacking the plasmid.

The $bla_{CTX\text{-}M\text{-}1}$ was the most detected gene conferring resistance to 3rd generation cephalosporin, followed by the $bla_{CTX\text{-}M\text{-}65}$ variant.

IncP (iterons associated with IncP plasmids), *mer*A and *ipf* were detected also in strains that didn´t carry the IncFIB(K)_1_Kpn3 plasmid.

The alignment of *S*. Infantis short read sequences revealed that all pESI-like reference genes showed more than 95% of identity except for *sog*S that displayed an identity of 94.2%.

To verify the real presence of the *sog*S gene, strains sequences were aligned against *sog*S gene primers proposed by Franco et al [3] confirming the presence of the genes in all strains harboring IncFIB(K)_1_Kpn3 plasmid via in silico PCR.

Out of all isolates, 26% (9/35) showed the presence of IncFIB(K)_1_Kpn3 plasmid but without carrying the ESBL encoding genes.

	ardA	I1 relaxase	sogS	trbA	IncP	pESI backbone	K88(fae)	Ybt	mer A	ipf	pilL	repB	blaCTX-M-1	blaCTX-M-65	blaTEM-1	IncFIB(K)
22-SA00386-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00387-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00388-0	+	+	+	+		+	+	+		+	+	+			+	+
22-SA00389-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00390-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00391-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00392-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00393-0	+	+	+	+		+	+	+		+		+	+			+
22-SA00394-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00395-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00396-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00397-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00398-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00399-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00400-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00401-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00402-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00403-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00404-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00405-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00406-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00407-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00408-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00409-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00446-0					+				+	+					+	
22-SA00447-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00448-0	+	+	+	+	+	+	+	+	+	+	+	+				+
22-SA00449-0	+	+	+	+	+		+	+	+	+	+	+		+		+
22-SA00450-0	+	+	+	+	+	+	+	+	+	+	+	+			+	+
22-SA00451-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00453-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00454-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00455-0	+	+	+	+	+		+	+	+	+	+	+		+		+
22-SA00456-0	+	+	+	+	+	+	+	+	+	+	+	+	+			+
22-SA00457-0	+	+	+	+	+	+	+	+	+	+	+	+				+

Table 6. Presence and absence of target gene used to predict the presence of pESI-like plasmid in the 35 *S*. Infantis isolated in Marche Region along the food chain.

3.3. Results of plasmids size determination via S1-PFGE

S1-PFGE analysis of the 35 *S*. Infantis strains confirmed the presence of plasmids with 224-310 kb size in 34/35 strains and the absence of any plasmid in one isolate. The presence of other plasmids was also confirmed in 10 isolates. Six Isolates that showed presence of genes of IncX incompatibility group in WGS plasmid typing, also showed bands of 33kb in S1-PFGE. Moreover 2 isolates showed presence of IncFIC and IncFII after WGS analysis and a band around 55 kb in the same 2 isolates, via S1-PFGE, were detected.

In two strains the presence of additional Col(MG828)_1 plasmids, that were detected via WGS analysis, could not be confirmed with S1-PFGE, probably due to their small size.

3.4. The cgMLST analysis

The cgMLST analysis revealed the presence of one main cluster, named Cluster 1, composed by strains isolated in environment, animal, food and human (in blue circles), based on the allele distance matrix (Fig. 1).

The majority of strains showed a pairwise genetic distance of 20 allelic difference (AD) as a maximum and belonged to the same cluster. The minimum distance matrix detected was 1 allelic difference between one environmental strain and one animal strain without *bla*$_{\text{CTX-M-1}}$ gene. Moreover, the minimal AD between a human strain and an animal strain carrying *bla*$_{\text{CTX-M-1}}$ was 3.

The strains outside the cluster are six and they didn't group together: 3 harbored plasmid carrying *bla*$_{\text{TEM-1}}$, 2 harbored plasmid carrying *bla*$_{\text{CTX-M-65}}$, 1 without plasmid and carrying *bla*$_{\text{TEM-1}}$.

The longest allelic distance of 93 AD was observed between strains outside to the Cluster 1.In particular it was detected between the only strain without a plasmid, isolated from clinical sample and a food isolate harbored *bla*$_{\text{TEM-1}}$

Excluding the only strains without the plasmid, the longest AD was 88 detected between one strains carrying *bla*$_{\text{CTX-M-65}}$ and one strains carrying *bla*$_{\text{TEM-1}}$ (distance matrix in supplement Fig.S1).

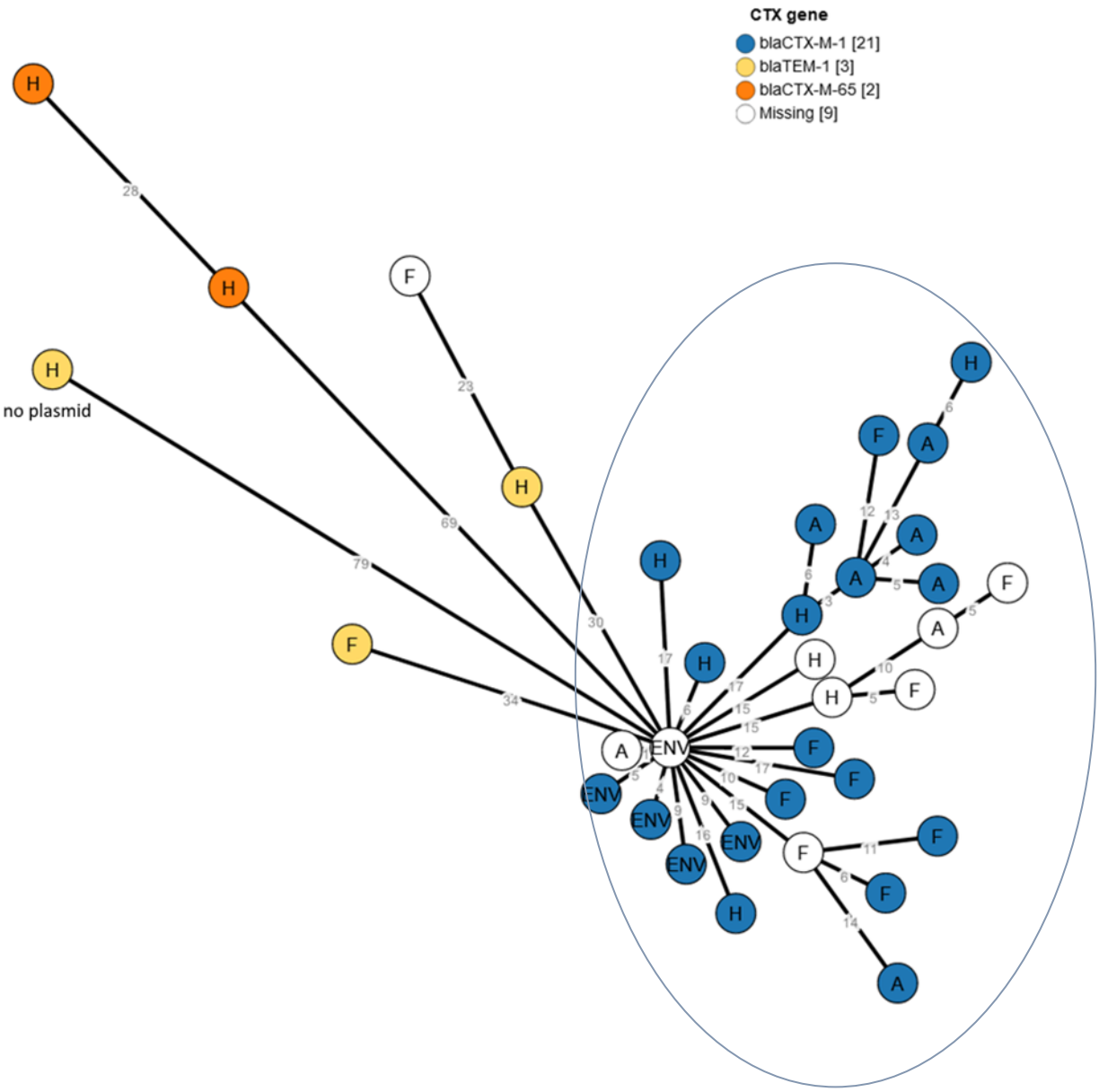


Fig. 1 Minimum spanning tree based on cgMLST analysis of 35 S. Infantis strains divided according to matrix source. Cluster 1, in blue circle, has been determined on the basis on a threshold of 20 allelic differences. Letters correspond to isolation sources: E= environment; A= animal; F= food; H= human. Allelic differences are labeld as numbers on the branches

3.5. SNP analysis

SNP analysis, based on the sequences of 35 *S*. Infantis strains, revealed a SNPs variation ranged from 1 to 314 using the internal reference 22-SA00401-0.

22-SA00401-0 was selected as internal reference because is the oldest strains harbored pESI-like plasmid carrying $bla_{\text{CTX-M-1}}$ and showed a complete pESI-like gene pattern (Table 6).

Minimum SNPs difference is 1 between two strains isolated from animal sources and 4 SNPs is the minimum distance between two strains isolated from different sources, one in animal and one in human.

The maximum SNPs distance is 314 between one strain carrying *bla*$_{TEM-1}$ and the only strain without plasmid. Excluding the only strain without plasmid, the maximum SNP distance is 286 between the same *bla*$_{TEM-1}$ strain and the strain carrying *bla*$_{CTX-M-65}$. These results confirming cgMLST analysis results where the same strains showed the maximum AD.

According to Pightling et al a SNP threshold of <21 serves as a feasible threshold and was thus selected for this study revealing a main Cluster 1 (Fig. 2) [23]. In particular, 71% (25/35) of *S*. Infantis strains were assigned to the Cluster 1 based on the SNP threshold of <21. Notably, this cluster contains strains harboring IncFIB(K)_1_Kpn3 plasmids with or without a *bla*$_{CTX-M-1}$ gene. Moreover, using SNP threshold<21, 10 strains were out the Cluster 1. Out of those, 3 isolates carried a *bla*$_{TEM-1}$ gene without any plasmid, 2 carried a *bla*$_{CTX-M-65}$, 2 carried pESI-like plasmid without ESBL/AmpC genes and 3 carried a pESI-like plasmid encoding a *bla*$_{CTX-M-1}$ gene.

