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Characteristics of clinical Nontyphoidal *Salmonella* isolates from a Historical Biobank with a focus on the invasive serotype Choleraesuis, Lombardy Region (2001-2016)

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ABSTRACT

Introduction. *Salmonella enterica* is a leading cause of foodborne infections worldwide, with clinical presentations ranging from self-limiting gastroenteritis to invasive systemic disease. While typhoidal serotypes are well recognized as agents of enteric fever, a subset of nontyphoidal serotypes also displays an unusually high invasiveness index, posing a major challenge for surveillance and control. In this context, analyses of historical biobanks of clinical *Salmonella* strains represent an invaluable tool to improve knowledge of the biological, clinical, and epidemiological characteristics of the pathogen, as well as to investigate pathogenicity and antimicrobial resistance mechanisms.

Aim. The aim of this study was to enhance and expand the potential of the historical *Salmonella enterica* preserved at the EpiSoMI laboratory (Center of Coordinate Research – “**E**pidemiologia e **S**orveglianza **M**olecolare delle **I**nfezioni”) and established during the operational period of the **C**entro **E**nterobatteri **P**atogeni per l’**I**talia **S**ettentrionale (CEPIS). Clinical *S. enterica* strains isolated in Lombardy between 2001 and 2016 and archived in our laboratory biobank were analyzed. Each isolate was associated with epidemiological, clinical, and demographic data corresponding to the related salmonellosis case. Furthermore, this thesis sought to better characterize the burden of invasive *Salmonella* serotypes in Lombardy, with a specific focus on *S. Choleraesuis*, a swine-adapted yet highly invasive human pathogen.

Materials and Methods. Datasheets from 6,624 laboratory-confirmed salmonellosis cases (2001–2016) were collected and analyzed to assess trends in incidence, demographics, clinical presentation, and hospitalization rates. Invasive serotypes were identified and characterized. Archived isolates underwent phenotypic assays to assess fitness and survival under acidic conditions. Particular attention was devoted to the *S. Choleraesuis* serotype due to its invasive properties. Genomic analyses included whole-genome sequencing (WGS), core-genome multilocus sequence typing (cgMLST), and in silico investigation of antimicrobial resistance determinants.

Results. A total of 172 nontyphoidal serotypes (NTS) were identified. The most prevalent were *S. Typhimurium* (41.8%, n = 2,772), *S. Enteritidis* (18.5%, n = 1,223), and *S. 1,4,[5],12:i:-* (9.8%, n = 649), which were primarily associated with gastroenteritis. In addition, a subset of invasive serotypes linked to systemic infections and higher hospitalization rates emerged. Bloodstream infections were identified in 244 patients, with *S. Choleraesuis*, *S. Typhimurium*, and *S. Napoli* being the most common serotypes (24.6%, n = 60; 22.5%, n = 55; 15.8%, n = 38, respectively). *S. Choleraesuis* displayed one of the highest invasive indexes (76.9%), disproportionately affecting older adults and frequently leading to severe extraintestinal disease. Phenotypic assays revealed comparable growth fitness across serotypes, but *S. Choleraesuis* exhibited enhanced acid tolerance, consistent with its ability to overcome the gastric barrier and disseminate systemically. Genomic analyses demonstrated that isolates of this serotype formed a highly clonal population, frequently harboring multidrug resistance genes, including determinants conferring resistance to aminoglycosides, sulfonamides, tetracyclines, and β -lactams.

Discussion. Although our biobank data consists of *Salmonella enterica* isolates collected over several years without any clear information as to how these samples and data were collected, it provides a realistic snapshot of the serotype's circulation. These findings highlight the dual nature of salmonellosis: a substantial burden of self-limiting gastroenteritis caused by common serotypes, alongside a smaller but clinically significant fraction of invasive infections associated with specific serotypes characterized by host adaptation and multidrug resistance. The persistence of clonal invasive lineages, such as *S. Choleraesuis*, underscores the zoonotic interface between swine and humans and illustrates the risks posed by antimicrobial resistance.

Conclusion. This study demonstrates the value of combining long-term biobank resources with clinical and demographic data to capture shifts in serovar distribution, antimicrobial resistance, and pathogenic potential over time, providing a comprehensive view of invasive *Salmonella* infections in Lombardy. The results emphasize the need for continuous One Health-based surveillance, targeting both common and invasive serotypes, to mitigate the impact of salmonellosis on human health.

ABSTRACT (Italian Version)

Introduzione. *Salmonella enterica* è una delle principali cause di infezioni a trasmissione alimentare a livello mondiale, con manifestazioni cliniche che spaziano da gastroenteriti autolimitanti a malattie sistemiche invasive. Sebbene i sierotipi tifoidi siano ben riconosciuti come agenti di febbre enterica, alcuni sierotipi non tifoidi mostrano un indice di invasività insolitamente elevato, rappresentando una sfida significativa per la sorveglianza e il controllo. In questo contesto, le analisi delle biobanche storiche di ceppi clinici di *Salmonella* costituiscono uno strumento prezioso per migliorare la conoscenza delle caratteristiche biologiche, cliniche ed epidemiologiche del patogeno, nonché per indagare i meccanismi di patogenicità e di resistenza antimicrobica.

Obiettivo. Lo scopo di questo studio è stato quello di valorizzare ed espandere il potenziale della biobanca storica di *Salmonella enterica* conservata presso il laboratorio EpiSoMI (“**E**pidemiologia e **S**orveglianza **M**olecolare delle **I**nfezioni”) e allestita durante il periodo di attività del **C**entro **E**nterobatteri **P**atogeni per l’**I**talia **S**ettentrionale (CEPIS). Sono stati analizzati i ceppi clinici di *S. enterica* isolati in Lombardia tra il 2001 e il 2016 e archiviati nella biobanca. Ogni isolato era associato a dati epidemiologici, clinici e demografici corrispondenti al caso di salmonellosi relativo. Infine, l’obiettivo di questa tesi è stato di caratterizzare meglio l’impatto dei sierotipi invasivi di *Salmonella* in Lombardia, con particolare attenzione a *S. Choleraesuis*, un patogeno adattato al suino ma altamente invasivo nell’uomo.

Materiali e Metodi. Sono state raccolte e analizzate le schede relative a 6.624 casi di salmonellosi confermati in laboratorio (2001–2016) per valutare i trend di incidenza, i dati demografici, la presentazione clinica e i tassi di ospedalizzazione. I sierotipi invasivi sono stati identificati e caratterizzati. Gli isolati archiviati nella biobanca sono stati sottoposti a saggi fenotipici per valutare la fitness e la sopravvivenza a pH acidi. Particolare attenzione è ricaduta sul sierotipo *S. Choleraesuis* per le sue caratteristiche invasive. Le analisi genomiche hanno incluso il sequenziamento dell’intero genoma (WGS), la tipizzazione multilocus del core-genoma (cgMLST) e lo studio *in silico* dei determinanti di resistenza antimicrobica.

Risultati. In totale sono stati identificati 172 sierotipi non tifoidi (NTS). I sierotipi prevalenti sono stati *S. Typhimurium* (41,8%, n = 2.772), *S. Enteritidis* (18,5%, n = 1.223) e *S. 1,4,[5],12:i:-* (9,8%, n = 649), principalmente associati a gastroenterite. Inoltre, dalle analisi sono emersi dei sierotipi invasivi correlati a infezioni sistemiche e a maggiori tassi di ospedalizzazione. Le infezioni sistemiche sono state identificate in 244 pazienti, con *S. Choleraesuis*, *S. Typhimurium* e *S. Napoli* come sierotipi più comuni (24,6%, n = 60; 22,5%, n = 55; 15,8%, n = 38, rispettivamente). *S. Choleraesuis* ha mostrato uno degli indici di invasività più elevati (76,9%), colpendo in modo sproporzionato gli adulti più anziani e portando frequentemente a gravi patologie extraintestinali. I saggi fenotipici hanno rivelato una fitness di crescita comparabile tra i sierotipi, ma *S. Choleraesuis* ha mostrato una maggiore tolleranza agli acidi, coerente con la sua capacità di superare la barriera gastrica e diffondersi sistemicamente. Le analisi genomiche hanno dimostrato che gli isolati di questo sierotipo formano una popolazione altamente clonale, portatrice frequente di geni di multiresistenza, inclusi determinanti di resistenza ad aminoglicosidi, sulfamidici, tetracicline e β -lattamici.

Discussione. Sebbene i dati della nostra biobanca si basino su isolati di *Salmonella enterica* raccolti in più anni senza informazioni precise sulle modalità di campionamento e raccolta, essi offrono una rappresentazione realistica dei sierotipi attualmente circolanti. I risultati evidenziano la duplice natura della salmonellosi: da un lato, un notevole carico di gastroenteriti autolimitanti causate dai sierotipi più comuni; dall'altro, una quota minore ma clinicamente rilevante di infezioni invasive associate a sierotipi specifici caratterizzati da adattamento all'ospite e multiresistenza. La persistenza di linee clonali invasive, come *S. Choleraesuis*, sottolinea l'interfaccia zoonotica tra suino e uomo e illustra i rischi posti dalla resistenza antimicrobica.

Conclusioni. Questo studio dimostra il valore della combinazione tra risorse biobancarie a lungo termine e dati clinici e demografici per rilevare cambiamenti nella distribuzione dei sierotipi, nella resistenza antimicrobica e nel potenziale patogenico nel tempo, fornendo una visione completa delle infezioni invasive da *Salmonella* in Lombardia. I risultati sottolineano la necessità di una sorveglianza continua basata su un approccio One Health, mirata sia ai sierotipi comuni che a quelli invasivi, per ridurre l'impatto della salmonellosi sulla salute umana.

INTRODUCTION

1.1 *Salmonella* genus

1.1.1 Microbiological background.

The *Salmonella* (S.) genus belongs to the *Enterobacteriaceae* family and includes facultative anaerobic, non-spore-forming, rod-shaped, non-lactose fermenting, Gram-negative microorganisms, generally motile due to the presence of peritrichous flagella, with the exception of serovar Gallinarum and serovar Pullorum, which are non-motile (Farhat M., *et al.*, 2023; Brown EW., *et al.*, 2021; Gut AM., *et al.*, 2018). *Salmonella* spp. exhibit relatively simple nutritional requirements and can multiply under a broad range of environmental conditions. Optimal growth occurs at 37 °C and pH 6.5-7.5, but proliferation is possible between 2-54 °C and pH 3.8-9.5 (Adley & Ryan, 2016). Cells can be rapidly inactivated by heat, being killed within minutes at 60 °C (Ebrahimi A, *et al.*, 2018). Unlike many other *Enterobacteriaceae*, *Salmonella* spp. are not inhibited by freezing in water or by certain chemical compounds such as brilliant green, sodium tetrathionate, or bile salts, properties that form the basis of selective isolation protocols from fecal material (Ryan MP., *et al.*, 2017). In liquid non-selective media such as Trypticase Soy Broth (TSB) and Brain Heart Infusion (BHI) broth, cultures typically exhibit uniform turbidity after 18-24 h of incubation at 37 °C.



Figure 1: Colonies appearance on Columbia agar with sheep blood, cultivated for 24 h at 37 °C (Microbiology in Pictures - 2015).

Growth in enrichment broths is comparable to, or slightly less than, that in non-selective broths, reaching 10^8 – 10^9 CFU/mL after 24 h at 37 °C. On non-selective solid media, colonies after 24 h at 37 °C are round (2-3 mm), well-defined, convex, translucent to colorless, smooth, and shiny (S phase), with rough, opaque variants (R phase) occurring rarely. On blood agar, colonies tend to be slightly larger than on standard solid media and do not exhibit hemolysis (Figure 1). In certain cases, slow-growing strains occur, producing colonies only 0.5-1 mm in diameter, which may remain small even after prolonged incubation. These so-called “dwarf colonies” are characteristic of host-adapted *Salmonella* serovars that are generally more difficult to culture (e.g., *S. Choleraesuis*, *S. Abortusequi*, *S. Abortusovis*, *S. Gallinarum*, *S. Pullorum*).

1.1.2 Discovery and history

Bacteria of the genus *Salmonella* owe their name to the American veterinary pathologist D. E. Salmon, who first isolated, in 1884, a strain from the intestine of a pig. This organism was initially named *Bacillus choleraesuis*, as it was believed to be the causative agent of swine cholera (Salmon D., *et al.*, 1885). In 1900, it was renamed *Salmonella* by the French bacteriologist J. L. M. Lignières, in honor of Salmon (Ryan MP., *et al.*, 2017). The taxonomy system of *Salmonella* has undergone numerous revisions and modifications, to the point of even debating the concept of “species” within the genus (Agbaje M., *et al.*, 2011). Beginning in the 1930s, thanks to the work of Philip B. White and especially Fritz Kauffmann, a classification system was established based on the remarkable antigenic diversity of somatic O antigens and flagellar H antigens. This formed the basis of the Kauffmann–White scheme, which characterizes members of the genus *Salmonella*. Classification according to O and H specificity (explained in detail in the following paragraph) defined, respectively, serogroups and serotypes within the genus, which was further subdivided, on a biochemical basis, into four subgenera (I, II, III, IV) (Kauffmann F., *et al.*, 1966). However, this classification system is highly complex, identifying more than 2,600 serotypes, often named after the geographic location of their first isolation (e.g., S. Napoli, S. London, S. Muenchen). This has led to confusion and to the incorrect attribution of a “species” status to the growing number of identified serovariants. A definitive clarification of *Salmonella* nomenclature was finally reached in 1982 with the publication of phenotypic and genotypic analyses by Le Minor and Popoff. These demonstrated that all serotypes, previously included in Kauffmann’s four subgenera, belong to a single species, designated (Le Minor L and Popoff MY, 1987). The correct denomination of isolates belonging to *Salmonella enterica* should therefore follow a complex formula, i.g.: subsp. serovar Choleraesuis. Nonetheless, in current usage, the binomial form (S. Typhi, S. Typhimurium, S. Derby, etc.) is accepted, with the serovar name capitalized but not italicized, as it does not represent a species name (Ryan MP., *et al.*, 2017; Su LH and Chiu CH., 2007). A proper serovar designation is available only for the subspecies, which is most frequently implicated in human pathology and epidemiology.

1.1.3 Taxonomy

According to the current taxonomy, the genus *Salmonella* is classified into two recognized species: *S. enterica* and *S. bongori* (Brown EW., et al., 2021; Gut AM., et al., 2018) (Table 1). *S. enterica* can be further divided into six subspecies and more than 2,600 serotypes, whereas *S. bongori* includes 22 serotypes (Gut AM., et al., 2018; Jajere SM., et al., 2019). Serotypes are differentiated according to the White–Kauffmann scheme, based on the variability of somatic O antigens, flagellar H antigens, and capsular K antigens (Kauffmann F., 1966; Brenner FW., et al., 2000; Ryan MP., et al., 2017). Based on O and H antigen specificity, *Salmonella* strains are classified into serogroups and serotypes, which are then assigned to six subspecies on a biochemical basis. These subspecies are denoted in nomenclature by Roman numerals:

- I.S. subsp. *enterica*;
- II. *S. subsp. salamae*;
- III. *S. subsp. Arizonae*;
- IIIa. *S. subsp. Diarizonae*;
- IV. *S. subsp. Houtenae*;
- V. *S. subsp. Indica*

(Jajere SM., et al., 2019; Brenner FW., et al., 2000; Langridge GC., et al., 2012).

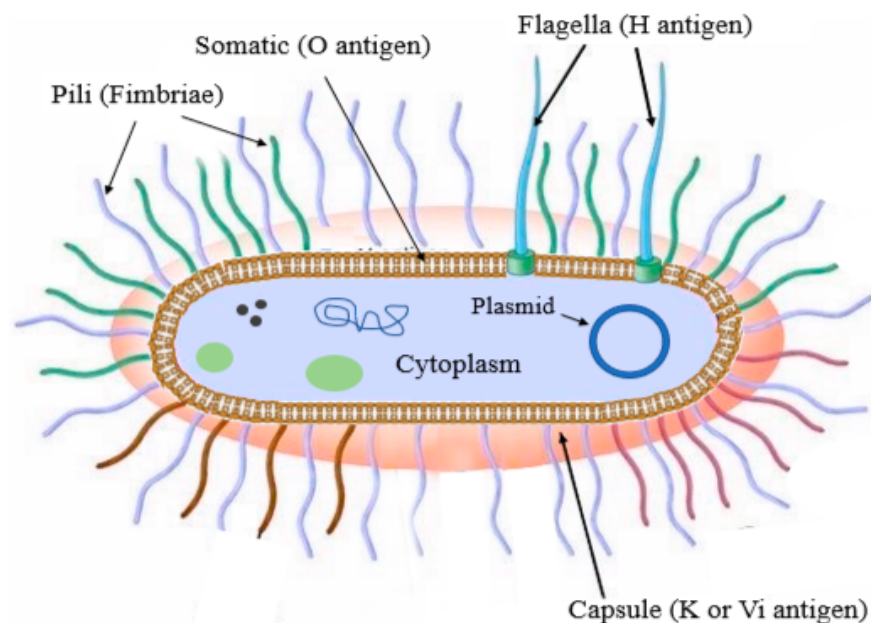


Figure 2: Model of O, H and K (Vi) antigens localization on cell. Vi antigen is present only in *Salmonella* Typhi (Teklemariam AD., et al., 2023).

The O antigen is of polysaccharide nature, covalently bound to a lipid (Lipid A) to form a lipopolysaccharide (LPS) complex, and is identified by the number corresponding to its major somatic antigen. In the original Kauffmann–White scheme, serogroups were defined according to O antigen specificity and designated by uppercase letters from A to Z, followed by numbers from 51 to 67. Today, they are more commonly identified by the number corresponding to their principal somatic antigen, ranging from O:2 to O:67. More than one O antigen can be expressed by a single *Salmonella* serotype. The H antigen is a protein that constitutes bacterial flagella filaments (flagellin), and it is possible to distinguish isolates that can present one or two distinct flagellar antigenic phases (monophasic or diphasic serotypes); triphasic serotypes are rare. Phase 1 H antigens are designated by lowercase letters (a, b, c, z, ...), while phase 2 antigens are indicated by arabic numbers and letters (1, 2, 3, e, n, x, ...). K antigens are polysaccharides and the best-known is the Vi antigen (from “virulence”), which is characteristic of *S. Typhi* but also present in *S. Paratyphi C* and *S. Dublin* (Ryan MP., *et al.*, 2017). In 2004, a third species, *Salmonella subterranea*, was described (Shelobolina ES., *et al.*, 2004); however, its taxonomic validity has been questioned, and it is not currently recognized as a legitimate member of the genus by major taxonomic authorities (Hata H., *et al.*, 2016). The classification of *S. enterica* serotypes according to the White–Kauffmann–Le Minor scheme is maintained by the WHO Collaborating Centre for Reference and Research on *Salmonella enterica* at the Institute Pasteur in Paris, in cooperation with various international reference laboratories. The most recent official publication of the scheme is the 9th edition (2007) by Grimont and Weill, listing 2,579 validated serotypes (including both *S. enterica* and *S. bongori*) (scacm.org, pasteur.fr). A subsequent supplement (Supplement No. 48, covering the years 2008–2010), published in 2014, added 63 new serotypes and 25 variants, but does not constitute a full, official new edition of the scheme, serving instead as a partial update (research.pasteur.fr, pubmed.ncbi.nlm.nih.gov). It is reported a total of 2,659 serotypes in the genus *Salmonella*: 2,639 in *S. enterica* and 22 in *S. bongori* (Ryan MP., *et al.*, 2017; Issenhuth-Jeanjean S., *et al.*, 2014). Although the White-Kauffmann-Le Minor scheme remains the international standard for *Salmonella* serotyping, molecular methods such as multilocus sequence typing (MLST) and whole-genome sequencing (WGS) are increasingly being adopted. These approaches provide higher resolution, allow for rapid outbreak investigation, and facilitate the identification of genetic determinants of virulence and antimicrobial resistance.

Table 1. Classification of *Salmonella* genus.

Genus <i>Salmonella</i>		Kauffmann-White scheme	N° serotypes	Reservoirs	Examples
Species					
<i>S. enterica</i>	subsp. <i>enterica</i> (I)	I subgenus	1586	Warm-blooded vertebrates (including humans)	Typhi, Typimurium, Enteritidis
	subsp. <i>salamae</i> (II)	II subgenus	522	Cold-blooded vertebrates and the environment	9,46;z:z39
	subsp. <i>arizonae</i> (IIIa)	III subgenus	102		43:z29:-
	subsp. <i>diarizonae</i> (IIIb)		308		6,7:l,v:1,5,7
	subsp. <i>houtanae</i> (IV)	IV subgenus	76		21:m,t:-
	subsp. <i>indica</i> (VI)	Not definable	13		59:z36:-
<i>S. bongori</i>	subsp. V	V subgenus	22		

1.2 Bacterial genetics

1.2.1 Comparative genomics and horizontal acquisition

Across *Salmonella*, genome-wide microarray and sequencing comparisons indicate that the core genome is highly conserved, while a substantial accessory fraction derives from horizontal gene transfer (HGT), with many laterally acquired regions corresponding to pathogenicity islands and other genomic islets that frequently integrate at tRNA loci (Porwollik S., *et al.*, 2002; Kushwaha SK., *et al.*, 2024; Sia CM., *et al.*, 2025; Hensel M., 2000). *S. Typhimurium* LT2 is the reference strain used for this comparison, and most annotated open reading frames show broad homology across sublineages. Each major clade retains 10-12% unique DNA, most of which is enriched in regions related to virulence and environmental fitness and implicated in host specificity and systemic spread (Kushwaha SK., *et al.*, 2024; Porwollik S., *et al.*, 2002; McClelland M., *et al.*, 2001). These regions correspond to the pathogenicity islands introduced in the preceding paragraph. Comparative work encompassing *S. Typhimurium*, *S. Typhi*, *S. Dublin*, *S. Enteritidis*, and *S. Paratyphi* has repeatedly underscored that SPI-1 and SPI-2 are universally present and centrally involved in invasion and intracellular survival, while numerous smaller islands and prophage cargo vary by serovar and correlate with host-range traits (Kushwaha SK., *et al.*, 2024; Porwollik S., *et al.*, 2002; McClelland M., *et al.*, 2001).

1.2.2 Architecture and relatedness of virulence plasmids

Subgroup I serotypes include 1,454 serotypes and at least 99% of clinical isolates (Popoff MY., *et al.*, 1998; Eng, S. K., *et al.*, 2015), however only a few of these serotypes harbor a virulence plasmid. The size of these plasmids varies with each serotype, ranging from 50 to 285 kb, (Hsu CH., *et al.*, 2019; Lee W., *et al.*, 2022) i.e., serotype *S. Choleraesuis* with a 50-kb virulence plasmid (pSCV) in pigs, serotype *S. Dublin* with an 80-kb virulence plasmid (pSDV) in cattle, serotype *S. Pullorum* with an 85-kb virulence plasmid (pSPV) in fowl, serotype *S. Typhimurium* with a 95-kb virulence plasmid (pSTV) and serotype *S. Enteritidis* with a 60-kb virulence plasmid (pSEV) in mice (Gulig PA., *et al.*, 1993; Chu C., *et al.*, 1999; Libby SJ., *et al.*, 1997 ; Haneda T., *et al.*, 2001). These plasmids typically carry the *spv* operon, its regulatory gene *spvR*, fimbrial loci like *pef*, and resistance genes such as *rck* (Lee W., *et al.*, 2022; Bentum KE., *et al.*, 2025; Ho DK., *et al.*, 2011). pSTV of *S. Typhimurium*, pSEV of *S. Enteritidis* and pSCV of *S. Choleraesuis* are in closely related group that share RepFIB backbones and the *pef* fimbrial region, with pSCV exhibiting characteristic deletions in the *pef/tra* region consistent with derivation from a pSTV-like ancestor (Chiu CH., *et al.*, 2004). Complete sequencing of the ~50-kb pSCV (pKDSC50) from *S. Choleraesuis* RF-1 identified 48 predicted ORFs including the *spvR-spvABCD* regulon and conserved backbone genes, and side-by-side comparisons with the ~94-kb pSTV support a common evolutionary origin with subsequent deletions and remodeling that may reflect adaptation to the porcine niche and systemic tropism (Haneda *et al.*, 2001; Chiu CH., *et al.*, 2004). In fact, all the virulence plasmids show a very close relationship indicating a common evolutionary origin with variations reflecting host adaptation (Lee W., *et al.*, 2022). Comparative genomic evidence also shows inter-serovar plasmid transfers mediated by mobile elements (e.g., IS26), emphasizing the dynamic nature of plasmid evolution in antibiotic resistance and virulence (La TM., *et al.*, 2025). A heteroduplex analysis indicates that the level of closeness runs, in descending order, from pSTV to pSEV to pSCV to pSDV . Analysis of the major variations in serum resistance genes *rsk* (for “resistance to serum killing”), *rck* (for “resistance to complement killing”), and *traT* and the minor fimbria genes, *pef*, *faeH*, *faeI* (Montenegro MA., *et al.*, 1985; Ho DK., *et al.*, 2011; Vandenbosch JL ., *et al.*, 1989; Bohn E., *et al.*, 2019), and *oriT* suggested the existence of at least two groups of virulence plasmids. Group I includes pSDV and pSPV, which contain *faeH* and *faeI* without *rsk*, *repA* of RepFIB, and *pef* (Khajanchi BK., *et al.*, 2017); in contrast, group II includes pSTV, pSEV, and pSCV, which contain *rsk* and *repA* of

RepFIB and *pef* but no *faeH* and *faeI* (Lee W., *et al.*, 2022). Beyond these canonical backbones, plasmid biology in *Salmonella* is dynamic, with self-transmissibility documented for *S. Typhimurium* virulence plasmids and frequent recombination with resistance and accessory modules that expand host range and therapeutic resilience, processes that have also been observed among *S. Choleraesuis* field isolates (La TM., *et al.*, 2025; Chiu *et al.*, 2004).

1.2.3 The *spv* regulon and systemic disease

All *Salmonella* virulence plasmids conserve the *spv* operon and the accessory virulence factor *mig-5* (Passaris I., *et al.*, 2018). The *spvR-spvABCD* regulon is induced during stationary phase and intracellular residence, with positive control by the LysR-type regulator SpvR and enhancement by the stationary-phase sigma factor RpoS, while SpvA provides negative feedback. Collectively these elements enhance bacterial proliferation within deep tissues and the reticuloendothelial system, which is essential for the septicemic phase characteristic of host-adapted serovars. Experimental and observational data therefore support a division of labor in which SPI-1/2 mediate entry and intracellular survival whereas plasmid-borne *spv* and associated serum resistance and adhesin modules potentiate dissemination and high bacterial burdens in spleen and liver, consistent with clinical patterns of invasive salmonellosis due to some serovars (Chiu *et al.*, 2004). Recent work further indicates that SpvB, one effector of the *spv* locus, inhibits the NF- κ B signaling pathway by targeting IKK β and promoting I κ B α degradation through upregulation of the E3 ubiquitin ligase KEAP1, thereby dampening host inflammatory responses and facilitating bacterial survival (Yang S., *et al.*, 2021). The *spv* operon is expressed rapidly after *Salmonella* is internalized by macrophages, even though in vitro under non-inducing conditions the plasmid genes are largely silent. The intracellular environment, which exposes the bacteria to nutrient limitation, oxidative stress, and host-derived signals, triggers the upregulation of *spvB*, *spvC* and related effectors, thereby promoting bacterial survival within macrophages and persistence in deep organs such as liver and spleen (Fierer J., *et al.*, 1993). Comparative genomics has shown that the *spv* operon is highly conserved in many host-adapted or invasive serovars, including *S. Typhimurium*, *S. Enteritidis*, *S. Choleraesuis* and *S. Dublin*, and that in some cases virulence plasmids simultaneously carry antimicrobial resistance determinants, giving rise to hybrid plasmids that combine resistance and virulence traits, with important implications for clinical management. In addition to SpvB, other plasmid-encoded effectors also contribute to immune modulation and systemic dissemination. SpvC, through its phosphothreonine lyase activity, has been shown to interfere with host MAPK signaling and inhibit pyroptosis, thereby reducing intestinal inflammation and favoring bacterial spread in experimental models (Zuo L., *et al.*, 2020). Moreover, in vitro assays correlating virulence gene content with phenotypic invasion potential suggest that the presence of plasmid-borne factors such as *spv*, *mig-5*, *rck* and *pef* increases

the probability of infection and dissemination, aligning with epidemiological observations of serovars that carry these plasmids being disproportionately associated with invasive disease (Kuijpers AFA., *et al.*, 2019). Taken together, these findings underscore the pivotal role of plasmid-borne virulence genes in the pathogenesis of invasive *Salmonella* infections. While SPI-1 and SPI-2 orchestrate invasion and intracellular survival, the *spv* operon and associated virulence plasmid loci act as key enhancers of systemic disease, shaping the clinical presentation of salmonellosis in host-adapted serovars and contributing to their persistence and epidemiological impact.