The cluster 1 could be divided into 6 subclusters, showed in Table 7, with strains isolated from different sources along the food chain, using a SNPs distance of ≤10. Moreover, sub-cluster 4 highlighted the presence of strains isolated in different steps along the meat production chain from livestock to food processing laboratory.

code	subcluster	matrix	year	IncFIB(K)	*bla*$_{CTX-M}$
22-SA00387-0	1	food:meat-based bovine products	2020	+	-
22-SA00402-0	1	animal: broiler	2020	+	-
22-SA00396-0	2	food:meat-based bovine products	2021	+	-
22-SA00457-0	2	human: faeces	2021	+	-
22-SA00394-0	3	food:meat-based chicken products	2021	+	-
22-SA00395-0	3	food:meat-based chicken products	2021	+	+
22-SA00397-0	4	environment: livestock	2018	+	+
22-SA00398-0	4	environment: livestock	2018	+	+
22-SA00399-0	4	environment: livestock	2020	+	-
22-SA00400-0	4	environment: food processing laboratory	2017	+	+
22-SA00401-0	4	environment: slaughterhouse	2016	+	+
22-SA00403-0	4	animal: broiler	2020	+	-
22-SA00406-0	5	animal: broiler livestock	2020	+	+
22-SA00454-0	5	human: faeces	2021	+	+
22-SA00404-0	6	animal: broiler	2020	+	+
22-SA00407-0	6	animal: broiler	2020	+	+
22-SA00408-0	6	animal: broiler	2020	+	+
22-SA00409-0	6	animal: broiler	2020	+	+
22-SA00456-0	6	human: faeces	2021	+	+

Table 7. *S*. Infantis strains of cluster 1, divided according to the sub-cluster association defined by a SNP threshold of ≤10 .

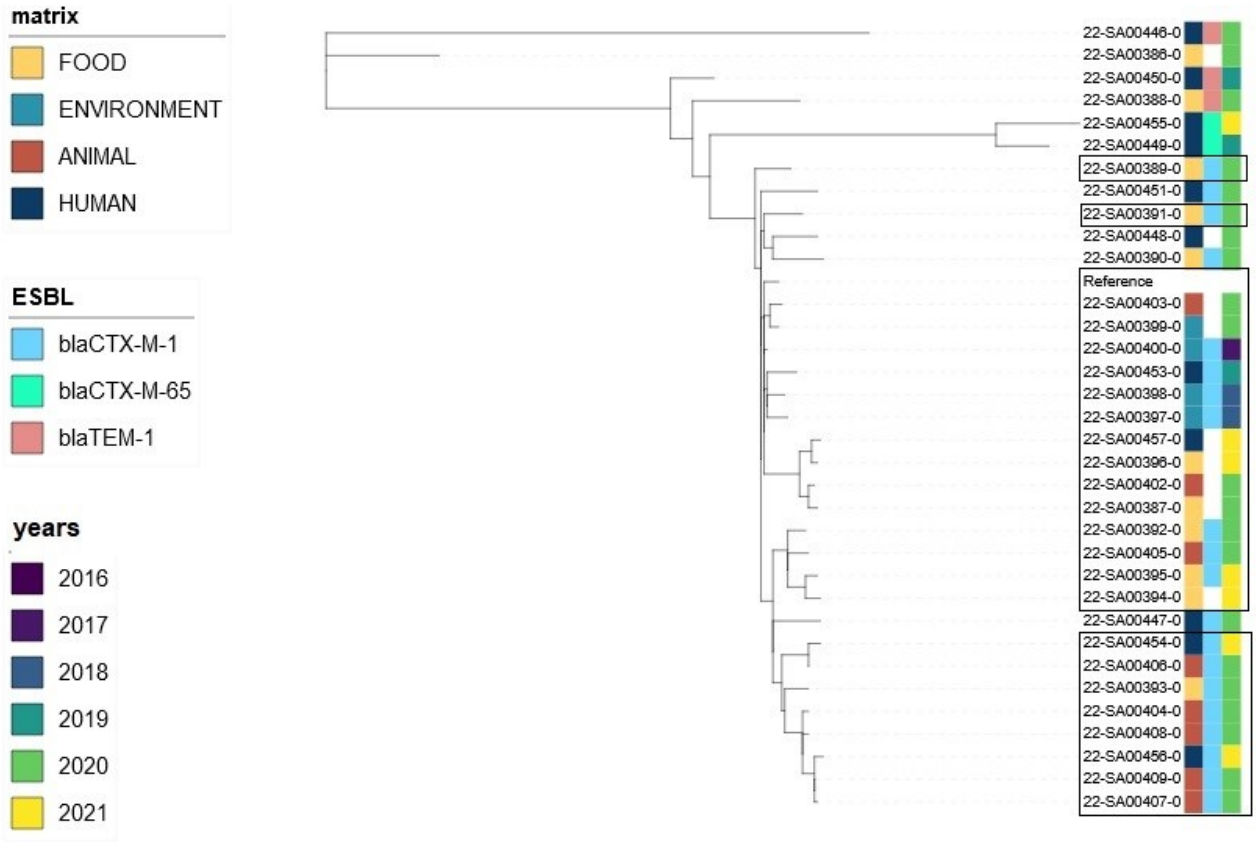


Fig.2 SNP-based phylogenetic tree based on the WGS of 35 *S*. Infantis. The strains are colored according to isolation source, ESBL content and year of isolation. Cluster 1 is highlighted in black box.

4. Discussion

S. Infantis is one of the five main causes of human salmonellosis [27]. In the last years an increase in isolation of MDR *S*. Infantis carrying *bla*$_{\text{CTX-M}}$ gene involved in 3rd generation cephalosporin resistance was noticed in the EU, including Italy [1] due to the spread of *S*. Infantis harboring a pESI-like plasmid [3].

In the present study we demonstrated the presence of *S*. Infantis pESI-like in Marche Region within strains isolated along the food chain, in particular from environment, animal, food and human samples. Moreover, an in depth molecular characterization allowed to study the possible correlation between isolated strains.

The presence of a plasmid with molecular size around 224-310 kb, thus compatible with the pESI-like, was confirmed by the S1-PFGE in 97% of samples and the WGS allowed the identification of a pESI-like gene pattern used to predict the presence of the plasmid and Inc group. In fact, two variants of S. Infantis pESI-like IncFIB(K)_1_Kpn3 were detected, one associated with the European clone carrying *bla*$_{\text{CTX-M-1}}$ [3] and the other associated with U.S. isolates carrying *bla*$_{\text{CTX-M-65}}$ [8].

Despite *bla*$_{CTX-M-1}$ is present in a large part of pESI-like plasmids [8], the absence of this gene is not associated to the absence of the plasmid [3,5,8]. The majority of our strains showed a partial or total pESI-like gene pattern, including strains without *bla*$_{CTX-M}$ gene.

In fact, within the 34 *S*. Infantis pESI-like strains not all of them carried *bla*$_{CTX-M.}$ This could depend on: (i) the loss of gene, due to the low exposition to the antibiotic [8]; (ii) the association of *bla*$_{CTX-M}$ gene with transposable elements that allow its loss and acquisition; the gene may be transposed within different strains, also of different species, improving the dissemination of 3rdgeneration cephalosporin resistance [28].

All genes used to detect the presence of pESI-like plasmid showed an identity <95% except for *sog*S, which showed high identity only in pESI-like *bla*$_{CTX-M-65.}$ Probably this gene carried by the Italian clone has different mutations in *sog*S gene comparing with the sequence proposed by Mc Millan underlining the divergent evolution from the US clone in Italian strains, confirmed also through the phylogenetic analysis in this study. However, to confirm *sog*S genes presence in isolates with less identity than 95%, the gene region was aligned with primer sequences proposed by Franco et al for the amplification of a *sog*S-specific fragment [3]. Results of this analysis showed 100% identity in the primer region without any mismatch confirming the presence of the gene.

The pESI backbone was not detected in the only two strains carrying *bla*$_{CTX-M-65}$ as already described being typical for the lineage circulating in the U.S. [8].

Notably, the two pESI-like U.S. variant *bla*$_{CTX-M-65}$ showed the presence of more ARGs , in particular *aac*(3)-IVa, *aph*(4), *floR* and *fosA3*, compared to the *bla*$_{CTX-M-1}$ EU variant [29,30].

Moreover, it was noticed that only 3 strains carried *bla*$_{TEM-1}$ which is a non-ESBL variant gene and two of them harbored an additional plasmid with IncX replicon, hinting towards the location of the *bla*$_{TEM-1}$ gene on this plasmid , as this association is already known [2].

According to Alba et al (2020), carbapenemases encoding genes were not detected [5].

Additionally, numerous virulence factors were detected. Ybt system and fimbriae coding genes, involved in the enhancement of *Salmonella* survival in adverse conditions and in the increase of the adhesion in human and animal cells, were found in the majority of strains. The Ybt operon, involved in iron acquisition, increases the possibility of *Salmonella* to growth in low iron conditions [8,31,32].

It was noticed that the only strain without the plasmid showed a lack of virulence genes as *fae*, *fyu, irp* and ybt operon [2,33] validating the association of certain virulence genes with the pESI-like plasmid.

WGS is an important tool used also by FDA to determine the genetic relatedness among isolates [23].

To estimate the similarity between two or more genomes, cgMLST can be used comparing allele profiles and calculating the total number of different alleles. Allele differences are first determined pairwise, a final distance matrix is derived by a comparison of all samples [20].

In this study, the cgMLST analysis revealed that most of the isolates belonged to one cluster that included strains isolated from different sources along the food chain, harboring pESI-like plasmid but not all strains carried $bla_{\text{CTX-M-1}}$ gene. To study in dept the phylogenetical relationship between our strains and to increase resolving power of WGS typing, SNP analysis was additionally carried out. The result verify the cgMLST analyses showing the cluster 1 grouping 25 strains isolated from different sources along the food chain. In this cluster, also isolates without the $bla_{\text{CTX-M-1}}$, were included, in line with cgMLST analysis results [2].

The majority of strains grouped together in this cluster with minimum of 1 and a maximum of 41 SNPs, regardless of the isolation source. Taking a threshold of 21 SNP, as proposed suitable for outbreaks analysis by Pightling et al (2018), we assume that the isolates from this study evolved from a common ancestor. Even using a stricter threshold of 10 SNPs, we observed 6 subclusters with isolates from different origins along the food chain. Since our strains were isolated in a restricted geographic area we assume their spread along the food chain [2,8] being responsible for their close genetic relationship. This is supported by the presence of other *S*. Infantis isolates in the outgroup of cluster 1, which also harbor the pESI like plasmids but show a genetic distance of a maximum of 314 SNPs in phylogenetic analysis. For those isolates we assume a varying origin and not the same ancestor as for the cluster 1 isolates.

According to Proietti et al (2020)[34], our results suggest that the spread could have happened in in the meat production chain, since genetically related strains were isolated in different points along the meat production line.

Food pathogen can undergo evolutionary changes in the path that they can take from farm to fork since they could acquire or loose genes or have genetic mutation, especially if it occurs over long

periods of time. For this reason, a phylogenetic study makes it possible to monitor the spread of pathogens and the correlation between them [23].

5. Conclusions

Our study reported the occurrence of *S*. Infantis pESI-like strains, carrying $bla_{\text{CTX-M-1}}$ gene responsible of resistance to 3rd generation cephalosporin, in Marche Region along the food chain. Genetic relationship of $bla_{\text{CTX-M-1}}$ *S*. Infantis harboring IncFIB(K)_1_Kpn3 megaplasmid, isolated from diverse sources, confirmed the spread of *S*. Infantis pESI-like $bla_{\text{CTX-M-1}}$ clone in some samples of the food chain of Marche Region.