1.2.4 Serum resistance, adhesins and host interaction functions on plasmids

Plasmids encode for genes that give serum resistance and adhesin factors that modulate early extracellular survival and mucosal colonization. Serum resistance genes, *rck*, *rsk*, and *traT*, are present in most of the virulence plasmids. The *rck* gene encodes an outer membrane protein, homologous to PagC (for “*phoP*-activated gene”) (Koczerka M., *et al.*, 2021) and Ail (for “attachment and invasion locus”) (Bohn E., *et al.*, 2019), which confers resistance to complement-mediated killing. The gene *traT* encodes for a surface lipoprotein originally characterized on F-like plasmids, that augments resistance in certain genetic backgrounds (Montenegro MA, *et al.*, 1985; Mambu J., *et al.*, 2017). *Rck* and *traT* genes are variably present across virulence plasmids with notable absence of *rck* from some pSCV variants that instead retain *traT*, a pattern consistent with functional shuffling during host adaptation (Lee W., *et al.*, 2022; Haneda *et al.*, 2001). The *rsk* gene is only 66 bp long and contains a series of direct 10-bp repeat in the 5'-noncoding region of the *repA* gene of RepFIB (Lee W., *et al.*, 2022; Ho DK., *et al.*, 2011; Bohn E., *et al.*, 2019). In addition, a gene, *spf* (for “stimulation of protein forty”), near *rsk* was recently found to be involved in the production of IL-12 p40 in *Salmonella*-infected macrophages. Adhesins are known to support the colonization of in the host alimentary tract, thereby increasing the bacterial load in the vicinity of the epithelial cell lining. The *pef* operon is a 7-kb region containing genes for plasmid-encoded fimbriae. It includes the *pefBACD*, *orf5*, *orf6*, and *pefI* genes (Bäumler AJ., *et al.*, 1996). It has been documented that the *pef* fimbriae mediate bacterial adhesion to murine intestinal epithelium, resulting in fluid accumulation in the gut (Bäumler AJ., *et al.*, 1996). pSCV has a deletion in the region behind *pefD*, and pSEV has sequence variations in *orf5* and *orf6*. These deletions and variations of *orf5*, *orf6*, and *rck* may be associated with host adaptation in *Salmonella*. *pef* adjacent ORFs on pSCV and pSEV offer a mechanistic route to serovar-specific colonization strategies without loss of systemic capability, aligning with the invasive clinical phenotype of *S. Choleraesuis* in both pigs and humans (Haneda *et al.*, 2001; Chiu *et al.*, 2004; Lee W., *et al.*, 2022).

1.2.5 Antimicrobial resistance

Antimicrobial resistance (AMR) in *S. enterica* has evolved from a localized clinical problem in the late 20th century to a sustained, globally distributed threat that links human, animal and environmental health. Prior to the 1990s/early 2000s, many non-typhoidal *Salmonella* (NTS) infections were treatable with conventional agents such as ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole; however, since the early 2000s the frequency and complexity of resistance phenotypes have risen sharply, driven by both clonal expansion and horizontal gene transfer (Wang Y., *et al.*, 2025). Between 2000 and the early 2010s the dominant global AMR concern in *S. enterica* was the emergence of multidrug-resistant (MDR) clones resistant to ampicillin, chloramphenicol and sulfonamides, often plasmid-associated phenotypes linked to livestock production and food-chain transmission. These early MDR lineages laid the groundwork for subsequent waves of resistance to critically important antimicrobials (Listorti V., *et al.*, 2022). From ~2010 onwards, two trends became prominent worldwide: (i) increasing decreased susceptibility to fluoroquinolones (often via chromosomal mutations and plasmid-mediated quinolone resistance determinants) (Figure 3), and (ii) dissemination of extended-spectrum β -lactamase (ESBL)–producing and AmpC–producing strains compromising third-generation cephalosporins. Both trends were amplified by the global movement of food animals, contaminated products, and mobile resistance elements (Wang Y., *et al.*, 2025). Global surveillance syntheses and genome-scale studies for the period 2000–2023 demonstrate widening geographic spread of high-risk resistance genes (e.g., blaCTX-M, qnr, mcr variants) and an increasing proportion of *S. enterica* genomes carrying multiple clinically relevant determinants. A recent comprehensive genomic atlas (covering genomes up to 2023) shows that plasmid-borne resistance has become a major driver of AMR in *Salmonella*, enabling rapid inter-serovar and inter-host dissemination and stabilizing MDR phenotypes even where antibiotic selection pressure is heterogeneous (Wang Y., *et al.*, 2025). Regionally, Europe experienced a generally lower prevalence of the most extreme resistance phenotypes compared with many low- and middle-income regions, but important upward shifts have been documented since 2000.

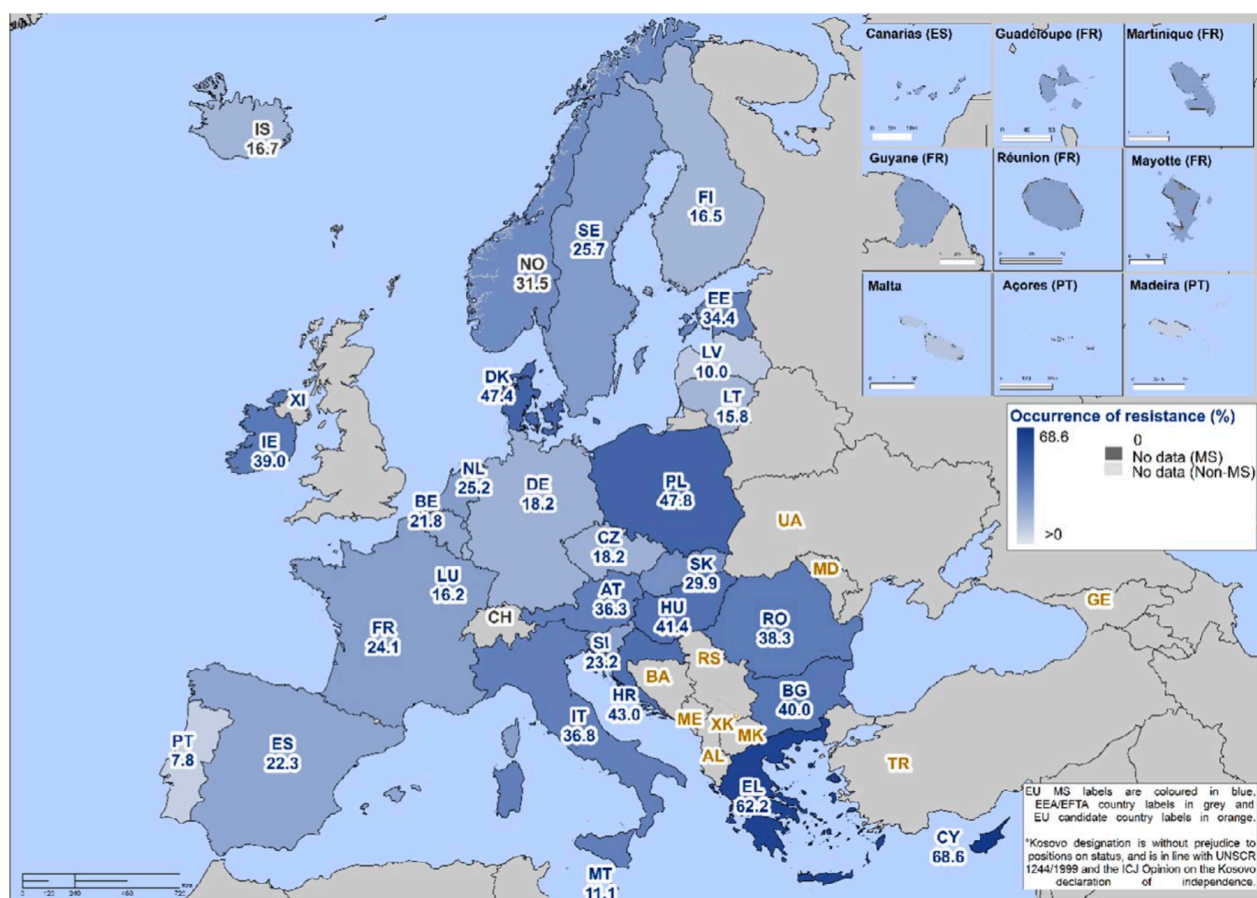


Figure 3: Spatial distribution of ciprofloxacin (fluoroquinolones) resistance among *S. Enteritidis* isolated from human cases, 2023 (EFSA/ECDC 2024).

European surveillance (harmonized data submissions and One Health reporting) reveal persistent hotspots in specific serovars and production sectors, notably *S. Infantis* and certain *S. Typhimurium* monophasic variants associated with poultry and pork production, that frequently carry large MDR plasmids including ESBL genes and other co-resistance loci. The EU One Health data for 2022-2023 indicate continued detection of ESBL-producing NTS in both human clinical isolates and in food-producing animals, with fluoroquinolone non-susceptibility increasing in several countries. These trends underscore that, although overall clinical resistance rates in many EU countries remain moderate for some drug classes, the burden of plasmid-driven resistance in the food chain represents a persistent cross-sector risk. (EFSA/ECDC 2024; ECDC 2025). In Italy, national and regional surveillance combining clinical, food- and animal-derived isolates show patterns that mirror broader European dynamics while also revealing localized features. Italian surveillance from the 2000s documented the early circulation of MDR *Salmonella* linked to livestock and food commodities; more recent regional studies (e.g., Piedmont, 2012–2021) and national

syntheses show ongoing detection of strains with decreased susceptibility to fluoroquinolones and occasional isolation of ESBL-producing NTS from humans and animal sources. The Italian datasets also illustrate the importance of subnational heterogeneity: some regions report higher proportions of resistant isolates tied to local production and processing practices. Overall, Italy has benefited from strengthened laboratory networks and integrated reporting, the data indicate persistent reservoirs of drug resistance in the food chain that require continued One Health action. (Pitti M., *et al.*, 2023; Alessiani A., *et al.*, 2022). Clinically important temporal patterns since 2000 include: (i) a shift from classical MDR to more complex resistance combinations that include reduced fluoroquinolone susceptibility and third-generation cephalosporin resistance; (ii) an increase in plasmid-mediated resistance determinants (including *mcr* and diverse *bla*CTX-M variants) that reduce the utility of last-line agents in severe infections; and (iii) episodic emergence of extensively drug-resistant (XDR) phenotypes in outbreak contexts driven by acquisition of multiple mobile elements. These dynamics have been documented repeatedly in global databases and modeled surveillance outputs linking antimicrobial consumption, agricultural practices and human clinical burden (WHO GLASS 2022; Wang Y., *et al.*, 2025). From a One Health standpoint, the implications are clear: reducing AMR in *S. enterica* requires coordinated interventions across clinical medicine, veterinary medicine and food production, including prudent antimicrobial use policies, targeted interventions in high-risk animal production systems (notably poultry and swine), improved sanitation and supply-chain controls, and sustained genomic surveillance to detect and interrupt the spread of high-risk plasmids and clones. Recent European and Italian surveillance reports emphasize that combined phenotypic testing and whole-genome sequencing (WGS) provides the resolution needed to assign transmission pathways and to prioritize interventions at the interface of humans, animals and environment (ECDC 2025; EFSA/ECDC 2024; Pitti M., *et al.*, 2023).

1.3 Salmonellosis

1.3.1 Clinical presentation

Salmonellosis refers to acute infections caused by *Salmonella*, which can manifest as enteric fever, gastroenteritis, bacteremia, or extraintestinal focal infections (Lamichhane B., *et al.*, 2024). Clinically, *S. enterica* serotypes are classified into Typhoidal *S. enterica* (TS) serotypes, which cause invasive extraintestinal disease (typhoid fever), and nontyphoidal *S. enterica* (NTS) serotypes, which usually cause self-limiting gastroenteritis (Schultz BM., *et al.*, 2021; Lamichhane B., *et al.*, 2024; Kumar G., *et al.*, 2025) (Figure 4). As in other infectious diseases, the course and outcome of infection depend on several factors, including the infecting organism, the infectious dose, and the immune status and genetic background of the host. NTS infections may be asymptomatic but frequently present as acute enterocolitis. In contrast, typhoid fever occurs following infection with typhoidal (*S. Typhi*, *S. Paratyphi* A, B, and C) strains that cross the intestinal barrier, reach the bloodstream, and cause systemic disease. Typhoid fever has an average incubation period of approximately 14 days (Schultz BM., *et al.*, 2021; Wain J., *et al.*, 2023). Symptoms may persist for up to three weeks and include high fever (39-40 °C), chills, abdominal pain, hepatosplenomegaly, rose-colored maculopapular skin eruptions (rose spots) on the abdomen, nausea, constipation, headache, and a dry cough (Wain J., *et al.*, 2023). Enterohemorrhagic complications of varying severity, and more rarely intestinal perforation, typically occur during the third week of illness. Overall, the clinical picture caused by *S. Typhi* is generally more severe and prolonged than that caused by paratyphoid serotypes; however, clinical features alone are insufficient to distinguish between them (Wain J., *et al.*, 2023). For NTS serotypes, infection can often be asymptomatic. When symptoms occur, the incubation period typically ranges from 6 to 72 hours after exposure (Lamichhane B., *et al.*, 2024). Common symptoms include headache, abdominal pain, diarrhea, nausea, and vomiting, often accompanied by fever (38-39 °C) (Lamichhane B., *et al.*, 2024). Clinical symptoms usually resolve within 4-5 days, but in some cases, fever and altered bowel habits can persist for 10-15 days (Lamichhane B., *et al.*, 2024).

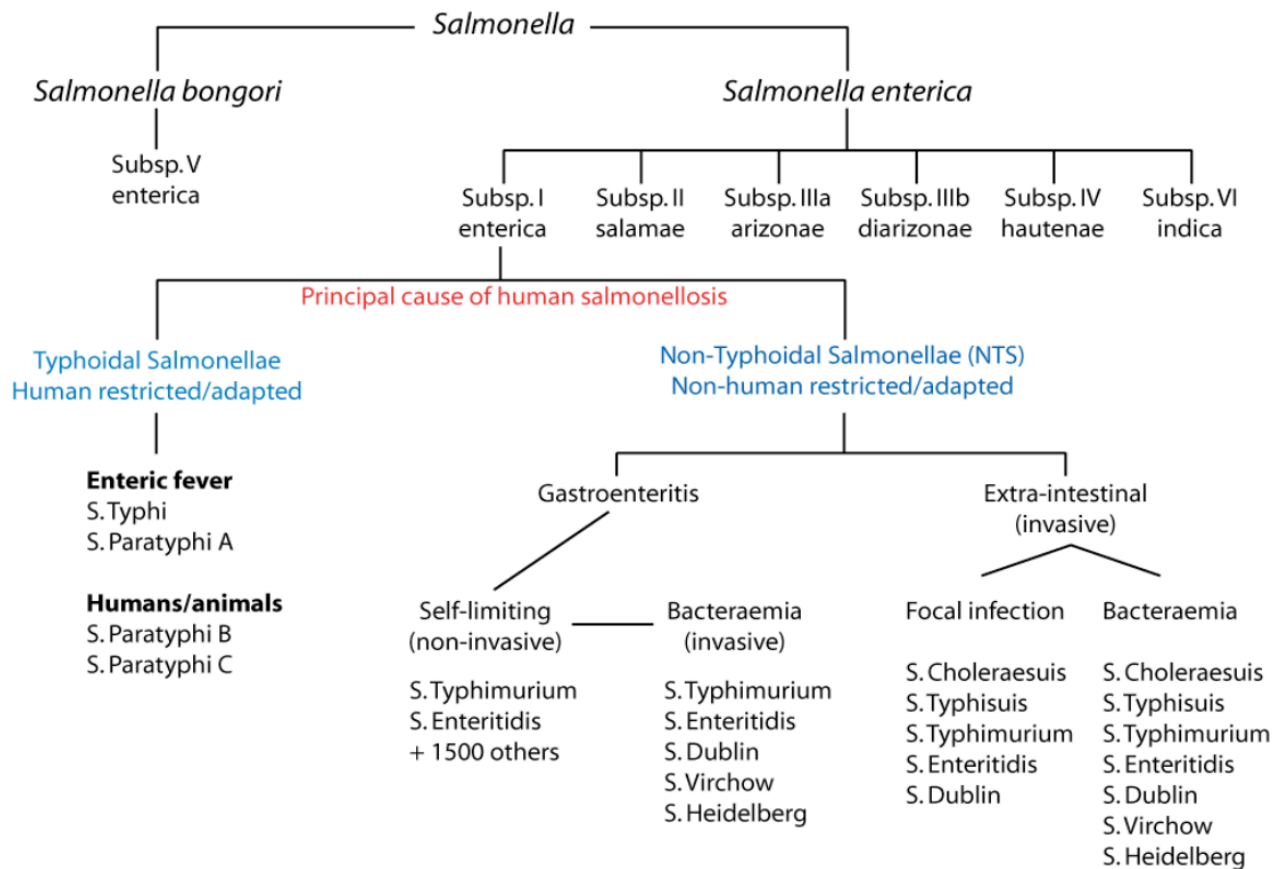


Figure 4: The genus classification of *Salmonella* and distinction of typhoidal and non-typhoidal *Salmonella* strains from a clinical point of view (Fowoyo P., et al., 2020).

Post-infectious complications may include reactive arthritis (Reiter's syndrome), most often in individuals carrying the HLA-B27 histocompatibility antigen (Lamichhane B., et al., 2024). During convalescence, prolonged shedding of *Salmonella* in stool is common, lasting up to one month in adults. In children under five years of age, fecal shedding can persist for up to seven weeks (Wain J., et al., 2023). The chronic carrier state is specifically defined as the excretion of *Salmonella* in stools for more than one year after the acute phase of infection. Because humans represent the only reservoir for typhoidal *Salmonella*, chronic carriers of *S. Typhi* and *S. Paratyphi* are responsible for maintaining transmission in endemic areas, mainly through contamination of food and water supplies (Bhan MK., et al., 2005). Approximately 4% of patients with enteric fever, predominantly infants, elderly individuals, and women develop this chronic carrier state (Gonzalez-Escobedo G., et al., 2011). In contrast, chronic carriage of NTS occurs much less frequently, with an estimated prevalence of 0.1% among patients with nontyphoidal salmonellosis, reflecting the fact that animals, rather than humans, serve as the principal reservoir for NTS (Hohmann, 2001). NTS serotypes rarely cause invasive disease in

healthy individuals. However, in vulnerable populations, including children under two years, elderly adults over 65 years, and patients with immunodeficiency, they can cause severe, typhoid-like illness requiring hospitalization (Marchello CS., *et al.*, 2022; Kumar G., *et al.*, 2025; Gal-Mor O., *et al.*, 2014). Approximately 5% of NTS infections progress to bacteremia, with higher rates in children with underlying conditions such as immunodeficiency, malignancies, immunosuppressive therapy, hemolytic anemia, or inflammatory bowel disease. In adults with comorbidities, bacteremia can lead to severe complications such as meningitis, septic arthritis, and osteomyelitis (Marchello CS., *et al.*, 2022, Chiu CH., *et al.*, 2004). Any organ system can be affected. The urinary tract is the most common extraintestinal site, but *Salmonella* bacteremia has a particular capacity to infect endovascular structures, leading to endocarditis, especially in the presence of preexisting valvular lesions (Dhayhi NS., *et al.*, 2021; Marchello CS., *et al.*, 2022). Central nervous system involvement may manifest as meningitis, ventriculitis, brain abscess, or subdural empyema (Santos E Silva A., *et al.*, 2025) The osteoarticular system is another relatively frequent site, presenting as septic arthritis or osteomyelitis (Marchello CS., *et al.*, 2022). Literature reports also indicate that individuals with inherited deficiencies in the IL-12/IL-23 immune pathway are particularly susceptible to infections caused by NTS serotypes, but not to those caused by typhoidal serotypes (Schultz BM., *et al.*, 2021). These findings support the hypothesis that typhoidal and nontyphoidal salmonellosis differ not only in their antigenic and epidemiological features but also in the inflammatory pathways they trigger (Schultz BM., *et al.*, 2021; Kumar G., *et al.*, 2025; Gal-Mor O., *et al.*, 2014).

1.3.2 Pathophysiology: Virulence factors and mechanisms of invasiveness

The pathogenicity of *Salmonella* spp. is the result of a sophisticated repertoire of virulence determinants that enable the bacterium to survive in hostile environments, colonize the host, invade tissues, and evade immune defenses. These include mechanisms to withstand acidic conditions, crucial for passage through the gastric barrier, as well as adhesins and invasion factors that promote attachment to the intestinal epithelium (Wang M., *et al.*, 2020). Following adhesion, *Salmonella* can penetrate intestinal cells either directly or via specialized M cells in the Peyer's patches, and to persist within professional phagocytes such as macrophages (Wang M., *et al.*, 2020). Many of these virulence factors are encoded on discrete chromosomal regions termed *Salmonella* pathogenicity islands (SPIs), while others reside on large virulence plasmids (Sia CM., *et al.*, 2025). Among these genomic regions, SPI-1 and SPI-2 are the best characterized. SPI-1 is primarily associated with the invasion of non-phagocytic intestinal epithelial cells, whereas SPI-2 is critical for systemic dissemination and intracellular survival within macrophages (Loul., *et al.*, 2019). Both SPI-1 and SPI-2 encode components of the Type III Secretion System (T3SS), a syringe-like macromolecular apparatus structurally related to bacterial flagella. T3SS-1 and T3SS-2, respectively, mediate the injection of bacterial effector proteins into the host cell cytoplasm, manipulating host signaling and cytoskeletal architecture (Bohn E., *et al.*, 2019). Within the gut, *Salmonella* can be taken up by CD18+ phagocytic cells extending dendrites between enterocytes, which capture luminal bacteria and transporting them to the lamina propria and, via lymphatic and hematogenous routes, to systemic sites such as the liver and spleen. T3SS-1 effectors, commonly known as *Salmonella* outer proteins (Sops), such as SopE, SopE2, SopD, and SopB, play pivotal roles in virulence. SopE triggers extensive actin polymerization, enabling bacterial internalization, while SopB (encoded on SPI-5) acts as an inositol phosphate phosphatase that disrupts chloride ion homeostasis, leading to fluid secretion and diarrhea (Lou L., *et al.*, 2019). These processes also induce pronounced inflammatory responses, marked by recruitment of neutrophils into the intestinal lumen, a hallmark of nontyphoidal salmonellosis (Wang M., *et al.*, 2020; Gal-Mor O., *et al.*, 2014). Once internalized, *Salmonella* switches to T3SS-2 activity, leading to the formation of the *Salmonella*-containing vacuole (SCV) (Figure 5).

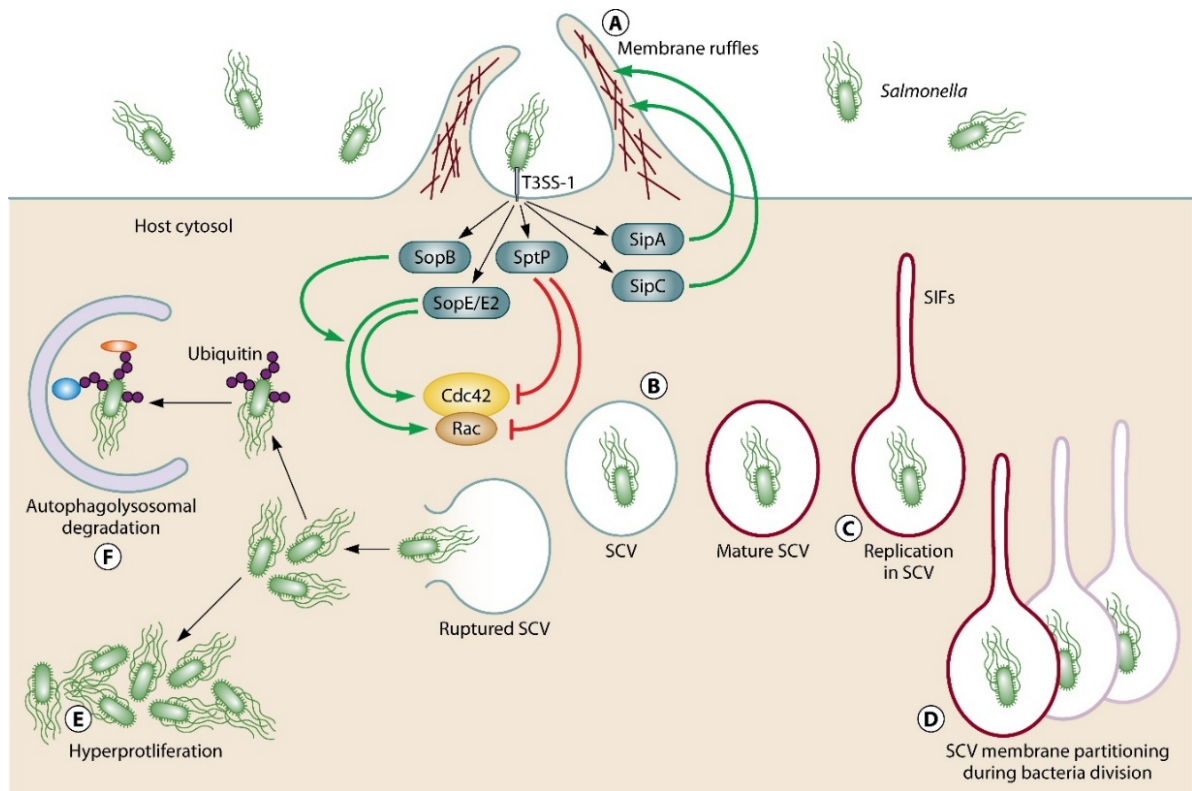


Figure 5: Adhesion and internalization of *Salmonella* spp. in the host (Han *et al.*, 2024).