The genetic homogeneity reported in our study among the majority of *S*. Infantis pESI-like that clustering together, suggested that, although *S*. Infantis population is heterogeneous in Europe, pESI-like variants are genetically similar each other. Those plasmids, once acquired, give the strains a selective advantage that enhance their colonization ability and fitness cost, and in this way their spread in other *S*. Infantis strains is facilitated [5,6,35].

In line with the WHO One Health approach [2,5], the control of the spread of *S*. Infantis pESI-like along the food chain is essential, since this clone can be resistant to 3rd generation cephalosporin considered CIAs for human health. The misuse of antibiotics or the extensive use of disinfectants, such as quaternary ammonium compounds or heavy metal used in agriculture, could increase the selection of resistant foodborne pathogens. For this reason, the monitoring of antibiotic use in livestock and the environment and the study of the mobile genetic elements involved in AMR is necessary to fight against the antimicrobial resistance threat.

SUPPLEMENTARY MATERIAL

S. Infantis code	sources	AMR profile
22-SA00386-0	FOOD	*aadA1;aph(3')-Ia;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00387-0	FOOD	*aph(3')-Ia;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00388-0	FOOD	*aph(3')-Ia;blaTEM-1;dfrA14;gyrA_D87G;mdsA;mdsB;tet(A)*
22-SA00389-0	FOOD	*aadA1;blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00390-0	FOOD	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00391-0	FOOD	*aph(3')-Ia;blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00392-0	FOOD	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00393-0	FOOD	*blaCTX-M-1;gyrA_D87G;mdsA;mdsB*
22-SA00394-0	FOOD	*dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00395-0	FOOD	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00396-0	FOOD	*aph(3')-Ia;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00397-0	ENVIRONMENT	*blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00398-0	ENVIRONMENT	*blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00399-0	ENVIRONMENT	*aph(3')-Ia;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00400-0	ENVIRONMENT	*aph(3')-Ia;blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00401-0	ENVIRONMENT	*aph(3')-Ia;blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00402-0	ANIMAL	*aph(3')-Ia;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00403-0	ANIMAL	*aph(3')-Ia;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00404-0	ANIMAL	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00405-0	ANIMAL	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00406-0	ANIMAL	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00407-0	ANIMAL	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00408-0	ANIMAL	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00409-0	ANIMAL	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00446-0	HUMAN	*ant(2'')-Ia;blaTEM-1;cmlA5;dfrA1;gyrA_D87Y;mdsA;mdsB;sul1;tet(A)*
22-SA00447-0	HUMAN	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00448-0	HUMAN	*dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00449-0	HUMAN	*aac(3)-IVa;aadA1;aph(3')-Ia;aph(4)-Ia;blaCTX-M-65;dfrA14;floR;gyrA_D87Y;mdsA;mdsB;sul1;tet(A)*
22-SA00450-0	HUMAN	*aadA1;aph(3')-Ia;blaTEM-1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00451-0	HUMAN	*blaCTX-M-1;dfrA1;gyrA_D87G;gyrA_S83Y;mdsA;mdsB;sul1;tet(A)*
22-SA00453-0	HUMAN	*blaCTX-M-1;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00454-0	HUMAN	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00455-0	HUMAN	*aac(3)-IVa;aadA1;aph(3')-Ia;aph(4)-Ia;blaCTX-M-65;dfrA14;floR;fosA3;gyrA_D87Y;mdsA;mdsB;sul1;tet(A)*
22-SA00456-0	HUMAN	*blaCTX-M-1;dfrA1;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*
22-SA00457-0	HUMAN	*aph(3')-Ia;dfrA1;dfrA14;gyrA_D87G;mdsA;mdsB;sul1;tet(A)*

Table S1 AMR profiles obtained by WGS and origin of *S*. Infantis isolates from this study. Bakcharak s was used to carry out the analysis and implement tool ncbi-amrfinderplus v3.9.3 detected antimicrobial resistance genes [17].

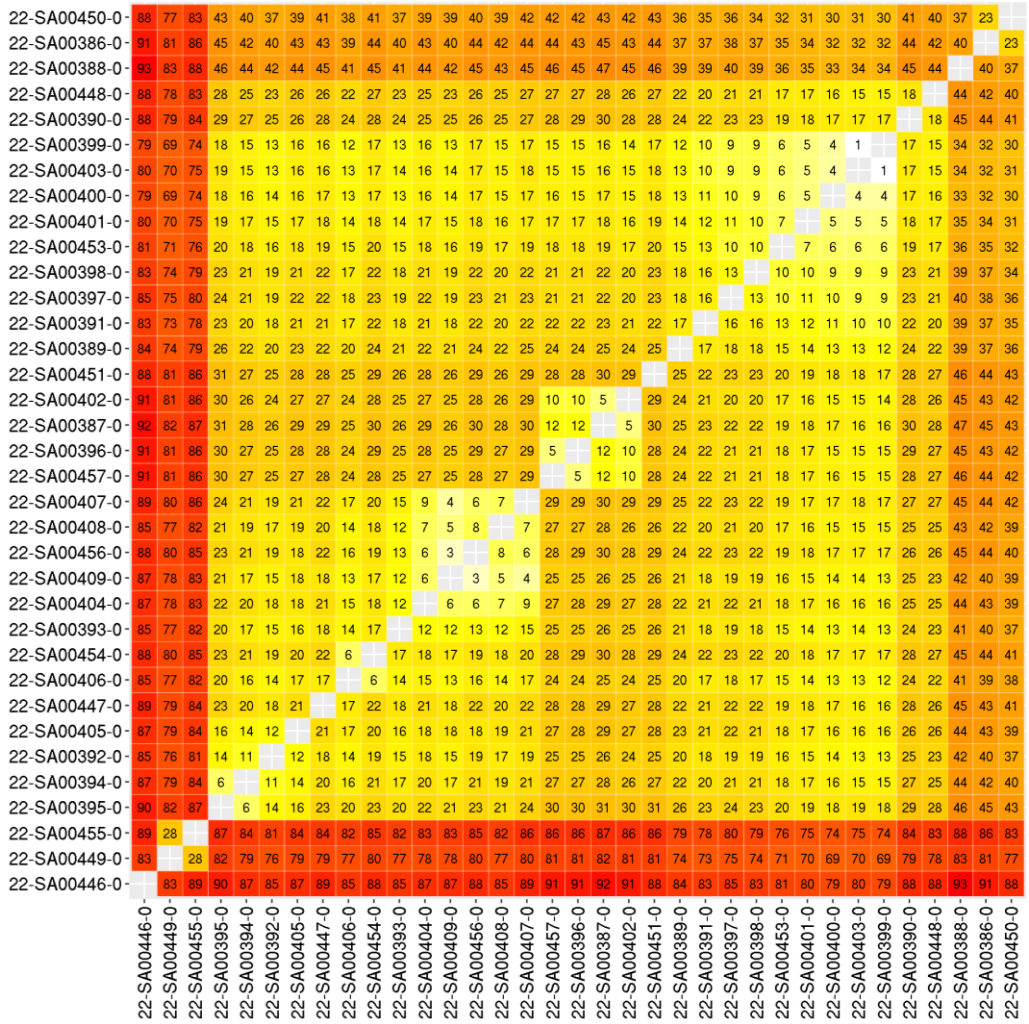


Fig. S1 Allelic differences table: the pairwise distance matrix has been clustered and colored to visualize similarity of groups of closely related strains. The chewieSnake pipeline v3.1.1 [20], which implements chewBBACA v2.0.12 [21] were used for the analysis. Allele profiles are generated and allele differences are calculated pairwise.

ADDITIONAL RESULTS

A total of 101 antibiotic resistant *Salmonella* strains of serovars *S*. Derby (n. 20), S. Typhimurium (n. 18), MVST (n. 28), and S. Infantis (n. 35) were analyzed for genes encoding resistance against 3rd generation cephalosporins (ESBL/AmpC) and carbapenems.

The AMR profile, previously investigated [26], showed that 78% (79/101) *Salmonella* strains, of serovars *S*. Derby (n. 6), S. Typhimurium (n. 17), monophasic variants of *S*. Typhimurium (MVST) (n. 28) and *S*. Infantis (n. 26) were resistant to at least one β-lactam, including ampicillin, cefotaxime and ceftazidime.

Notably, 25% (26/101) *Salmonella* strains, exhibited phenotypical resistance to 3rd generation cephalosporins, in particular, 23/26 were *S*. Infantis.

A group of 79 β-lactams resistant strains were selected for ESBL/AmpC detection by multiplex real-time PCR [12].

Real-time PCR analysis showed that 30% (24/79) of isolates carried a member of the $bla_{\text{CTX-A/B}}$ gene family, 50% (40/79) bla_{TEM} gene family and in only one strain bla_{CMY} gene variant was detected.

In detail, 6/6 *S*. Derby isolates carried the bla_{TEM} variant; 1/17 *S*. Typhimurium isolates carried $bla_{\text{CTX-A/B}}$ and 4/17 bla_{TEM}.

On the other hand, MVST strains showed a high proportion of bla_{TEM} variants (27/28) and only one strain carried the bla_{CMY} variant.

S. Infantis strains showed the highest proportion (23/26) of $bla_{\text{CTX-A/B}}$ positive strains, while 3 isolates carried bla_{TEM}.

35 S. Infantis and 28 MVST strains were selected for an in-depth molecular characterization by WGS. Results revealed that 26/35 *S*. Infantis strains carried $bla_{\text{CTX-M-1}}$ and 27 MVST carried $bla_{\text{TEM-1}}$ variant.

Moreover, 82% (25/28) MVST showed a typical ASSuT phenotype with resistance to ampicillin, streptomycin, sulphisoxazole and harbored *tet*(B) gene involved in tetracycline resistance.

Discussion

A strong correlation between serovars and ESBL/AmpC variants was noticed after multiplex real-time PCR amplification. *S*. Infantis and MVST showed the highest proportion of genes involved in beta-lactams resistance. In particular, *S*. Infantis isolates showed the presence of $bla_{\text{CTX-M-1}}$ mostly associated with *S*. Infantis pESI-like clone diffused in Europe, including Italy [3,5].

On the other hand, MVST showed a typical ASSuT phenotype, derived from a stabilization of an IncH1 plasmid integrated into the bacterial chromosome, and the presence of *tet*(B) involved in tetracycline resistance. All these characteristics were associated to the MVST ASSuT clone that spread in Europe [36,37].

FUTURE WORK

To study in dept the genetic structure of pESI-like plasmid, third generation sequence technology could be used, in particular long-reads sequencing (Illumina-Oxford Nanopore Technologies) [29]. The use of this approach allow a complete resolution of plasmid structure [38]. A future work will be to fully reconstruct the plasmid and study pESI-like plasmids found in our *S*. Infantis strains. Moreover a comparison with pESI plasmid isolated in Israel [6] and pESI-like plasmids found in our study will allow to investigate the differences and the evolution of pESI-like, along the time, in contrast with the original pESI.