This compartment shields the bacteria from toxic host defenses and provides a niche for replication (Holden DW., 2002). Inside macrophages, plasmid-borne *spv* genes (*spvA*, *spvB*, *spvC*, *spvD*, and *spvR*) enhance survival. *SpvB* and *SpvC* are delivered into the macrophage cytosol via T3SS-2, where they impair NADPH oxidase recruitment to the phagosomal membrane, reducing superoxide production, inhibiting pro-inflammatory cytokine release, and promoting macrophage apoptosis before bacterial clearance can occur (Guiney DG., *et al.*, 2011). These apoptotic macrophages are then engulfed by newly recruited phagocytes, enabling bacterial spread. In nontyphoidal infections, this cycle is typically confined to the intestinal mucosa. However, typhoidal serotypes (*S. Typhi*, *S. Paratyphi* A, B, C) are adapted for systemic infection. After breaching the intestinal epithelium, they pass through mesenteric lymph nodes and enter the bloodstream via the thoracic duct, causing an initial transient bacteremia that is partially controlled by macrophages in the liver and spleen (Raffatellu M., *et al.*, 2005; Sia CM., *et al.*, 2025). Despite this, *Salmonella* persists and replicates in these organs, producing a sustained bacteremia that coincides with the onset of clinical symptoms. During later stages, bacteria reach the gallbladder, from which they are reintroduced into the intestinal tract. There, lymphoid tissues such as Peyer's patches and solitary follicles exhibit

marked inflammatory infiltration by lymphocytes and histiocytes, followed by necrosis, epithelial sloughing, and the formation of characteristic typhoid ulcers (Sia CM., *et al.*, 2025). Additional research has highlighted the interplay between *Salmonella* virulence programs and host immunity. The secretion of inflammatory cytokines such as IFN- γ during infection promotes the recruitment of Th1 lymphocytes, dendritic cells, and neutrophils to the infection site. This immune activation is a double-edged sword, limiting bacterial replication locally but also contributing to tissue pathology and transmission (Griffin AJ., *et al.*, 2011; Wang M., *et al.*, 2020).

1.3.3 Treatment options

Non-typhoidal *Salmonella* (NTS) gastroenteritis is generally a self-limiting illness in otherwise healthy individuals. In most cases, mild infections resolve without the need for antimicrobial therapy, and management focuses on supportive care primarily oral or intravenous rehydration to correct fluid deficits and electrolyte imbalances (Sima CM., *et al.*, 2024). Antibiotic therapy is usually not advised, as they do not shorten illness duration and may prolong bacterial shedding (Leinert JL., *et al.*, 2021). Treatment is reserved for patients belonging to specific high-risk groups, such as neonates, immunocompromised individuals, and those with pre-existing endovascular abnormalities (cardiovascular disease) in whom the risk of invasive disease or complications is significantly higher (Sima C., *et al.*, 2024). Some reports in the literature have noted that antimicrobial treatment may prolong fecal excretion of organisms (Gal-Mor O *et al.*, 2014), although the evidence remains inconclusive and results across studies are somewhat contradictory. This uncertainty underscores the need for careful risk-benefit evaluation before prescribing antibiotics in uncomplicated NTS gastroenteritis. Bacteremia is the most common complication of *Salmonella* gastroenteritis, occurring in approximately 1-4% of immunocompetent patients (Chiu C., *et al.*, 2004). However, the incidence is markedly higher in individuals with underlying immunodeficiencies, such as those with HIV infection, where recurrent *Salmonella* bacteremia is considered an AIDS-defining condition (Tack B., *et al.*, 2025). When antimicrobial therapy is indicated, the choice of agent depends on patient age, immune status, comorbidities, and local antimicrobial resistance patterns. Prompt empirical antibiotic and supportive therapy are crucial in NTS bloodstream infections, particularly in high-risk settings such as sub-Saharan Africa, where mortality is high and resistance to third-generation cephalosporins is widespread (Tack B., *et al.*, 2025). Fluoroquinolones, such as ciprofloxacin, are widely used as first-line agents in adults due to their excellent oral bioavailability, intracellular penetration, and broad Gram-negative coverage, including activity against most *S. enterica* serovars (Crump JA., *et al.*, 2015). In pediatric populations, fluoroquinolones are generally avoided because of concerns over potential cartilage toxicity, although short courses may be considered in severe or refractory cases (Nelson JD, Bradley JS, 2021). In children, third-generation cephalosporins such as ceftriaxone or cefotaxime are preferred due to their favorable safety profile and proven efficacy against invasive *Salmonella* infections (Shane AL., *et al.*, 2017). Azithromycin has also emerged as a useful alternative in

both adults and children, particularly in settings where fluoroquinolone resistance is prevalent or when prolonged intracellular activity is desired (Sjölund-Karlsson M., *et al.*, 2011). In enteric fever, antibiotic treatment usually lasts 5-10 days with fluoroquinolones or azithromycin, or 7-14 days with third-generation cephalosporins; however, in patients with significant comorbidities or in the presence of deep-seated complications (such as endovascular, osteoarticular, or biliary involvement), therapy may need to be prolonged for several weeks, often in combination with further diagnostic or surgical interventions (Parry CM., *et al.*, 2019). In patients with enteric fever complicated by infected prosthetic vascular grafts, endocarditis, or mycotic aneurysms, combination antibiotic therapy and surgical intervention, including debridement or graft replacement, are often required, with prolonged antimicrobial treatment for at least 4-8 weeks and sometimes lifelong suppression if devices cannot be removed (Guo Y., *et al.*, 2018; Sörelus K, *et al.*, 2019). The selective use of antimicrobials in NTS infection is rooted in the balance between therapeutic benefit and potential drawbacks. In healthy individuals with uncomplicated disease, antibiotics offer minimal impact on symptom duration while potentially disrupting the gut microbiota and fostering antimicrobial resistance (Onwuezobe IA., *et al.*, 2012). Additionally, prolonged fecal shedding may increase the risk of transmission within households and communities. Conversely, in high-risk patients, the potential to prevent systemic complications and reduce mortality outweighs these concerns, making prompt initiation of appropriate antibiotic therapy essential. The global rise of multidrug-resistant *Salmonella* strains, particularly those resistant to fluoroquinolones and extended-spectrum cephalosporins, poses a growing challenge to empirical treatment strategies (Eng SK., *et al.*, 2015). This trend reinforces the importance of antimicrobial stewardship, local resistance surveillance, and the development of novel therapeutic options (Jung Ls., *et al.*, 2017; Sadhu S., *et al.*, 2021).

1.4 Epidemiology

1.4.1 Global data

Salmonellosis is among the most common foodborne bacterial infections worldwide, causing an estimated 94 million cases and approximately 150,000 deaths each year (Nazir J., *et al.*, 2025; Achtman *et al.*, 2021; Besser JK., *et al.*, 2017). It affects all age groups, but children under five years of age and elderly individuals experience the highest incidence rates and the most severe outcomes (Gut AM., *et al.*, 2018; Teklemariam AD., *et al.*, 2023; Weinberger M., *et al.*, 2004). The disease burden, however, is distributed unevenly between low-/middle-income countries (LMICs) and high-income countries, reflecting disparities in infrastructure, sanitation, and food safety practices. In LMICs, the incidence of non-typhoidal *Salmonella* (NTS) gastroenteritis frequently exceeds 100 cases per 100,000 population per year, with invasive non-typhoidal *Salmonella* (iNTS) particularly prevalent in sub-Saharan Africa. Here, iNTS incidence can surpass 100 per 100,000 person-years, with case fatality rates reaching 20-85% among children, and a global burden estimated at 595,000 iNTS cases annually, resulting in around 79,000 deaths and over 6 million disability-adjusted life years (DALYs) (WHO. Immunization, Vaccines and Biologicals; Kim S., *et al.*, 2024). Children under five account for over one-third of all NTS deaths and nearly half of iNTS-related deaths (Kim S., *et al.*, 2024). The impact is magnified by comorbidities such as malaria, HIV infection, and malnutrition, which increase susceptibility and mortality risk. In high-income countries, salmonellosis remains one of the most frequently reported foodborne diseases, but mortality rates are generally lower due to early diagnosis and supportive care. Whereas non-typhoidal salmonellosis is endemic worldwide (Majowicz SE., *et al.*, 2010), in high-income countries typhoid fever is usually sporadic and linked to travel to endemic regions. Typhoid fever remains a major public health concern worldwide, with an estimated 9 million cases and approximately 110,000 deaths occurring annually (WHO, 2023; GBD 2017). The highest incidence rates (>100 cases per 100,000 population per year) are concentrated in South Asia, which continues to account for the majority of global cases and deaths, followed by parts of Southeast Asia and sub-Saharan Africa (WHO, 2023; GBD 2017). Being a pathogen transmitted via the fecal-oral route, the spread of serovar Typhi is strongly associated with sanitary conditions and with access to safe drinking water. For this reason, infection is uncommon in industrialized countries. In endemic regions, transmission occurs mainly in densely populated urban areas and disproportionately

affects the pediatric age group (Gal-Mor O., *et al.*, 2014). Major risk factors include the consumption of inadequately monitored food products along the supply chain, improper food handling and storage, and poor personal hygiene practices. Globalization and international travel further influence iNTS epidemiology, enabling the rapid spread of outbreak strains across borders through the movement of both travelers and globally traded food products (Tauxe RV., *et al.*, 2010). In high-income settings, imported foods, exotic produce, and processed products have occasionally been implicated in large-scale outbreaks. Conversely, in LMICs, informal food markets, inadequate cold chain systems, and limited access to potable water remain central to the persistence of transmission cycles. Globally, LMICs bear over 90% of the foodborne disease burden, making salmonellosis a priority for international food safety initiatives (Grace D, 2023). Addressing the epidemiological challenge of *Salmonella* requires integrated surveillance, risk-based food safety interventions, improved water and sanitation infrastructure, and strengthened cross-border outbreak investigation systems, particularly in an era where the interplay between climate change, global trade, and human mobility is reshaping the landscape of infectious disease transmission.

1.4.2 European and international Data

Data from the latest EU One Health 2023 zoonoses report confirm that salmonellosis is the second most reported zoonotic disease in the European Union, with 77,486 cases in 2023, surpassed only by campylobacteriosis (ECDC and EFSA, 2023). In 2022, 66,721 cases were reported across 30 EU/EEA countries, corresponding to an overall notification rate of 15.5 cases per 100,000 population, with hospitalization in 39.3% of cases (ECDC, Annual Epidemiological Report 2022). The age-specific distribution shows a striking peak in children aged 0-4 years, with notification rates exceeding 80 cases per 100,000, nearly ten times higher than in adults aged 25-64. In Italy, salmonellosis has been the most frequently reported zoonosis in recent years, with 3,333 confirmed cases between 2019 and 2023. In 2023 alone, 171 foodborne outbreaks involving *Salmonella* were reported, resulting in 1,271 human cases, 349 hospitalizations, and three deaths (ECDC and EFSA, 2023). These figures reflect both ongoing endemic transmission and the country's robust surveillance system. Salmonellosis displays a pronounced seasonal trend, with incidence peaking during the summer months in temperate regions. This is likely linked to higher ambient temperatures favoring bacterial multiplication in foods, as well as seasonal patterns in food handling, outdoor dining, and tourism-related exposure (ECDC and EFSA, 2023). From a public health perspective, salmonellosis imposes a substantial economic burden in the EU alone, the estimated annual cost is around €3 billion, driven by healthcare expenses, productivity losses, and trade impacts (ECDC and EFSA, 2023).

1.4.3 Way of transmission

Salmonella species are harbored in the intestinal tract of humans and animals, which represent a great variety of reservoirs facilitating their transmission. While typhoid fever recognizes humans (patients or carriers) as the only source of infection, and contagion is therefore strictly related to the fecal-oral route (Schneider KR., *et al.*, 2016), non-typhoidal salmonellosis are zoonoses, that can be transmitted directly or indirectly between animals and humans (Zizza A., *et al.*, 2024). It has been estimated that in the vast majority (95%) of cases human infection is foodborne (Sodagari HR., *et al.*, 2020). Many animals can act as *Salmonella* reservoirs, including poultry, cattle, and wild animals, which can contaminate food products and the environment. Among the over 2,600 identified serotypes, some are host-restricted, while most can infect various species, affecting both livestock and humans. Some *Salmonella* serotypes are host-specific, while others can infect more than one type of warm-blooded animal (Feasey NA., *et al.*, 2012). The *S. Gallinarum* and *S. Pullorum* serovars are restricted to poultry, while *S. Dublin*, *S. Choleraesuis* and *S. Abortusequi* to cattle, pigs, and horses respectively. However all can sometimes infect other warm-blooded animals (Ali S., *et al.*, 2022; Jajere SM., *et al.*, 2019). Other serovars, such as *S. Typhimurium* and *S. Enteritidis*, are generalists and can infect any warm-blooded animal (Feasey NA., *et al.*, 2012) (Figure 6). Not by chance, *S. Typhimurium* and *S. Enteritidis* are the two most widespread *S. enterica* subsp. serotypes, responsible for most clinical cases of salmonellosis in most parts of the world (WHO, *Salmonella* non-typhoidal fact sheet, 2018). Therefore, in the case of non-typhoidal salmonellosis, the transmission chain is particularly complex, involving not only a wide range of animal reservoirs but also different food vehicles which can have both animal and plant origin (e.g., eggs, poultry meat, pork, beef, nuts, vegetables), water and the environment (Brown EW., *et al.*, 2021; Gut AM., *et al.*, 2018). Moreover, infection may also be acquired through direct or indirect contact with animals (farm or domestic animals), improper handling and storage of food, or nosocomial infection (WHO non-typhoidal *Salmonella* fact sheet, 2018).

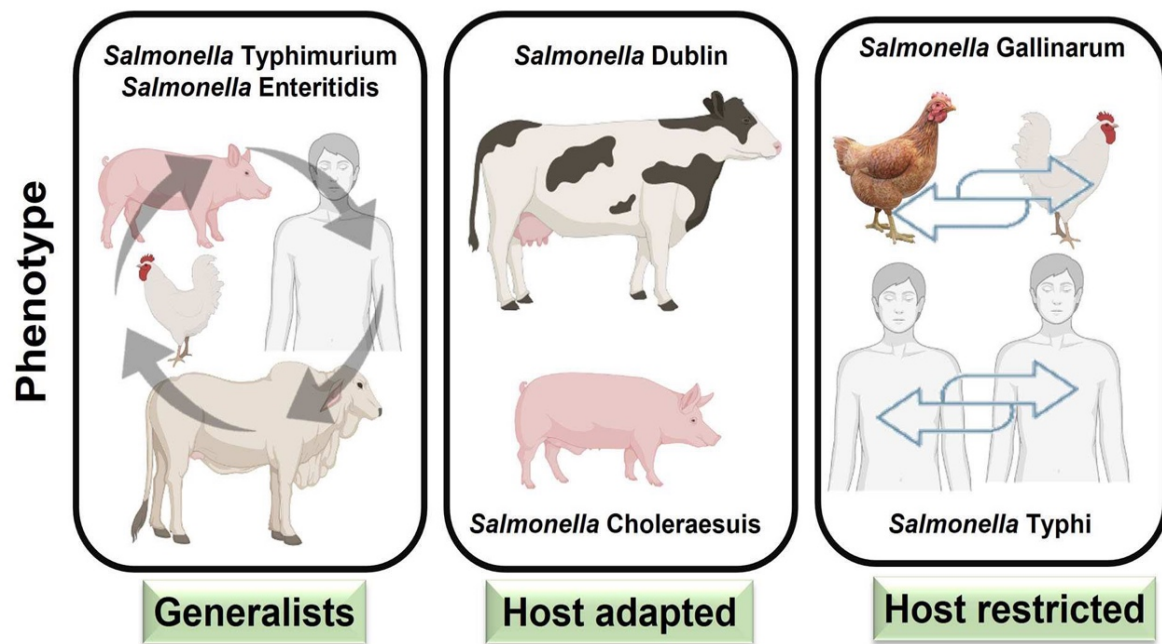


Figure 6: *Salmonella* serotypes can be classified into three groups according to their ability to infect different hosts: host-restricted, host-adapted, and generalist (Ali., *et al.*, 2022).

The infective dose for salmonellosis that can establish infection in the mucosa of the small intestine ranges from 10^5 to 10^6 CFU/mL (Xu H *et al.*, 2010). The ingestion of high doses of microorganisms is generally associated with a shorter duration of illness and with more severe clinical manifestations. In the literature it has been reported that, in some cases, ingestion of 10^2 - 10^3 bacteria are sufficient to cause disease, particularly in patients with low gastric pH, in neonates, and in individuals taking antacid drugs (Zizza A *et al.*, 2024; Chlebicz A & Śliżewska K, 2018). Most infections are foodborne in origin, often due to the prolonged interval between food preparation, cooking, and consumption, which favors bacterial multiplication, increases the infective dose, and thus raises the probability of infection. The role of food-chain operators is also relevant. Meat contamination occurs in different ways: the presence of bacteria is generally caused by fecal contamination or is directly proportional to the extent of infection in the animal and to hygienic deficiencies during slaughter. Contamination at the level of muscle tissue is usually infrequent but increases following processing to produce ground meat or fresh sausages. In poultry meat, *Salmonella* contamination depends mainly on farming methods. Each farm may house thousands of animals, and this concentration of potential hosts provides the opportunity for extremely rapid spread of infection among animals. The same condition is observed during transport from farm to slaughterhouse, which always takes place under overcrowded conditions. During slaughter, bacteria can then pass from one carcass to another

through cross-contamination occurring during processing. Eggs and egg products represent the main source of human infection, accounting for about 50% of outbreaks, mostly due to *S. Enteritidis* (EFSA and ECDC, 2016; Whiley H., et al., 2015). This serotype can colonize the reproductive organs of hens. Egg contamination can occur in the ovary by vertical transmission, in the cloaca, and at the time of laying, mainly due to fecal contamination of egg conveyor belts (Whiley H., et al., 2015; Khatun MF., et al., 2022). In the latter two cases, bacteria are found on the eggshell surface and may penetrate the egg through microlesions of the shell itself, or via pores that allow gaseous exchange between the exterior and the interior. Pathogen penetration into eggs is facilitated by the presence of moisture on the surface of the eggshell, which modifies surface tension (Whiley H et al., 2015). These phenomena are the basis for the European Commission's decision not to make refrigeration of eggs mandatory during commercialization, to avoid that interruptions in the cold chain could cause condensation on the shell, thereby facilitating the penetration of microorganisms present on its surface (Whiley H et al., 2015) (EFSA BIOHAZ Panel, 2014). Inside the egg, contamination is localized at the vitelline membrane and in the layer of albumen surrounding it. In fresh eggs the number of *Salmonella* bacteria is extremely low, and since albumen is an iron-poor substrate, multiplication occurs only after the yolk is penetrated, because of changes in vitelline membrane permeability. These changes are directly proportional to storage time and temperature: in contaminated eggs stored at temperatures below 20 °C, yolk invasion begins after about three weeks, while at temperatures between 20 and 30 °C microbial growth occurs rapidly, within a few days (Whiley H., et al., 2015). From these observations, the importance of egg storage temperature as a critical factor. In addition to the most common animal-derived foods, several outbreaks have been associated with unusual foods, including plant-derived products, such as the epidemic in England and Wales between 1994 and 1995 caused by a corn-based snack contaminated with *S. Agona* (Killalea D., et al., 1996), and the one caused by soybean sprouts contaminated with *S. Saintpaul*, which occurred in the United Kingdom in 1988 (O'Mahoney M., et al., 1990). Although animals and animal-derived foods represent the main vehicles of *Salmonella* bacteria, they are also found in the environment through fecal contamination from both human and animal origin. Environmental reservoirs (water, soil, plant foods) represent excellent reservoirs for many serotypes, even for those not normally found in livestock or humans, playing important roles. *Salmonella* is very common in wastewater, through which it can spread into aquatic environments such as streams, rivers, and lakes,

becoming a source of soil contamination and consequently of vegetables (Liu H., *et al.*, 2018). The use of wastewater for irrigation represents a direct source of contamination, favored in leafy vegetables, which protect microorganisms from exposure to environmental factors such as solar radiation, high temperatures, and desiccation, and offer them an optimal surface for growth (Liu H., *et al.*, 2018; Iwu CD., *et al.*, 2019). Leafy vegetables, especially, may protect pathogens from desiccation and UV exposure (Iwu CD., *et al.*, 2019). Grazing animals also contribute to direct soil contamination, which can extend to water basins. Fish and shellfish are not affected by *Salmonella*-related disease themselves; instead, contamination occurs via external routes, primarily through contact with contaminated water, processing environments, and handling practices. Aquatic environments, such as grow-out ponds or harvesting waters, can be contaminated by fecal-origin bacteria including *Salmonella*, which then serve as vectors of contamination (Sheng L., *et al.*, 2021). In tilapia sashimi processing plants, *Salmonella* was isolated at various stages, freezing, scaling, bleeding, and evisceration, with contamination rates rising in less sanitary facilities, and sometimes appearing even in final ready-to-eat products, underscoring the role of processing practices as key contamination points (Reis JO., *et al.*, 2025; Chiu CH., *et al.*, 2019). Furthermore, an integrative review of aquaculture-related studies (2000-2020) reports that *Salmonella spp.* has been isolated from fish skin, gills, and viscera, indicating cross-contamination facilitated by inadequate hygiene, water quality, and unsanitary equipment or surfaces (Rebouças RH., *et al.*, 2023). A systematic integrative review (2000-2020) confirms *Salmonella spp.* has been isolated from fish skin, gills, and viscera, often due to inadequate hygiene, poor water quality, and unsanitary equipment or surfaces (Porto YD., *et al.*, 2022). Even when the fish appear healthy, the detection of *Salmonella* in surrounding water and rearing environments confirms that fish do not develop disease but acquire contamination through external exposure to polluted aquatic systems (Sheng L., *et al.*, 2021). It is also important to consider that many of these foods are unsafe because they are often consumed undercooked or even raw, thereby increasing the likelihood of introducing a higher bacterial load into the human body. Other kind of products usually considered “safe” foods, as chocolate, because of their low water activity (aw), have caused major salmonellosis outbreaks. Between 1973 and 1974 in Canada and the United States, an epidemic was caused by chocolate balls contaminated with *S. Eastbourne* (Craven PC., *et al.*, 1975), while in 1982 an epidemic occurred in England and Wales, associated with the presence of *S. Napoli* in chocolate bars of Italian origin (Gill ON., *et al.*, 1983). An interesting observation

in both cases is the extremely low infective dose compared to that normally associated with infection, which can be explained by the protective effect of the food matrix, particularly rich in lipids, against gastric acidity (Craven PC., *et al.*, 1975; Gill ON., *et al.*, 1983).

1.4.4 Vaccination strategies

INTS vaccination in animals. In the veterinary field, vaccination has been investigated as a critical strategy to reduce burden in livestock, thereby limiting zoonotic transmission to humans through the food chain. Several vaccines are currently available or under development, including inactivated, subunit, and live-attenuated formulations. These vaccines primarily target non-typhoidal *Salmonella* serovars such as *S. Enteritidis* and *S. Typhimurium*, which are the leading causes of foodborne salmonellosis worldwide. Studies in poultry have demonstrated that both live-attenuated and killed vaccines can significantly reduce intestinal colonization, fecal shedding, and egg contamination, thus improving food safety (Alborali GL., *et al.*, 2017; Chang YF., *et al.*, 2019). More recent advances include recombinant vector vaccines and subunit formulations that elicit both humoral and cell-mediated responses, further reducing transmission risk (Desin TS., *et al.*, 2013). In cattle and swine, vaccination has been shown to lower clinical disease incidence and shedding, although variability in efficacy across farms highlights the need for integrated control measures (Barrow PA., *et al.*, 2020). Despite these promising results, widespread adoption is still challenged by issues of cost, strain coverage, and the need for herd-specific vaccination programs.

Typhoid vaccination in humans. In humans, vaccination is primarily directed against typhoid fever caused by *S. Typhi*. The older parenteral killed whole-cell vaccine, although effective, caused severe adverse reactions due to lipopolysaccharide toxicity (WHO. Background document: the diagnosis, treatment and prevention of typhoid fever, 2003). Today, two safer and widely available vaccines are licensed: the Vi capsular polysaccharide (Vi-PS) vaccine and the oral live-attenuated *Ty21a* vaccine. The Vi-PS vaccine induces a protective antibody response but has limited efficacy in children under two years of age and does not confer long-term protection. To overcome these limitations, newer Vi-conjugate vaccines (TCVs), in which the Vi polysaccharide is conjugated to a carrier protein, have been developed. These vaccines provide longer-lasting immunity, are effective in infants, and are recommended by the World Health Organization for inclusion in routine immunization programs in endemic countries (Shakya M., *et al.*, 2019). Field trials in Asia and Africa have demonstrated high efficacy, with reductions of up to 80% in typhoid incidence (Lo NC., *et al.*, 2021). Importantly, widespread use of TCVs is expected not only to reduce disease burden but also to slow the emergence of antimicrobial resistance in *S. Typhi*, which remains a major global concern (WHO, 2019).

Nonetheless, challenges related to vaccine coverage, delivery, and affordability persist, particularly in resource-limited settings.

1.4.5 Systems of surveillance

The prevention of Foodborne Diseases (FBD) remains a major public health priority for the European Union (EU), requiring a coordinated and harmonized approach among Member States. The legislative framework has evolved over time: Decision No. 2119/98/EC, which established the first European network for epidemiological surveillance, was replaced by Decision No. 1082/2013/EU and, more recently, by Regulation (EU) 2022/2371 on serious cross-border threats to health. This regulation currently provides the legal basis for the European Early Warning and Response System (EWRS) and for coordinated surveillance of communicable diseases. The European Centre for Disease Prevention and Control (ECDC) is responsible for collecting, analysing, and disseminating surveillance data on foodborne infections, as well as promoting technical and scientific collaboration among Member States. In parallel, the European Food Safety Authority (EFSA) gathers, through national monitoring systems, data on the prevalence of major zoonotic agents in food and animal reservoirs, as well as data on foodborne outbreak investigations. The evolution of the European legal framework for communicable disease surveillance has progressively introduced substantial changes in both organization and operational scope.

- ◆ Decision 2119/98/EC (1998–2013): established the first European network for epidemiological surveillance. Its focus was the coordinated collection of case-based data, aiming to ensure timely communication among Member States. However, the approach remained largely descriptive, centered on case notifications, and lacked binding mechanisms for rapid response.
- ◆ Decision 1082/2013/EU (2013–2022): marked a significant step forward by introducing the concept of “serious cross-border threats to health”, thereby including not only infectious diseases but also chemical, environmental, and bioterrorism-related threats. It formalized the role of the ECDC as the coordinating body for surveillance and strengthened the Early Warning and Response System (EWRS), enabling improved information sharing and greater harmonization across Member States.

- ◆ Regulation (EU) 2022/2371 (from 2022): represents a further milestone, since, unlike decisions, a regulation is directly binding across all Member States. It now provides the legal basis for integrated surveillance and a more effective and rapid alert system. The framework is aligned with the One Health approach, integrating data from human, animal, and food sectors, and aims to ensure coordinated and timely responses at the EU level.