Moreover, after the study of pESI-like plasmid in our S. Infantis isolates, others *Salmonella* serovar, belonging to the *Salmonella* collection composed of 102 strains isolated in Marche Region along the food chain and all of them AMR, will considered for and in dept investigation. In particular, we will continue with the investigation of the MVST and the possible spread MVST ASSuT clone that are previously sequenced with NGS approace.

102 *Salmonella* strains collection includes also: 18 *S*. Typhimurium and 20 *S*. Derby that will be characterize for the gene involved in the AMR.

References

1. Food, E.; Authority, S. The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2019–2020. *EFSA Journal* **2022**, *20*, doi:10.2903/j.efsa.2022.7209.

2. Di Marcantonio, L.; Romantini, R.; Marotta, F.; Chiaverini, A.; Zilli, K.; Abass, A.; Di Giannatale, E.; Garofolo, G.; Janowicz, A. The Current Landscape of Antibiotic Resistance of Salmonella Infantis in Italy: The Expansion of Extended-Spectrum Beta-Lactamase Producers on a Local Scale. *Frontiers in Microbiology* **2022**, *13*, 1–12, doi:10.3389/fmicb.2022.812481.

3. Franco, A.; Leekitcharoenphon, P.; Feltrin, F.; Alba, P.; Cordaro, G.; Iurescia, M.; Tolli, R.; D'Incau, M.; Staffolani, M.; Di Giannatale, E.; et al. Emergence of a Clonal Lineage of Multidrug-Resistant ESBL-Producing Salmonella Infantis Transmitted from Broilers and Broiler Meat to Humans in Italy between 2011 and 2014. *PLoS ONE* **2015**, *10*, 1–15, doi:10.1371/journal.pone.0144802.

4. EMA REVISED REFLECTION PAPER ON THE USE OF 3rd AND 4th GENERATION CEPHALOSPORINS IN FOOD PRODUCING ANIMALS IN THE EUROPEAN UNION: DEVELOPMENT OF RESISTANCE AND IMPACT ON HUMAN AND ANIMAL HEALTH DRAFT AGREED BY SAGAM (SCIENTIFIC ADVISORY GROUP ON ANTIMICROBIAL. **2009**, 1–37.

5. Alba, P.; Leekitcharoenphon, P.; Carfora, V.; Amoruso, R.; Cordaro, G.; Di Matteo, P.; Ianzano, A.; Iurescia, M.; Diaconu, E.L.; Pedersen, S.K.; et al. Molecular Epidemiology of Salmonella Infantis in Europe: Insights into the Success of the Bacterial Host and Its Parasitic Pesi-like Megaplasmid. *Microbial Genomics* **2020**, *6*, 1–12, doi:10.1099/mgen.0.000365.

6. Aviv, G.; Tsyba, K.; Steck, N.; Salmon-Divon, M.; Cornelius, A.; Rahav, G.; Grassl, G.A.; Gal-Mor, O. A Unique Megaplasmid Contributes to Stress Tolerance and Pathogenicity of an Emergent Salmonella Enterica Serovar Infantis Strain. *Environmental Microbiology* **2014**, *16*, 977–994, doi:10.1111/1462-2920.12351.

7. Vázquez, X.; Fernández, J.; Rodríguez-Lozano, J.; Calvo, J.; Rodicio, R.; Rodicio, M.R. Genomic Analysis of Two MDR Isolates of Salmonella Enterica Serovar Infantis from a Spanish Hospital Bearing the BlaCTX-M-65 Gene with or without FosA3 in PESI-like Plasmids. *Antibiotics* **2022**, *11*, 1–12, doi:10.3390/antibiotics11060786.

8. McMillan, E.A.; Wasilenko, J.L.; Tagg, K.A.; Chen, J.C.; Simmons, M.; Gupta, S.K.; Tillman, G.E.; Folster, J.; Jackson, C.R.; Frye, J.G. Carriage and Gene Content Variability of the Pesi-like Plasmid Associated with Salmonella Infantis Recently Established in United States Poultry Production. *Genes* **2020**, *11*, 1–15, doi:10.3390/genes11121516.

9. McMillan, E.A.; Weinroth, M.D.; Frye, J.G. Increased Prevalence of Salmonella Infantis Isolated from Raw Chicken and Turkey Products in the United States Is Due to a Single Clonal Lineage Carrying the PESI Plasmid. *Microorganisms* **2022**, *10*, doi:10.3390/microorganisms10071478.

10. Badescu, B.; Buda, V.; Romanescu, M.; Lombrea, A.; Danciu, C.; Dalleur, O.; Dohou, A.M.; Dumitrascu, V.; Cretu, O.; Licker, M.; et al. Current State of Knowledge Regarding WHO Critical Priority Pathogens: Mechanisms of Resistance and Proposed Solutions through Candidates Such as Essential Oils. *Plants* **2022**, *11*, doi:10.3390/plants11141789.

11. *Critically Important Antimicrobials for Human Medicine 6th Revision 2018 Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-Human Use*;

12. Roschanski, N.; Fischer, J.; Guerra, B.; Roesler, U. Development of a Multiplex Real-Time PCR for the Rapid Detection of the Predominant Beta-Lactamase Genes CTX-M, SHV, TEM and CIT-Type Ampcs in Enterobacteriaceae. *PLoS ONE* **2014**, *9*, doi:10.1371/journal.pone.0100956.

13. Swayne, R.L.; Ludlam, H.A.; Shet, V.G.; Woodford, N.; Curran, M.D. Real-Time TaqMan PCR for Rapid

Detection of Genes Encoding Five Types of Non-Metallo- (Class A and D) Carbapenemases in Enterobacteriaceae. *International Journal of Antimicrobial Agents* **2011**, *38*, 35–38, doi:10.1016/j.ijantimicag.2011.03.010.

14. Van der Zee, A.; Roorda, L.; Bosman, G.; Fluit, A.C.; Hermans, M.; Smits, P.H.M.; van der Zanden, A.G.M.; te Witt, R.; Bruijnesteijn van Coppenraet, L.E.S.; Cohen Stuart, J.; et al. Multi-Centre Evaluation of Real-Time Multiplex PCR for Detection of Carbapenemase Genes OXA-48, VIM, IMP, NDM and KPC. *BMC Infectious Diseases* **2014**, *14*, 1–5, doi:10.1186/1471-2334-14-27.

15. Roschanski, N.; Friese, A.; von Salviati- Claudius, C.; Hering, J.; Kaesbohrer, A.; Kreienbrock, L.; Roesler, U. Prevalence of Carbapenemase Producing Enterobacteriaceae Isolated from German Pig-Fattening Farms during the Years 2011–2013. *Veterinary Microbiology* **2017**, *200*, 124–129, doi:10.1016/j.vetmic.2015.11.030.

16. Deneke, C.; Brendebach, H.; Uelze, L.; Borowiak, M.; Malorny, B.; Tausch, S.H. Species-Specific Quality Control, Assembly and Contamination Detection in Microbial Isolate Sequences with Aquamis. *Genes* **2021**, *12*, doi:10.3390/genes12050644.

17. Feldgarden, M.; Brover, V.; Haft, D.H.; Prasad, A.B.; Slotta, D.J.; Tolstoy, I.; Tyson, G.H.; Zhao, S.; Hsu, C.H.; McDermott, P.F.; et al. Validating the AMRFINder Tool and Resistance Gene Database by Using Antimicrobial Resistance Genotype-Phenotype Correlations in a Collection of Isolates. *Antimicrobial Agents and Chemotherapy* **2019**, *63*, doi:10.1128/AAC.00483-19.

18. Carattoli, A.; Zankari, E.; Garciá-Fernández, A.; Larsen, M.V.; Lund, O.; Villa, L.; Aarestrup, F.M.; Hasman, H. In Silico Detection and Typing of Plasmids Using Plasmidfinder and Plasmid Multilocus Sequence Typing. *Antimicrobial Agents and Chemotherapy* **2014**, *58*, 3895–3903, doi:10.1128/AAC.02412-14.

19. Yoshida, C.E.; Kruczkiewicz, P.; Laing, C.R.; Lingohr, E.J.; Gannon, V.P.J.; Nash, J.H.E.; Taboada, E.N. The Salmonella in Silico Typing Resource (SISTR): An Open Web-Accessible Tool for Rapidly Typing and Subtyping Draft Salmonella Genome Assemblies. *PLoS ONE* **2016**, *11*, 1–17, doi:10.1371/journal.pone.0147101.

20. Deneke, C.; Uelze, L.; Brendebach, H.; Tausch, S.H.; Malorny, B. Decentralized Investigation of Bacterial Outbreaks Based on Hashed CgMLST. *Frontiers in Microbiology* **2021**, *12*, 1–12, doi:10.3389/fmicb.2021.649517.

21. Silva, M.; Machado, M.P.; Silva, D.N.; Rossi, M.; Moran-Gilad, J.; Santos, S.; Ramirez, M.; Carriço, J.A. ChewBBACA: A Complete Suite for Gene-by-Gene Schema Creation and Strain Identification. *Microbial genomics* **2018**, *4*, 1–7, doi:10.1099/mgen.0.000166.

22. Lüth, S.; Deneke, C.; Kleta, S.; Dahouk, S. Al Translatability of Wgs Typing Results Can Simplify Data Exchange for Surveillance and Control of Listeria Monocytogenes. *Microbial Genomics* **2021**, *7*, 1–12, doi:10.1099/mgen.0.000491.

23. Pightling, A.W.; Pettengill, J.B.; Luo, Y.; Baugher, J.D.; Rand, H.; Strain, E. Interpreting Whole-Genome Sequence Analyses of Foodborne Bacteria for Regulatory Applications and Outbreak Investigations. *Frontiers in Microbiology* **2018**, *9*, 1–13, doi:10.3389/fmicb.2018.01482.

24. Rodríguez, I.; Barownick, W.; Helmuth, R.; Mendoza, M.C.; Rodicio, M.R.; Schroeter, A.; Guerra, B. Extended-Spectrum β-Lactamases and AmpC β-Lactamases in Ceftiofur-Resistant Salmonella Enterica Isolates from Food and Livestock Obtained in Germany during 2003-07. *Journal of Antimicrobial Chemotherapy* **2009**, *64*, 301–309, doi:10.1093/jac/dkp195.

25. Franco, A.; Leekitcharoenphon, P.; Feltrin, F.; Alba, P.; Cordaro, G.; Iurescia, M.; Tolli, R.; D'Incau, M.; Staffolani, M.; Di Giannatale, E.; et al. Emergence of a Clonal Lineage of Multidrug-Resistant ESBL-Producing Salmonella Infantis Transmitted from Broilers and Broiler Meat to Humans in Italy between 2011 and 2014. *PLoS ONE* **2015**, *10*, doi:10.1371/journal.pone.0144802.