In summary, the regulatory pathway has evolved from a system of basic notification and data collection (2119/98/EC) to harmonization and expansion of health threats (1082/2013/EU), culminating in today's model of binding, integrated, and One Health-oriented surveillance (Reg. 2022/2371). The reference framework for zoonoses surveillance is laid down in Directive 2003/99/EC, transposed into Italian legislation by Legislative Decree No. 191/2006. This directive defines the principles and priorities for data collection on zoonotic agents, their reservoirs, and foodborne outbreaks. Case definitions for the main zoonoses are harmonized at EU level through Implementing Decision (EU) 2018/945. The list of foodborne pathogens under surveillance includes, among others, *Salmonella spp.*, *Campylobacter spp.*, *Shiga toxin-producing Escherichia coli (STEC)*, *Listeria monocytogenes*, *Shigella spp.*, and *Yersinia spp.* Each year, EFSA and ECDC jointly publish an epidemiological report that integrates data from human, animal, and food surveillance. This report represents a key instrument for trend analysis, risk assessment, and the development of prevention and control strategies at the EU level. Since 2007, the surveillance of food- and waterborne diseases in the European Union has been coordinated by the ECDC through the Food- and Waterborne Diseases (FWD) program. This program acts as the central “digital framework” integrating monitoring, data analysis, and information exchange on the major foodborne pathogens. Within this structure, a key component is TESSy (The European Surveillance System), the database platform that compiles case notifications from Member States. TESSy allows comprehensive analysis of infections, combining epidemiological data (incidence, risk factors, geographic distribution) with microbiological information, including phenotypic and genotypic strain typing. To complement these activities, the EPIS (Epidemic Intelligence Information System) was introduced in 2010 as a rapid alert and communication network. EPIS facilitates real-time exchange of information on outbreaks and cross-border risks, transforming surveillance from a merely descriptive activity into a proactive tool that strengthens Europe's capacity for coordinated public health

response. Laboratories have a central role in the surveillance, prevention, and control of foodborne diseases because phenotypic and genotypic typing of the causative agents has therefore become indispensable for the early recognition of emerging threats. In fact, while routine diagnosis may only require the detection of pathogens in clinical specimens from suspected cases, tracing the contaminated food vehicle and the original source of infection demands more advanced strain characterization through molecular biology techniques. Large-scale analysis of isolates obtained from both human and non-human sources has greatly enhanced the ability to monitor epidemiological trends of *Salmonella*, for which it is essential to identify the serotype to identify outbreaks and study trends. In fact, this integrated approach enables the assessment of infection dynamics across time and geographic regions in human populations and allows these to be correlated with findings from food products and animal reservoirs. Estimating the true global burden of foodborne diseases, and salmonellosis in particular, remains a major challenge due to systematic underreporting at multiple levels of surveillance. One of the most influential studies, conducted by Majowicz *et al.* (2010), estimated that *non-typhoidal Salmonella* infections cause approximately 93.8 million cases of gastroenteritis per year (1,140 cases per 100,000 population), of which around 80.3 million are foodborne, resulting in an estimated 155,000 deaths annually (Majowicz SE., *et al.*, 2010). These data highlighted a striking variability across regions, with incidence rates ranging from 140 cases per 100,000/year in North Africa and the Middle East to 3,280 cases per 100,000/year in Asia-Pacific, Central and Eastern Asia, and Oceania. More recent analyses by the WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) and the Global Burden of Disease (GBD) study confirm and expand upon these findings. According to WHO estimates, unsafe food is responsible for 600 million cases of foodborne illness and 420,000 deaths each year, accounting for more than 33 million disability-adjusted life years (DALYs) globally, with children under five years representing almost one third of fatalities (WHO, 2015). The invasive form (iNTS) remains of particular concern in sub-Saharan Africa, where incidence exceeds 100 cases per 100,000 annually and case fatality rates can approach 20-25%, especially in children with comorbidities such as HIV or malaria (GBD, 2019). The GBD 2019 analysis further estimated 594,000 iNTS cases, 79,000 deaths, and 6.11 million DALYs worldwide. At the European level, the latest ECDC surveillance report (2023) indicates that salmonellosis continues to be the second most frequently reported zoonosis in the EU/EEA, with an overall notification rate of 17.4 cases per 100,000 population in 2022, and more than

65,000 laboratory-confirmed cases (ECDC, 2023). These numbers, however, capture only the “tip of the iceberg” compared to the much higher burden suggested by global estimates. Many infections are mild and do not require medical attention; when healthcare is sought, diagnostic testing is not always performed; when performed, test sensitivity and laboratory capacity vary across settings; and finally, reporting by laboratories and physicians remains inconsistent. The result is a steep “surveillance pyramid” where the officially reported cases represent only a small fraction of the true incidence of salmonellosis and other foodborne diseases.

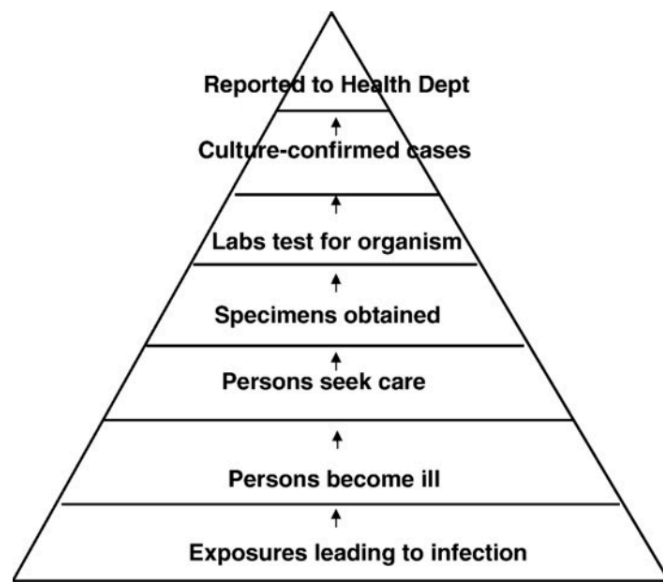


Figure 7: Surveillance pyramid (Tauxe RV., *et. al.*, 2010).

1.4.6 Systems of notification

It is essential to recall that notification of salmonellosis cases is mandatory across the European Union, as highlighted in the One Health 2022 Zoonoses Report by EFSA and ECDC (EFSA & ECDC, 2022). In Italy, this obligation is governed by the Decreto Ministeriale of 15 December 1990, which requires healthcare providers to report confirmed cases within 48 hours (Colarusso G., *et al.*, 2022). The decree classifies major bacterial foodborne diseases into Class II (sporadic cases) and Class IV (epidemic outbreaks), and the resulting flow of information serves as the primary data source for the European TESSy (The European Surveillance System). Complementing this notification path, Enter-Net (Enteric pathogen Network) Italia, coordinated by the Italian National Institute of Health (Istituto Superiore di Sanità), contributes detailed clinical, epidemiological, and microbiological data, such as serotyping and genetic characterization of isolates, from human and environmental samples. These enriched data feed into TESSy to enhance surveillance of key pathogens, including *Salmonella*, *Campylobacter*, *Listeria*, and STEC (Shiga toxin-producing *E. coli*). The ISS acts as the national focal point for the ECDC within the FWD program, supporting both the Italian Ministry of Health and the broader European surveillance infrastructure. The Enter-Net Italia surveillance system was established to provide timely and detailed information on the circulation of major enteric pathogens, including *Salmonella*, *Campylobacter*, *Listeria monocytogenes*, and other enteropathogenic bacteria, across the Italian territory. Its activities encompass the rapid collection of descriptive data on pathogen isolations following diagnosis, coupled with the phenotypic and genotypic characterization of strains through standardized protocols. These characterizations are subsequently transmitted to the European TESSy system, ensuring harmonized integration into the broader surveillance network. By systematically analyzing these data, Enter-Net Italia enhances the capacity to detect and investigate epidemic events at the national level, with the additional benefit of enabling comparisons with surveillance outcomes from other European countries participating in the ECDC's FWD network. Furthermore, the system actively supports the identification of cases potentially linked to cross-border outbreaks, thus contributing to the timely recognition of events with transnational implications. Finally, Enter-Net Italia promotes the development and dissemination of reference materials and harmonized phenotypic and genotypic

methodologies, aligned with the veterinary sector, thereby fostering integrated approaches and comparative analyses within the framework of One Health surveillance.

Enter-Net is an international surveillance network established to monitor human gastrointestinal infections caused primarily by *Salmonella*, VTEC O157, and other enteric pathogens, with a particular focus on antimicrobial resistance patterns (Fisher IS., *et al.*, 1999). The system was developed in response to the increasing globalization of food production and distribution, as well as the growing role of international travel in the dissemination of foodborne diseases (Fisher IS., *et al.*, 1999). Initially involving all 15 EU Member States together with Switzerland and Norway, Enter-Net has since expanded to include a total of 23 European and 3 non-European countries. In each country, the network operates through a coordinated structure that brings together the national reference laboratory microbiologist and the national public health epidemiologist, ensuring rapid access to high-quality and interpretable data.

Italy has participated in Enter-Net since 1997 and maintains one of the most extensive national surveillance systems within the network. The Italian component consists of referral laboratories coordinated by the Istituto Superiore di Sanità (ISS) (Tozzi A.E.; *et al.*, 2001). The network comprises approximately 468 local microbiology diagnostic laboratories and 24 Regional Reference Laboratories. In the veterinary sector, surveillance is conducted through the Enter-Vet network, active since 2002 and coordinated by the National Reference Center for Salmonellosis at the Istituto Zooprofilattico Sperimentale delle Venezie. Enter-Vet monitors *Salmonella* isolations from animals, food, and environmental sources. These laboratories routinely isolate *Salmonella*, VTEC O157, and other enteric pathogens from human, animal, food, and environmental sources, and collect standardized clinical, epidemiological, and microbiological information. Data are transmitted monthly to the ISS for central analysis, enabling timely detection of unusual temporal or geographical clusters, as well as evaluation of regional and national trends. Aggregated reports are then shared internationally with the Public Health Laboratory Service (PHLS) in London, which coordinates the global database and disseminates alerts and surveillance outputs to participating countries (Fisher IS., *et al.*, 1999). In Italy, *S. enterica* represents most notifications, with approximately 12,000 estimated cases annually (Demicheli V.; *et al.*, 1994). Surveillance data reveal seasonal trends, typically peaking in summer and early autumn, and highlight the predominance of serotypes such as *S.*

Typhimurium and *S. Enteritidis* which circulate across humans, animals, food, and environmental reservoirs. The integrated human–animal–food dataset allows identification of ubiquitous serotypes and those confined to more restricted ecological niches. Although Enter-Net does not provide incidence estimates nor always detect outbreaks with optimal timeliness due to variability in data submission, the system remains crucial for recognizing multinational events, harmonizing laboratory methods, and promoting coherent public health action across Europe. It is important to note, however, that any surveillance system is inevitably subject to a degree of underestimation of the true incidence.

In the Lombardy region, the Enter-Net surveillance framework was complemented by the activities of the regional reference center CEPIS, located within the Department of Public Health–Microbiology/Virology at the University of Milan. CEPIS served as the central hub for the collection and characterization of bacterial isolates submitted by the network of local diagnostic laboratories. Its role included second-level testing, such as serotyping and, when required, molecular analyses for selected *Salmonella* serotypes or isolates of epidemiological relevance (<https://www.epicentro.iss.it/ben/2010/settembre/2>). Within this regional system, CEPIS enhanced data quality by integrating laboratory-based microbiological information with clinical notifications, thereby improving the identification of circulating serotypes, antimicrobial resistance trends, and potential outbreak clusters. For other pathogens of public health concern (such as *Listeria monocytogenes*), CEPIS also acted as the designated center for mandatory regional submission of isolates, providing an additional layer of microbiological surveillance. Overall, the inclusion of CEPIS within the surveillance architecture strengthened the ability of the Lombardy region to conduct structured, high-resolution monitoring of enteric pathogens.

1.4.7 One Health

Salmonella is a globally distributed zoonotic pathogen comprising thousands of serovars that cause a spectrum of human disease ranging from self-limiting gastroenteritis to invasive typhoid fever, and which colonizes a broad range of animal hosts and environmental reservoirs (Jajere SM., *et al.*, 2019). The widespread occurrence of *S. enterica* across the farm-to-fork continuum makes it a paradigmatic organism for One Health approaches that integrate human, animal and environmental surveillance (Mattock J., *et al.*, 2024). Over the past decade, the epidemiology of *S. enterica* has been profoundly reshaped by the emergence and global dissemination of antimicrobial-resistant lineages. Multidrug resistance (MDR), extended-spectrum beta-lactamase (ESBL) production and fluoroquinolone non-susceptibility have been increasingly reported among both typhoidal and non-typhoidal serovars, complicating clinical management and increasing the risk of treatment failure (Browne AJ., *et al.*, 2020; GRAM Typhoid Collaborators 2024; Egorova A., *et al.*, 2023). Horizontal gene transfer mediated by plasmids and other mobile genetic elements is a dominant mechanism underlying the rapid acquisition and spread of resistance determinants in *S. enterica*. Plasmid-borne genes conferring resistance to critically important antimicrobials, including ESBLs, plasmid-mediated quinolone resistance (PMQR) determinants and *mcr*-mediated colistin resistance, have been documented in isolates from humans, food animals, retail meats and environmental samples, underscoring the interconnected reservoirs that drive onward transmission (Lima T., *et al.*, 2019; Hassan ER., *et al.*, 2022; Egorova A., *et al.*, 2023). Experimental and genomic studies illuminate how large MDR plasmids persist and evolve within *Salmonella* populations under variable antibiotic selection pressures, thereby stabilizing resistance traits even when direct selection is intermittent (Cheng M., *et al.*, 2024). A One Health framing highlights key transmission routes and intervention points: clonal expansion of resistant serovars in food animal production (notably poultry), cross-sectoral movement of mobilizable plasmids, and contamination of shared environments (water, soil) which act as secondary reservoirs. Genome-scale analyses of emerging clones, for example the pESI-associated *S. Infantis* lineage, provide concrete examples of plasmid-driven MDR that links animal production with human infections (Mattock J., *et al.*, 2024). These observations argue for integrated laboratory networks that combine phenotypic surveillance with whole-genome sequencing across human, veterinary and environmental sectors. Given this

landscape, the research priorities are clear: (i) resolve the eco-evolutionary drivers of plasmid acquisition and maintenance in *S. enterica*; (ii) quantify cross-sector transmission pathways that sustain MDR lineages; and (iii) design evidence-based stewardship and biosecurity interventions targeted at the most influential nodes in the One Health network. Operationalizing these priorities requires harmonized surveillance, data sharing, and coordinated antimicrobial stewardship across clinical, agricultural and environmental domains to prevent regression to eras when effective therapy was limited (GRAM Typhoid Collaborators 2024).