26. Region, M.; Russo, I.; Bencardino, D.; Napoleoni, M.; Andreoni, F.; Schiavano, F.; Baldelli, G.; Brandi, G.; Amagliani, G. Salmonella Strains among Serovars Mainly Isolated from Food. **2022**, 1–15.

27. Food, E.; Authority, S. The European Union One Health 2020 Zoonoses Report. *EFSA Journal* **2021**, *19*, doi:10.2903/j.efsa.2021.6971.

28. Irrgang, A.; Hammerl, J.A.; Falgenhauer, L.; Guiral, E.; Schmoger, S.; Imirzalioglu, C.; Fischer, J.; Guerra, B.; Chakraborty, T.; Käsbohrer, A. Diversity of CTX-M-1-Producing E. Coli from German Food Samples and Genetic Diversity of the BlaCTX-M-1 Region on IncI1 ST3 Plasmids. *Veterinary Microbiology* **2018**, *221*, 98–104, doi:10.1016/j.vetmic.2018.06.003.

29. Alba, A.P.; Carfora, V.; Feltrin, F.; Iurescia, M.; Diaconu, E.L.; Aira, E.D.; Marani, I.; Giacomi, A.; Franco, A.; Battisti, A. Evidence of Structural Rearrangements and Variability in PESI(like) Megaplasmids of S.Infantis by Resolving the PESI-like-BlaCTX-M-1 Positive Variant. **2021**.

30. Roca, I.; Akova, M.; Baquero, F.; Carlet, J.; Cavaleri, M.; Coenen, S.; Cohen, J.; Findlay, D.; Gyssens, I.; Heure, O.E.; et al. The Global Threat of Antimicrobial Resistance: Science for Intervention. *New Microbes and New Infections* **2015**, *6*, 22–29, doi:10.1016/j.nmni.2015.02.007.

31. Foley, S.L.; Lynne, A.M.; Nayak, R. Salmonella Challenges: Prevalence in Swine and Poultry and Potential Pathogenicity of Such Isolates. *Journal of animal science* **2008**, *86*, doi:10.2527/jas.2007-0464.

32. Kang, H.; Loui, C.; Clavijo, R.I.; Riley, L.W.; Lu, S. Survival Characteristics of Salmonella Enterica Serovar Enteritidis in Chicken Egg Albumen. *Epidemiology and Infection* **2006**, *134*, 967–976, doi:10.1017/S0950268806006054.

33. García-Soto, S.; Abdel-Glil, M.Y.; Tomaso, H.; Linde, J.; Methner, U. Emergence of Multidrug-Resistant Salmonella Enterica Subspecies Enterica Serovar Infantis of Multilocus Sequence Type 2283 in German Broiler Farms. *Frontiers in Microbiology* **2020**, *11*, doi:10.3389/fmicb.2020.01741.

34. Proietti, P.C.; Stefanetti, V.; Musa, L.; Zicavo, A.; Dionisi, A.M.; Bellucci, S.; Mensa, A. La; Menchetti, L.; Branciari, R.; Ortenzi, R.; et al. Genetic Profiles and Antimicrobial Resistance Patterns of Salmonella Infantis Strains Isolated in Italy in the Food Chain of Broiler Meat Production. *Antibiotics* **2020**, *9*, 1–12, doi:10.3390/antibiotics9110814.

35. Carfora, V.; Alba, P.; Leekitcharoenphon, P.; Ballarò, D.; Cordaro, G.; Matteo, P. Di; Donati, V.; Ianzano, A.; Iurescia, M.; Stravino, F.; et al. Colistin Resistance Mediated by Mcr-1 in ESBL-Producing, Multidrug Resistant Salmonella Infantis in Broiler Chicken Industry, Italy (2016-2017). *Frontiers in Microbiology* **2018**, *9*, doi:10.3389/fmicb.2018.01880.

36. García, P.; Hopkins, K.L.; García, V.; Beutlich, J.; Mendoza, M.C.; Threlfall, J.; Mevius, D.; Helmuth, R.; Rodicio, M.R.; Guerra, B. Diversity of Plasmids Encoding Virulence and Resistance Functions in Salmonella Enterica Subsp. Enterica Serovar Typhimurium Monophasic Variant 4,[5],12:I:- Strains Circulating in Europe. *PLoS ONE* **2014**, *9*, doi:10.1371/journal.pone.0089635.

37. Sun, H.; Wan, Y.; Du, P.; Bai, L. The Epidemiology of Monophasic Salmonella Typhimurium. *Foodborne Pathogens and Disease* **2020**, *17*, 87–97, doi:10.1089/fpd.2019.2676.

38. George, S.; Pankhurst, L.; Hubbard, A.; Votintseva, A.; Stoesser, N.; Sheppard, A.E.; Mathers, A.; Norris, R.; Navickaite, I.; Eaton, C.; et al. Resolving Plasmid Structures in Enterobacteriaceae Using the MinION Nanopore Sequencer: Assessment of MinION and MinION/Illumina Hybrid Data Assembly Approaches. *Microbial Genomics* **2017**, *3*, 1–8, doi:10.1099/mgen.0.000118.

CONCLUSION

The antibiotic resistance is becoming an alarming public health problem that is destined to worsen in the near future increasing morbidity and mortality. The misuse of antibiotic in veterinary medicine, in concert with human medicine, contributes to the development of antibiotic resistant or MDR microorganisms that can spread along the food chain disseminating antibiotic resistance determinants.

In conclusion, this study shows that a heterogeneous *Salmonella* serovars population colonizes the food chain with the ability to reach consumers and cause serious illnesses.

Isolates analyzed here were characterized by a high rate of resistance to a wide panel of antibiotics. In the first part, a particular attention was focused on tetracyclines resistant gene (*tet*) considering the extensive use of this agent in veterinary medicine.

As well know, mobile genetic elements are involved in the AMR spread and the presence of MDR strains carrying genetic determinants on mobile genetic elements is a matter of concern for the spread of resistance in food settings.

Our study reported the occurrence of a clone of *S*. Infantis harboring pESI-like, carrying $bla_{\text{CTX-M-1}}$ gene responsible of resistance to 3rd generation cephalosporin, in Marche Region along the food chain.

Moreover, genetic homogeneity reported in our study among the majority of $bla_{\text{CTX-M-1}}$ *S*. Infantis pESI-like, isolated from diverse sources, confirmed the spread of this clone along the food chain.

In line with the One Health approach, the control of the spread of *S*. Infantis pESI-like along the food chain is essential, since this clone can be resistant to 3rd generation cephalosporin considered CIA for human health.

The fact that the majority of *Salmonella* serovars can infect both humans and animals and was detected also in environmental samples, consolidate the use of “One Health” approach to control the spread of *Salmonella* and AMR in environment-animal-human system.

Greater coordination across all sectors of the food chain is needed to fight antibiotic resistance, and the implementation of molecular typing methods within routinary screening programs can become an important tool of the One Health preventive approach.

REFERENCES

1. DiFonzo, N.; Bordia, P. Reproduced with Permission of the Copyright Owner . Further Reproduction Prohibited Without. *Journal of Allergy and Clinical Immunology* **1998**, *130*, 556.

2. Eng, S.K.; Pusparajah, P.; Ab Mutalib, N.S.; Ser, H.L.; Chan, K.G.; Lee, L.H. Salmonella: A Review on Pathogenesis, Epidemiology and Antibiotic Resistance. *Frontiers in Life Science* **2015**, *8*, 284–293, doi:10.1080/21553769.2015.1051243.

3. Schulz, S.M.; Köhler, G.; Schütze, N.; Knauer, J.; Straubinger, R.K.; Chackerian, A.A.; Witte, E.; Wolk, K.; Sabat, R.; Iwakura, Y.; et al. Protective Immunity to Systemic Infection with Attenuated Salmonella Enterica Serovar Enteritidis in the Absence of IL-12 Is Associated with IL-23-Dependent IL-22, but Not IL-17 . *The Journal of Immunology* **2008**, *181*, 7891–7901, doi:10.4049/jimmunol.181.11.7891.

4. Food, E.; Authority, S. The European Union One Health 2020 Zoonoses Report. *EFSA Journal* **2021**, *19*, doi:10.2903/j.efsa.2021.6971.

5. Hurley, D.; McCusker, M.P.; Fanning, S.; Martins, M. Salmonella-Host Interactions - Modulation of the Host Innate Immune System. *Frontiers in Immunology* **2014**, *5*, 1–11, doi:10.3389/fimmu.2014.00481.

6. Jajere, S.M. A Review of Salmonella Enterica with Particular Focus on the Pathogenicity and Virulence Factors, Host Specificity and Adaptation and Antimicrobial Resistance Including Multidrug Resistance. *Veterinary World* **2019**, *12*, 504–521, doi:10.14202/vetworld.2019.504-521.

7. Brenner F.W, Villar R.G, Angulo F.J, Tauxe R, Swaminathan B. *Salmonella* nomenclature. *J. Clin. Microbiol.* **2000**;38(7):2465–2467, https://doi.org/10.1128/JCM.38.7.2465-2467.2000

8. Lamas, A.; Miranda, J.M.; Regal, P.; Vázquez, B.; Franco, C.M.; Cepeda, A. A Comprehensive Review of Non-Enterica Subspecies of Salmonella Enterica. *Microbiological Research* **2018**, *206*, 60–73, doi:10.1016/j.micres.2017.09.010.

9. Gal-Mor, O.; Boyle, E.C.; Grassl, G.A. Same Species, Different Diseases: How and Why Typhoidal and Non-Typhoidal Salmonella Enterica Serovars Differ. *Frontiers in Microbiology* **2014**, *5*, 1–10, doi:10.3389/fmicb.2014.00391.

10. Majowicz, S.E.; Musto, J.; Scallan, E.; Angulo, F.J.; Kirk, M.; O'Brien, S.J.; Jones, T.F.; Fazil, A.; Hoekstra, R.M. The Global Burden of Nontyphoidal Salmonella Gastroenteritis. *Clinical Infectious Diseases* 2010, *50*, 882–889.

11. Yue, M.; Schifferli, D.M. Allelic Variation in Salmonella: An Underappreciated Driver of Adaptation and Virulence. *Frontiers in Microbiology* **2013**, *4*, 1–10, doi:10.3389/fmicb.2013.00419.

12. Ohl, M.E.; Miller, S.I. Salmonella: A Model for Bacterial Pathogenesis. *Annual Review of Medicine* **2001**, *52*, 259–274, doi:10.1146/annurev.med.52.1.259.

13. Foley, S.L.; Lynne, A.M.; Nayak, R. Salmonella Challenges: Prevalence in Swine and Poultry and Potential Pathogenicity of Such Isolates. *Journal of animal science* **2008**, *86*, doi:10.2527/jas.2007-0464.

14. Sabbagh, S.C.; Forest, C.G.; Lepage, C.; Leclerc, J.M.; Daigle, F. So Similar, yet so Different: Uncovering Distinctive Features in the Genomes of Salmonella Enterica Serovars Typhimurium and Typhi. *FEMS Microbiology Letters* **2010**, *305*, 1–13, doi:10.1111/j.1574-6968.2010.01904.x.

15. Hensel, M. Evolution of Pathogenicity Islands of Salmonella Enterica. *International Journal of Medical Microbiology* **2004**, *294*, 95–102, doi:10.1016/j.ijmm.2004.06.025.

16. Coburn, B.; Grassl, G.A.; Finlay, B.B. Salmonella, the Host and Disease: A Brief Review. *Immunology and Cell Biology* **2007**, *85*, 112–118, doi:10.1038/sj.icb.7100007.

17. Lou, L.; Zhang, P.; Piao, R.; Wang, Y. Salmonella Pathogenicity Island 1 (SPI-1) and Its Complex Regulatory Network. *Frontiers in Cellular and Infection Microbiology* **2019**, *9*, 1–12, doi:10.3389/fcimb.2019.00270.

18. Van Asten, A.J.A.M.; Van Dijk, J.E. Distribution of "Classic" Virulence Factors among Salmonella Spp. *FEMS Immunology and Medical Microbiology* **2005**, *44*, 251–259, doi:10.1016/j.femsim.2005.02.002.

19. Rychlik, I.; Barrow, P.A. Salmonella Stress Management and Its Relevance to Behaviour during Intestinal Colonisation and Infection. *FEMS Microbiology Reviews* **2005**, *29*, 1021–1040, doi:10.1016/j.femsre.2005.03.005.

20. Kasturi P.K, Panigrahi D, Ganguly N.K, Nayak N, Ayyagari A, Khuller M. Enterotoxin production and mouse virulence of clinical isolates of *Salmonella* Typhimurium strains. *Indian J. Med. Res.* **1991**,*93*(1):166–170.

21. Dufresne, K.; Daigle, F. Salmonella Fimbriae: What Is the Clue to Their Hairdo? *Current Topics in Salmonella and Salmonellosis* **2017**, doi:10.5772/67189.

22. Das, C.; Mokashi, C.; Mande, S.S.; Saini, S. Dynamics and Control of Flagella Assembly in Salmonella Typhimurium. *Frontiers in Cellular and Infection Microbiology* **2018**, *8*, 1–13, doi:10.3389/fcimb.2018.00036.

23. Wang, F.; Deng, L.; Huang, F.; Wang, Z.; Lu, Q.; Xu, C. Flagellar Motility Is Critical for Salmonella Enterica Serovar Typhimurium Biofilm Development. *Frontiers in Microbiology* **2020**, *11*, 1–12, doi:10.3389/fmicb.2020.01695.

24. Barbosa, F. de O.; Freitas Neto, O.C. de; Batista, D.F.A.; Almeida, A.M. de; Rubio, M. da S.; Alves, L.B.R.; Vasconcelos, R. de O.; Barrow, P.A.; Berchieri Junior, A. Contribution of Flagella and Motility to Gut Colonisation and Pathogenicity of Salmonella Enteritidis in the Chicken. *Brazilian Journal of Microbiology* **2017**, *48*, 754–759, doi:10.1016/j.bjm.2017.01.012.

25. Zamora-Sanabria, R.; Alvarado, A.M. Preharvest Salmonella Risk Contamination and the Control Strategies. *Current Topics in Salmonella and Salmonellosis* **2017**, doi:10.5772/67399.

26. Brenneman, K.E.; Willingham, C.; Kong, W.; Curtiss, R.; Roland, K.L. Low-Ph Rescue of Acid-Sensitive Salmonella Enterica Serovar Typhi Strains by a Rhamnose-Regulated Arginine Decarboxylase System. *Journal of Bacteriology* **2013**, *195*, 3062–3072, doi:10.1128/JB.00104-13.

27. Takaya, A.; Suzuki, M.; Matsui, H.; Tomoyasu, T.; Sashinami, H.; Nakane, A.; Yamamoto, T. Lon, a Stress-Induced ATP-Dependent Protease, Is Critically Important for Systemic Salmonella Enterica Serovar Typhimurium Infection of Mice. *Infection and Immunity* **2003**, *71*, 690–696, doi:10.1128/IAI.71.2.690-696.2003.

28. Monack, D.M.; Mueller, A.; Falkow, S. Persistent Bacterial Infections: The Interface of the Pathogen and the Host Immune System. *Nature Reviews Microbiology* **2004**, *2*, 747–765, doi:10.1038/nrmicro955.

29. Urdaneta, V.; Casadesús, J. Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts. *Frontiers in Medicine* **2017**, *4*, 1–13, doi:10.3389/fmed.2017.00163.

30. Connor, B.A.; Schwartz, E. Typhoid and Paratyphoid Fever in Travellers. *The Lancet Infectious Diseases* **2005**, *5*, 623–628, doi:10.1016/S1473-3099(05)70239-5.

31. Patel, T.A.; Armstrong, M.; Morris-Jones, S.D.; Wright, S.G.; Doherty, T. Imported Enteric Fever: Case Series from the Hospital for Tropical Diseases, London, United Kingdom. *American Journal of Tropical Medicine and Hygiene* **2010**, *82*, 1121–1126, doi:10.4269/ajtmh.2010.10-0007.

32. Kuvandik, C.; Karaoglan, I.; Namiduru, M.; Baydar, I. Predictive Value of Clinical and Laboratory Findings in the Diagnosis of the Enteric Fever. *New Microbiologica* **2009**, *32*, 25–30.

33. Gal-Mor, O. Persistent Infection and Long-Term Carriage of Typhoidal and Nontyphoidal Salmonellae. **2018**, doi:10.1128/CMR.

34. Ehuwa, O.; Jaiswal, A.K.; Jaiswal, S. Salmonella, Food Safety and Food Handling Practices. *Foods* **2021**, *10*, doi:10.3390/foods10050907.

35. Keithlin, J.; Sargeant, J.M.; Thomas, M.K.; Fazil, A. Systematic Review and Meta-Analysis of the Proportion of Non-Typhoidal Salmonella Cases That Develop Chronic Sequelae. *Epidemiology and Infection* **2014**, *760*, 1333–1351, doi:10.1017/S0950268814002829.

36. Haselbeck, A.H.; Panzner, U.; Im, J.; Baker, S.; Meyer, C.G.; Marks, F. Current Perspectives on Invasive Nontyphoidal Salmonella Disease. *Current Opinion in Infectious Diseases* 2017, *30*, 498–503.

37. Ao, T.T.; Feasey, N.A.; Gordon, M.A.; Keddy, K.H.; Angulo, F.J.; Crump, J.A. Global Burden of Invasive Nontyphoidal Salmonella Disease, 2010. *Emerging Infectious Diseases* **2015**, *21*, 941–949, doi:10.3201/eid2106.140999.

38. Guiney, D.G.; Fierer, J. The Role of the Spv Genes in Salmonella Pathogenesis. *Frontiers in Microbiology* **2011**, *2*, 1–10, doi:10.3389/fmicb.2011.00129.

39. Lesnick, M.L.; Reiner, N.E.; Fierer, J.; Guiney, D.G. The Salmonella SpvB Virulence Gene Encodes an Enzyme That ADP-Ribosylates Actin and Destabilizes the Cytoskeleton of Eukaryotic Cells. *Molecular Microbiology* **2001**, *39*, 1464–1470, doi:10.1046/j.1365-2958.2001.02360.x.

40. Haneda, T.; Ishii, Y.; Shimizu, H.; Ohshima, K.; Iida, N.; Danbara, H.; Okada, N. Salmonella Type III Effector SpvC, a Phosphothreonine Lyase, Contributes to Reduction in Inflammatory Response during Intestinal Phase of Infection. *Cellular Microbiology* **2012**, *14*, 485–499, doi:10.1111/j.1462-5822.2011.01733.x.

41. Grabe, G.J.; Zhang, Y.; Przydacz, M.; Rolhion, N.; Yang, Y.; Pruneda, J.N.; Komander, D.; Holden, D.W.; Hare, S.A. The Salmonella Effector SpvD Is a Cysteine Hydrolase with a Serovar-Specific Polymorphism Influencing Catalytic Activity, Suppression of Immune Responses, and Bacterial Virulence. *Journal of Biological Chemistry* **2016**, *291*, 25853–25863, doi:10.1074/jbc.M116.752782.

42. Gonzalez-Escobedo, G.; Marshall, J.M.; Gunn, J.S. Chronic and Acute Infection of the Gall Bladder by Salmonella Typhi: Understanding the Carrier State. *Nature Reviews Microbiology* **2011**, *9*, 9–14, doi:10.1038/nrmicro2490.

43. Hohmann, E.L. Nontyphoidal Salmonellosis. *Clinical Infectious Diseases* **2001**, *32*, 263–269, doi:10.1086/318457.

44. Besser, J.M. Salmonella Epidemiology: A Whirlwind of Change. *Food Microbiology* **2018**, *71*, 55–59, doi:10.1016/j.fm.2017.08.018.

45. Lee, W.H.; Jeon, Y.; Chung, J.S.; Shim, W.J.; Shin, M.J.; Han, S.G.; Baek, S.; Kim, J.; Kim, J.; Chung, P.S. Global Genomic Variations of Pathogenic Bacteria <I>Salmonella</I> Reading. *Computational Molecular Bioscience* **2021**, *11*, 57–68, doi:10.4236/cmb.2021.113004.

46. World Health Organization. WHO estimates of the global burden of foodborne diseases: foodborne

disease burden epidemiology reference group 2007-2015, **2015**,World Health Organization.

47. Linam, W.M.; Gerber, M.A. Changing Epidemiology and Prevention of Salmonella Infections. *Pediatric Infectious Disease Journal* **2007**, *26*, 747–748, doi:10.1097/INF.0b013e3181376abc.

48. Koutsoumanis, K.; Allende, A.; Álvarez-Ordóñez, A.; Bolton, D.; Bover-Cid, S.; Chemaly, M.; Davies, R.; De Cesare, A.; Herman, L.; Hilbert, F.; et al. Role Played by the Environment in the Emergence and Spread of Antimicrobial Resistance (AMR) through the Food Chain. *EFSA Journal* **2021**, *19*, doi:10.2903/j.efsa.2021.6651.

49. Centro di Referenza Nazionale per le Salmonellosi Enter-Vet Report Dati 2018. **2018**, 1–33.

50. *WHO List of Critically Important Antimicrobials for Human Medicine (WHO CIA List)*; **2019**.

51. Franco, A.; Leekitcharoenphon, P.; Feltrin, F.; Alba, P.; Cordaro, G.; Iurescia, M.; Tolli, R.; D'Incau, M.; Staffolani, M.; Di Giannatale, E.; et al. Emergence of a Clonal Lineage of Multidrug-Resistant ESBL-Producing Salmonella Infantis Transmitted from Broilers and Broiler Meat to Humans in Italy between 2011 and 2014. *PLoS ONE* **2015**, *10*, 1–15, doi:10.1371/journal.pone.0144802.

52. Collignon, P.C.; Conly, J.M.; Andremont, A.; McEwen, S.A.; Aidara-Kane, A.; Griffin, P.M.; Agerso, Y.; Dang Ninh, T.; Donado-Godoy, P.; Fedorka-Cray, P.; et al. World Health Organization Ranking of Antimicrobials According to Their Importance in Human Medicine: A Critical Step for Developing Risk Management Strategies to Control Antimicrobial Resistance from Food Animal Production. *Clinical Infectious Diseases* **2016**, *63*, 1087–1093, doi:10.1093/cid/ciw475.

53. Anderson, A.D.; Nelson, J.M.; Rossiter, S.; Angulo, F.J. Public Health Consequences of Use of Antimicrobial Agents in Food Animals in the United States. *Microbial Drug Resistance* **2003**, *9*, 373–379, doi:10.1089/107662903322762815.

54. Lathers, C.M. Risk Assessment in Regulatory Policy Making for Human and Veterinary Public Health. *Journal of Clinical Pharmacology* **2002**, *42*, 846–866, doi:10.1177/009127002401102768.

55. Wierup, M. The Control of Microbial Diseases in Animals: Alternatives to the Use of Antibiotics. *International Journal of Antimicrobial Agents* **2000**, *14*, 315–319, doi:10.1016/S0924-8579(00)00143-6.

56. Regulation (EC) No 1831/2003 of the European Parliament and of the Council of 22 September 2003 on additives for use in animal nutrition. OJ L 268, 18.10.2003, p. 29.

57. Roca, I.; Akova, M.; Baquero, F.; Carlet, J.; Cavaleri, M.; Coenen, S.; Cohen, J.; Findlay, D.; Gyssens, I.; Heure, O.E.; et al. The Global Threat of Antimicrobial Resistance: Science for Intervention. *New Microbes and New Infections* **2015**, *6*, 22–29, doi:10.1016/j.nmni.2015.02.007.

58. Naziri, Z.; Poormaleknia, M.; Ghaedi Oliyaei, A. Risk of Sharing Resistant Bacteria and/or Resistance Elements between Dogs and Their Owners. *BMC Veterinary Research* **2022**, *18*, 1–8, doi:10.1186/s12917-022-03298-1.

59. Gambino, D.; Gargano, V.; Butera, G.; Sciortino, S.; Pizzo, M.; Oliveri, G.; Cardamone, C.; Piraino, C.; Cassata, G.; Vicari, D.; et al. Food Is Reservoir of MDR Salmonella: Prevalence of ESBLs Profiles and Resistance Genes in Strains Isolated from Food. *Microorganisms* **2022**, *10*, 4–11, doi:10.3390/microorganisms10040780.

60. Castro-Vargas, R.E.; Herrera-Sánchez, M.P.; Rodríguez-Hernández, R.; Rondón-Barragán, I.S. Antibiotic Resistance in Salmonella Spp. Isolated from Poultry: A Global Overview. *Veterinary World* **2020**, *13*, 2070–2084, doi:10.14202/vetworld.2020.2070-2084.

61. Castanon, J.I.R. History of the Use of Antibiotic as Growth Promoters in European Poultry Feeds. *Poultry Science* **2007**, *86*, 2466–2471, doi:10.3382/ps.2007-00249.

62. European Medicines Agency; Agency, E.M. *Sales of Veterinary Antimicrobial Agents in 31 European Countries in 2017 (EMA/294674/2019)*; **2019**; ISBN 9789291550685.

63. *Piano Nazionale Di Contrasto de Ll ' Antimicrobico -Resistenza (PNCAR)*; **2020**; ISBN 9789241564748.

64. Bennett, P.M. Plasmid Encoded Antibiotic Resistance: Acquisition and Transfer of Antibiotic Resistance Genes in Bacteria. *British Journal of Pharmacology* **2008**, *153*, 347–357, doi:10.1038/sj.bjp.0707607.

65. Frost, L.S.; Leplae, R.; Summers, A.O.; Toussaint, A. Mobile Genetic Elements: The Agents of Open Source Evolution. *Nature Reviews Microbiology* **2005**, *3*, 722–732, doi:10.1038/nrmicro1235.

66. San Millan, A. Evolution of Plasmid-Mediated Antibiotic Resistance in the Clinical Context. *Trends in Microbiology* **2018**, *26*, 978–985, doi:10.1016/j.tim.2018.06.007.

67. Goyal, G.; Ekedahl, E.; Nyblom, M.; Krog, J.; Fröbrant, E.; Brander, M.; Sewunet, T.; Tangkoskul, T.; Giske, C.G.; Sandegren, L.; et al. A Simple Cut and Stretch Assay to Detect Antimicrobial Resistance Genes on Bacterial Plasmids by Single-Molecule Fluorescence Microscopy. *Scientific Reports* **2022**, *12*, 1–12, doi:10.1038/s41598-022-13315-w.

68. Virolle, C.; Goldlust, K.; Djermoun, S.; Bigot, S.; Lesterlin, C. Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level. *Genes* **2020**, *11*, 1–33, doi:10.3390/genes11111239.

69. Brolund, A. Overview of ESBL-Producing Enterobacteriaceae from a Nordic Perspective. *Infection Ecology and Epidemiology* **2014**, *4*, 1–9, doi:10.3402/iee.v4.24555.

70. Madec, J.Y.; Haenni, M. Antimicrobial Resistance Plasmid Reservoir in Food and Food-Producing Animals. *Plasmid* **2018**, *99*, 72–81, doi:10.1016/j.plasmid.2018.09.001.

71. Francia, M.V.; Varsaki, A.; Garcillán-Barcia, M.P.; Latorre, A.; Drainas, C.; De La Cruz, F. A Classification Scheme for Mobilization Regions of Bacterial Plasmids. *FEMS Microbiology Reviews* **2004**, *28*, 79–100, doi:10.1016/j.femsre.2003.09.001.

72. Villa, L.; García-Fernández, A.; Fortini, D.; Carattoli, A. Replicon Sequence Typing of IncF Plasmids Carrying Virulence and Resistance Determinants. *Journal of Antimicrobial Chemotherapy* **2010**, *65*, 2518–2529, doi:10.1093/jac/dkq347.

73. Carattoli, A.; Bertini, A.; Villa, L.; Falbo, V.; Hopkins, K.L.; Threlfall, E.J. Identification of Plasmids by PCR-Based Replicon Typing. *Journal of Microbiological Methods* **2005**, *63*, 219–228, doi:10.1016/j.mimet.2005.03.018.

74. Carattoli, A. Resistance Plasmid Families in Enterobacteriaceae. *Antimicrobial Agents and Chemotherapy* 2009, *53*, 2227–2238.

75. Carloni, E.; Andreoni, F.; Omiccioli, E.; Villa, L.; Magnani, M.; Carattoli, A. Comparative Analysis of the Standard PCR-Based Replicon Typing (PBRT) with the Commercial PBRT-KIT. *Plasmid* **2017**, *90*, 10–14, doi:10.1016/j.plasmid.2017.01.005.

76. Carattoli, A.; Zankari, E.; Garciá-Fernández, A.; Larsen, M.V.; Lund, O.; Villa, L.; Aarestrup, F.M.; Hasman, H. In Silico Detection and Typing of Plasmids Using Plasmidfinder and Plasmid Multilocus Sequence Typing. *Antimicrobial Agents and Chemotherapy* **2014**, *58*, 3895–3903, doi:10.1128/AAC.02412-14.

77. García-Fernández, A.; Fortini, D.; Veldman, K.; Mevius, D.; Carattoli, A. Characterization of Plasmids Harbouring QnrS1, QnrB2 and QnrB19 Genes in Salmonella. *Journal of Antimicrobial Chemotherapy* **2009**, *63*, 274–281, doi:10.1093/jac/dkn470.

78. García-Fernández, A.; Villa, L.; Carta, C.; Venditti, C.; Giordano, A.; Venditti, M.; Mancini, C.; Carattoli, A. Klebsiella Pneumoniae ST258 Producing KPC-3 Identified in Italy Carries Novel Plasmids and OmpK36/OmpK35 Porin Variants. *Antimicrobial Agents and Chemotherapy* **2012**, *56*, 2143–2145, doi:10.1128/AAC.05308-11.

79. Babakhani, S.; Oloomi, M. Transposons: The Agents of Antibiotic Resistance in Bacteria. *Journal of Basic Microbiology* **2018**, *58*, 905–917, doi:10.1002/jobm.201800204.

80. Bennett, P.M.; Livesey, C.T.; Nathwani, D.; Reeves, D.S.; Saunders, J.R.; Wise, R. An Assessment of the Risks Associated with the Use of Antibiotic Resistance Genes in Genetically Modified Plants: Report of the Working Party of the British Society for Antimicrobial Chemotherapy. *Journal of Antimicrobial Chemotherapy* **2004**, *53*, 418–431, doi:10.1093/jac/dkh087.

81. Akrami, F.; Rajabnia, M.; Pournajaf, A. Resistance Integrons; A Mini Review. *Caspian Journal of Internal Medicine* **2019**, *10*, 370–376, doi:10.22088/cjim.10.4.370.

82. Domingues, S.; da Silva, G.J.; Nielsen, K.M. Integrons. *Mobile Genetic Elements* **2012**, *2*, 211–223, doi:10.4161/mge.22967.

83. Rowe-Magnus, D.A.; Mazel, D. The Role of Integrons in Antibiotic Resistance Gene Capture. *International Journal of Medical Microbiology* **2002**, *292*, 115–125, doi:10.1078/1438-4221-00197.

84. Abraham, E.P. The Antibiotics. *Comprehensive Biochemistry* **1963**, *11*, 181–224, doi:10.1016/B978-1-4831-9711-1.50022-3.

85. Zaman, S. Bin; Hussain, M.A.; Nye, R.; Mehta, V.; Mamun, K.T.; Hossain, N. A Review on Antibiotic Resistance: Alarm Bells Are Ringing. *Cureus* **2017**, *9*, doi:10.7759/cureus.1403.

86. Hernando-Amado, S.; Coque, T.M.; Baquero, F.; Martínez, J.L. Defining and Combating Antibiotic Resistance from One Health and Global Health Perspectives. *Nature Microbiology* **2019**, *4*, 1432–1442, doi:10.1038/s41564-019-0503-9.

87. Zinsstag, J.; Schelling, E.; Waltner-Toews, D.; Tanner, M. From “One Medicine” to “One Health” and Systemic Approaches to Health and Well-Being. *Preventive Veterinary Medicine* **2011**, *101*, 148–156, doi:10.1016/j.prevetmed.2010.07.003.

88. Ramankutty, N.; Mehrabi, Z.; Waha, K.; Jarvis, L.; Kremen, C.; Herrero, M.; Rieseberg, L.H. Trends in Global Agricultural Land Use: Implications for Environmental Health and Food Security. *Annual Review of Plant Biology* **2018**, *69*, 789–815, doi:10.1146/annurev-arplant-042817-040256.

89. Pehrsson, E.C.; Tsukayama, P.; Patel, S.; Mejía-Bautista, M.; Sosa-Soto, G.; Navarrete, K.M.; Calderon, M.; Cabrera, L.; Hoyos-Arango, W.; Bertoli, M.T.; et al. Interconnected Microbiomes and Resistomes in Low-Income Human Habitats. *Nature* **2016**, *533*, 212–216, doi:10.1038/nature17672.

90. Salerno, B.; Furlan, M.; Sabatino, R.; Di Cesare, A.; Leati, M.; Volanti, M.; Barco, L.; Orsini, M.; Losasso, C.; Cibin, V. Antibiotic Resistance Genes Load in an Antibiotic Free Organic Broiler Farm. *Poultry Science* **2022**, *101*, 101675, doi:10.1016/j.psj.2021.101675.

91. Farooq, M.; Smoglica, C.; Ruffini, F.; Soldati, L.; Marsilio, F.; Di Francesco, C.E. Antibiotic Resistance Genes Occurrence in Conventional and Antibiotic-Free Poultry Farming, Italy. *Animals* **2022**, *12*, doi:10.3390/ani12182310.

92. Kumarasamy, K.K.; Toleman, M.A.; Walsh, T.R.; Bagaria, J.; Butt, F.; Balakrishnan, R.; Chaudhary, U.; Doumith, M.; Giske, C.G.; Irfan, S.; et al. Emergence of a New Antibiotic Resistance Mechanism in India, Pakistan, and the UK: A Molecular, Biological, and Epidemiological Study. *The Lancet Infectious Diseases* **2010**, *10*, 597–602, doi:10.1016/S1473-3099(10)70143-2.

93. European Union (EU). Commission Implementing Decision (EU) 2020/1729 of 17 November 2020 on the Monitoring and Reporting of Antimicrobial Resistance in Zoonotic and Commensal Bacteria and

Repealing Implementing Decision 2013/652/EU (Notified under Document C(2020) 7894) (Only the English Version Is Authentic). Available online: https://eur-lex.europa.eu/legal-content/en/TXT/?uri=CELEX%3A32020D1729.

94. Food, E.; Authority, S. The European Union Summary Report on Antimicrobial Resistance in Zoonotic and Indicator Bacteria from Humans, Animals and Food in 2019–2020. *EFSA Journal* **2022**, *20*, doi:10.2903/j.efsa.2022.7209.

95. Ministero della Salute Piano Nazionale Di Controllo Delle Salmonellosi Nei Riproduttori. **2016**, *1*, 1–23.

96. Nógrády, N.; Király, M.; Davies, R.; Nagy, B. Multidrug Resistant Clones of Salmonella Infantis of Broiler Origin in Europe. *International Journal of Food Microbiology* **2012**, *157*, 108–112, doi:10.1016/j.ijfoodmicro.2012.04.007.

97. Acar, J.F.; Moulin, G. Antimicrobial Resistance at Farm Level. *Revue scientifique et technique (International Office of Epizootics)* **2006**, *25*, 775–792.

98. Founou, L.L.; Founou, R.C.; Essack, S.Y. Antibiotic Resistance in the Food Chain: A Developing Country-Perspective. *Frontiers in Microbiology* 2016, *7*.

99. WFS, 1996 (and 2002) FOOD AND AGRICULTURE ORGANISATION OF THE UNITED NATIONS (FAO) World Food Summit: Five Years' Later (Rome, 10 to 13 June 2002) The World Food Summit: Five Years Later ("WFS: **2002**, 12–14.

100. Verraes, C.; Van Boxstael, S.; Van Meervenne, E.; Van Coillie, E.; Butaye, P.; Catry, B.; de Schaetzen, M.A.; Van Huffel, X.; Imberechts, H.; Dierick, K.; et al. Antimicrobial Resistance in the Food Chain: A Review. *International Journal of Environmental Research and Public Health* 2013, *10*, 2643–2669.

101. Chang, Q.; Wang, W.; Regev-Yochay, G.; Lipsitch, M.; Hanage, W.P. Antibiotics in Agriculture and the Risk to Human Health: How Worried Should We Be? *Evolutionary Applications* **2015**, *8*, 240–247, doi:10.1111/eva.12185.

102. Price, L.B.; Stegger, M.; Hasman, H.; Aziz, M.; Larsen, J.; Andersen, P.S.; Pearson, T.; Waters, A.E.; Foster, J.T.; Schupp, J.; et al. Staphylococcus Aureus CC398: Host Adaptation and Emergence of Methicillin Resistance in Livestock. *mBio* **2012**, *3*, 1–6, doi:10.1128/mBio.00305-11.

103. Vanderhaeghen, W.; Dewulf, J. Antimicrobial Use and Resistance in Animals and Human Beings. *The Lancet Planetary Health* **2017**, *1*, e307–e308, doi:10.1016/S2542-5196(17)30142-0.

104. Nair, D.V.T.; Venkitanarayanan, K.; Johny, A.K. Antibiotic-Resistant Salmonella in the Food Supply and the Potential Role of Antibiotic Alternatives for Control. *Foods* **2018**, *7*(10),167.

105. Alali, W.Q.; Thakur, S.; Berghaus, R.D.; Martin, M.P.; Gebreyes, W.A. Prevalence and Distribution of Salmonella in Organic and Conventional Broiler Poultry Farms. *Foodborne Pathogens and Disease* **2010**, *7*, 1363–1371, doi:10.1089/fpd.2010.0566.

106. Gebreyes, W.A.; Thakur, S.; Morrow, W.E.M. Comparison of Prevalence, Antimicrobial Resistance, and Occurrence of Multidrug-Resistant Salmonella in Antimicrobial-Free and Conventional Pig Production. *Journal of Food Protection* **2006**, *69*, 743–748, doi:10.4315/0362-028X-69.4.743.

107. Thakur, S.; Tadesse, D.A.; Morrow, M.; Gebreyes, W.A. Occurrence of Multidrug Resistant Salmonella in Antimicrobial-Free (ABF) Swine Production Systems. *Veterinary Microbiology* **2007**, *125*, 362–367, doi:10.1016/j.vetmic.2007.05.025.

108. Done, H.Y.; Venkatesan, A.K.; Halden, R.U. Does the Recent Growth of Aquaculture Create Antibiotic Resistance Threats Different from Those Associated with Land Animal Production in Agriculture? *AAPS Journal* **2015**, *17*, 513–524, doi:10.1208/s12248-015-9722-z.

109. Guillon, F.; Chasset, P.; Le Hello, S.; Granier, S.A. Investigation Épidémiologique Du Premier Foyer Lié à Salmonella Kentucky Hautement Résistante Aux Fluoroquinolones Détecté En Élevage Avicole En France | Bulletin Épidémiologique. *Bulletin épidémiologique, santé animale et alimentation* **2013**, *57*, 22–23.

110. Wu, X.; Suksawat, F.; Richards, A.L.; Phommachanh, S.; Phongaran, D.; Angkititrakul, S. Evaluation of the Containment of Antimicrobial-Resistant Salmonella Species from a Hazard Analysis and Critical Control Point (HACCP) and a Non-HACCP Pig Slaughterhouses in Northeast Thailand. *Pathogens* **2020**, *9*, doi:10.3390/pathogens9010020.

111. Di Marcantonio, L.; Romantini, R.; Marotta, F.; Chiaverini, A.; Zilli, K.; Abass, A.; Di Giannatale, E.; Garofolo, G.; Janowicz, A. The Current Landscape of Antibiotic Resistance of Salmonella Infantis in Italy: The Expansion of Extended-Spectrum Beta-Lactamase Producers on a Local Scale. *Frontiers in Microbiology* **2022**, *13*, 1–12, doi:10.3389/fmicb.2022.812481.

112. EMA REVISED REFLECTION PAPER ON THE USE OF 3rd AND 4th GENERATION CEPHALOSPORINS IN FOOD PRODUCING ANIMALS IN THE EUROPEAN UNION: DEVELOPMENT OF RESISTANCE AND IMPACT ON HUMAN AND ANIMAL HEALTH DRAFT AGREED BY SAGAM (SCIENTIFIC ADVISORY GROUP ON ANTIMICROBIAL. **2009**, 1–37.

113. Alba, P.; Leekitcharoenphon, P.; Carfora, V.; Amoruso, R.; Cordaro, G.; Di Matteo, P.; Ianzano, A.; Iurescia, M.; Diaconu, E.L.; Pedersen, S.K.; et al. Molecular Epidemiology of Salmonella Infantis in Europe: Insights into the Success of the Bacterial Host and Its Parasitic Pesi-like Megaplasmid. *Microbial Genomics* **2020**, *6*, 1–12, doi:10.1099/mgen.0.000365.

114. Aviv, G.; Tsyba, K.; Steck, N.; Salmon-Divon, M.; Cornelius, A.; Rahav, G.; Grassl, G.A.; Gal-Mor, O. A Unique Megaplasmid Contributes to Stress Tolerance and Pathogenicity of an Emergent Salmonella Enterica Serovar Infantis Strain. *Environmental Microbiology* **2014**, *16*, 977–994, doi:10.1111/1462-2920.12351.

115. McMillan, E.A.; Weinroth, M.D.; Frye, J.G. Increased Prevalence of Salmonella Infantis Isolated from Raw Chicken and Turkey Products in the United States Is Due to a Single Clonal Lineage Carrying the PESI Plasmid. *Microorganisms* **2022**, *10*, doi:10.3390/microorganisms10071478.

116. Vázquez, X.; Fernández, J.; Rodríguez-Lozano, J.; Calvo, J.; Rodicio, R.; Rodicio, M.R. Genomic Analysis of Two MDR Isolates of Salmonella Enterica Serovar Infantis from a Spanish Hospital Bearing the BlaCTX-M-65 Gene with or without FosA3 in PESI-like Plasmids. *Antibiotics* **2022**, *11*, 1–12, doi:10.3390/antibiotics11060786.

117. Badescu, B.; Buda, V.; Romanescu, M.; Lombrea, A.; Danciu, C.; Dalleur, O.; Dohou, A.M.; Dumitrascu, V.; Cretu, O.; Licker, M.; et al. Current State of Knowledge Regarding WHO Critical Priority Pathogens: Mechanisms of Resistance and Proposed Solutions through Candidates Such as Essential Oils. *Plants* **2022**, *11*, doi:10.3390/plants11141789.

118. World Health Organization. Critically Important Antimicrobials for Human Medicine 6th Revision. Ranking of Medically Important Antimicrobials for Risk Management of Antimicrobial Resistance Due to Non-Human Use, **2018**.