1.4.8 Biobanks

Over recent decades, the emergence and re-emergence of pathogenic agents have highlighted the need to better understand microbial evolution and adaptation. In this context, historical biobanks storing microbial strains from clinical, environmental, and food sources are invaluable for molecular epidemiology, evolutionary surveillance, and the study of pathogenicity and antimicrobial resistance mechanisms (De Paoli P., *et al.*, 2005, Muzambi R., *et al.*, 2022). These collections offer a unique opportunity to compare current strains with those isolated in the past, enabling retrospective analyses that inform both scientific understanding and public health strategies (De Paoli P., *et al.*, 2005; Medina PB., *et al.*, 2022). In addition to supporting evolutionary studies, biobanks contribute to improving surveillance systems by allowing assessment of data collection practices (e.g., standardized case definitions, harmonized antimicrobial susceptibility testing, consistent metadata reporting) and their impact on pathogen monitoring and disease characterization at the population level (Delpy L., *et al.*, 2024; Gentile B., *et al.*, 2020; Postiglione U., *et al.*, 2023). Moreover, historical biobanks provide a basis for evaluating the effectiveness of interventions aimed at reducing pathogen spread, such as food safety regulations, animal vaccination programs, and hygiene policies (Siddique A., *et al.*, 2024). Biobanks have played a particularly important role in the study of a zoonotic pathogen with significant public health relevance worldwide. Large-scale strain collections have been used to reconstruct the evolutionary trajectories of epidemic clones, to investigate the emergence of multidrug resistance, and to link genomic variations with changes in agricultural practices and antimicrobial usage. For instance, retrospective analysis of archived *S. enterica* serovar Typhimurium isolates has allowed the reconstruction of plasmid-mediated antimicrobial resistance evolution across decades, revealing how resistance determinants such as ESBL and PMQR genes became established and disseminated (Alikhan *et al.*, 2018; Petrovska L., *et al.*, 2016). Similarly, studies of *S. enterica* serovar Infantis strains preserved in European biobanks have traced the emergence of the globally distributed pESI megaplasmid, highlighting the role of historical collections in identifying the drivers of MDR clone expansion (Mattock J., *et al.*, 2024). Beyond resistance, *Salmonella* biobanks have facilitated genomic epidemiology by allowing direct comparisons between contemporary outbreak strains and historical isolates, thereby clarifying the origins of epidemic lineages and informing source attribution models (Achtman M., *et al.*, 2012; Deng X., *et al.*, 2016). These

investigations underscore the importance of long-term preservation infrastructures, which not only safeguard microbial biodiversity but also provide a platform for modern genomic and phenotypic investigations using advanced technologies. Taken together, microbial biobanks represent a cornerstone for One Health–oriented pathogen research. By bridging past and present through preserved isolates, they enable a deeper understanding of microbial adaptation and provide a foundation for robust surveillance systems, evidence-based interventions, and preparedness for future infectious disease challenges.

1.5 Molecular epidemiology

Traditional serotyping of based on slide agglutination has been used for decades worldwide (Shi C., *et al.*, 2015), and it is still considered the gold standard method for serotyping. According to this phenotype-based approach, the surface antigens are detected by agglutination of bacterial cells using specific antisera (Shi C., *et al.*, 2015). Traditional serotyping is labor intensive, and it requires trained technicians to provide valuable data (Shi C., *et al.*, 2015). Another limitation of this method, which leads to inconclusive results, is a possible loss of expression of antigens required for definitive serovar identification (for example rough strains) (Fitzgerald C, *et al.*, 2007). For all these reasons, molecular methods for serotyping have been developed. The primary goal of molecular typing is to distinguish genetically distinct strains that are indistinguishable by conventional phenotypic methods. This differentiation is crucial for molecular epidemiology, specifically for outbreak investigation and understanding pathogen population dynamics. Epidemiological analysis lacking a molecular component is considered incomplete and insufficient to define the epidemiological landscape accurately. Modern molecular techniques offer an integrated approach, surpassing traditional methods by enabling precise characterization of pathogen clonality, traceability, and evolution. Species-level identification and subtyping inform diagnostics, treatment, and surveillance. It is essential that typing methods utilize molecular markers that are epidemiologically relevant and evolve at appropriate rates. Two key performance metrics in molecular epidemiology are:

1. Discriminatory power, which reflects a method's ability to differentiate unrelated isolates sampled randomly from the same species.
2. Epidemiological concordance, the likelihood that epidemiologically linked isolates are grouped within the same clone.

A method with excessive discriminatory power may over-separate strains from the same outbreak, while insufficient concordance could obscure true epidemiological linkage. Therefore, both conventional and molecular data should align for robust conclusions. Optimal molecular markers should be selected based on their relevance to epidemic events.

In salmonellosis, molecular typing methods span restriction enzyme-based (e.g., PFGE), gene amplification techniques (e.g., MLST, CRISPR-MVLST), and sequencing approaches (e.g., WGS, SNP-based typing). These strategies vary significantly in resolution, speed, and applicability. Recent evaluations demonstrate that whole-genome sequencing (WGS) based methods provide superior discriminatory power and outbreak detection compared to traditional typing, and have become fit-for-purpose tools in public health surveillance (Chattaway MA., *et al.*, 2023). Instead, comparative analyses confirm that CRISPR-MVLST offers high discrimination and is practical for routine surveillance along food production chains (Pan H., *et al.*, 2022). Overall reviews reaffirm WGS as the current gold standard for subtyping in both clinical and food safety contexts (Deng X., *et al.*, 2016; Tang S., *et al.*, 2019).

1.5.1 Restriction enzyme-based methods

Among molecular typing methods based on DNA restriction digestion, the most traditional include Restriction Fragment Length Polymorphism (RFLP), ribotyping, and Pulsed-Field Gel Electrophoresis (PFGE). RFLP, often involving Southern blotting of rRNA gene fragments, is laborious, time-consuming, and requires large quantities of DNA, making it poorly suited for rapid outbreak investigations. Classic ribotyping analyzes restriction patterns around ribosomal operons hybridized with rRNA-targeted probes, and strains classification is based on ribopatterns. This technique has a good discriminatory power, is reproducible and broadly applicable, but equally slow and manual. Automated ribotyping platforms exist, yet their high equipment cost limits widespread use. PFGE has long been considered the gold standard for subtyping many foodborne pathogens. It involves digesting the entire bacterial genome with rare-cutting restriction enzymes to generate large DNA fragments (8-15), which are separated using pulsed-field electrophoresis to produce strain-specific banding patterns. PFGE offers strong discrimination and sensitivity to genomic rearrangements but suffers from extended processing times (≈ 72 hours) and requires high technical expertise (Nadon C., *et al.*, 2017; Deng X., *et al.*, 2016).

1.5.2 Genetic amplification methods

The most widely used molecular typing method in this category is Random Amplified Polymorphic DNA (RAPD), which employs PCR with arbitrary short primers under low-stringency conditions to amplify multiple random genomic regions, yielding a unique electrophoretic band profile per isolate. Its discriminative power derives from sensitivity to reaction conditions. While simplicity, low cost, speed, and broad applicability are advantages, RAPD suffers from poor reproducibility. In fact, profiles are unstable unless strains are processed concurrently under identical conditions and being comparable in the same session. Nevertheless, when optimized and used in controlled settings, RAPD remains useful for targeted outbreak analysis (Sultana KF., *et al.*, 2021; Martinez-Puchol S., *et al.*, 2021).

1.5.3 Sequencing methods

Sequencing is a powerful molecular technique for characterizing genetic polymorphisms across conserved and variable genomic regions. Its greatest advantage over band-based profiling methods lies in reproducibility and standardization. Then, the sequences obtained are unambiguous, can be stored in online databases, and easily compared across laboratories. Multiple Locus Variable Number of Tandem Repeat Analysis (MLVA) is a sequencing-based subtyping method that examines variability in tandem repeat counts at specific genomic loci. MLVA is relatively simple, cost-effective, discriminative, and its standardizable nature ensures reproducibility and cross-lab comparability. This technique remains valuable for targeted outbreak investigations due to its reproducibility and high throughput capacity, even as Whole Genome Sequencing (WGS) becomes more prevalent. It provides rapid results at lower cost and is particularly well-suited for resource-constrained settings (Tang S., *et al.*, 2019; Ikeuchi, *et al.* 2024). Regarding another important technique, Multi-Locus Sequence Typing (MLST), was used for the first time in *Neisseria meningitidis*, quickly became a benchmark molecular method for both epidemiological investigations and studies of pathogen evolution (Maiden MCJ., *et al.*, 1998). MLST sequences fragments (~500 bp each) of approximately ten *housekeeping* genes, assigning allele types per locus and encoding strain genotypes as numeric allele profiles. Sequence data are digitally portable, enabling the development of global, publicly accessible databases that facilitate inter-laboratory comparison. MLST continues to serve as a robust framework for evolutionary studies and global epidemiologic surveillance, especially when complemented by WGS which enhances resolution while retaining data portability (Achtman M., *et al.* 2012; Xu, *et al.* 2022).

1.5.4 Whole-Genome Sequencing

Whole Genome Sequencing (WGS) has emerged as a transformative technology in microbiology, providing complete genomic information from bacterial isolates. Its versatility makes it applicable across multiple domains, including foodborne disease surveillance, food inspection, outbreak investigations, and source attribution studies. A major advantage of WGS is its capacity to simultaneously support species identification, high-resolution subtyping, detection of virulence factors, and antimicrobial resistance (AMR) profiling within a single workflow (Sammarco ML., *et al.*, 2014). Importantly, datasets generated by WGS can be retrospectively reanalyzed, which is critical for monitoring the emergence of new pathogens and understanding their evolutionary dynamics over time. Numerous studies have confirmed the superior discriminatory power of WGS compared with conventional subtyping methods, allowing for the detection of fine-scale genomic variation that often remains undetectable with traditional typing approaches (Scaltriti E., *et al.*, 2015; Ronholm J., *et al.*, 2016; Allard MW., *et al.*, 2018). The development of WGS is tightly linked to the advances in next-generation sequencing (NGS) platforms. The first NGS technology introduced to the market was the 454 FLX pyrosequencing system, released by 454 Life Sciences in 2005. Illumina launched the Solexa Genome Analyzer in 2007, followed shortly by the SOLiD platform from Applied Biosystems. Since then, several other platforms have been developed, including Helicos' HeliScope, the Ion Torrent PGM (Life Technologies), and Pacific Biosciences' long-read sequencing technology. These platforms differ significantly in sequencing chemistry, read length, throughput, and error profiles. For instance, SOLiD technology offers extremely high throughput but generates shorter reads, which limits its utility in complete genome assemblies. Conversely, platforms such as 454 and Illumina provide longer reads, making them more suitable for metagenomic applications and *de novo* genome projects (Sabat AJ., *et al.*, 2013; Sammarco ML., 2014; Goodwin S., *et al.*, 2016; Logsdon GA., *et al.*, 2020). The continuous refinement of these technologies, particularly in long-read sequencing from Pacific Biosciences and Oxford Nanopore, has expanded the ability to resolve complex genomic structures, plasmids, and mobile genetic elements, which are key in understanding bacterial pathogenicity and resistance mechanisms. Over the last decade, WGS has proven invaluable as a complement to phenotypic antimicrobial susceptibility testing (AST) in AMR surveillance. By identifying antimicrobial resistance genes (ARGs), WGS enables the prediction of resistance

phenotypes across a broad range of bacterial species (Sørensen LH., *et al.*, 2024; Ellington MJ., *et al.*, 2017). While genotypic data often correlate well with resistance phenotypes, phenotypic testing still plays an important role, as it captures resistance mechanisms that may be independent of genetic determinants, for example, those driven by environmental conditions, regulatory changes, or epigenetic factors (Hughes and Andersson, 2017; Doyle RM., *et al.*, 2020). Conversely, genotypic predictions provide crucial insights into the molecular mechanisms underlying AMR and can help identify possible pathways through which resistance spreads across bacterial populations (Price JR., *et al.*, 2023). The application of WGS in AMR surveillance has not only improved our understanding of resistance at the strain level but has also provided insights into the global dissemination of resistance determinants (Argimón S., *et al.*, 2020). By mapping mobile genetic elements such as plasmids, transposons, and integrons, WGS allows researchers to track how resistance genes move within and between bacterial species, which is fundamental for implementing effective public health interventions. WGS has revolutionized outbreak detection and source attribution in food safety. Its unprecedented resolution enables the differentiation of closely related isolates, which is essential for identifying outbreak strains and tracing them back to their sources. For instance, WGS has been successfully applied in the investigation of *Listeria monocytogenes*, Shiga toxin-producing *Escherichia coli* (STEC), and *Salmonella* spp., outperforming pulsed-field gel electrophoresis (PFGE) and other conventional subtyping methods (Allard MJ., *et al.*, 2018). The capacity to detect virulence genes and AMR determinants in the same dataset further enhances its utility in public health response strategies. In Canada, the integration of WGS into national surveillance systems significantly improved the detection of epidemiological clusters for *L. monocytogenes*, STEC, and *Shigella* (Morton V., *et al.*, 2024). This enhanced discriminatory power, however, also increased the number of identified clusters, thereby imposing a heavier workload on public health authorities. The adoption of a One Health framework has been instrumental in addressing these challenges by facilitating data integration across human, animal, and environmental health sectors (Morton V., *et al.*, 2024). Globally, countries such as the United States, members of the European Union, Australia, and China have adopted WGS as a cornerstone of their food safety surveillance frameworks. The World Health Organization (WHO) has also recognized the transformative potential of WGS, incorporating it into the Global Antimicrobial Resistance Surveillance System (GLASS) and publishing a dedicated guide in 2023 to support its integration into

foodborne disease surveillance and response systems (WHO, GLASS; 2023). WGS-based AMR surveillance has revealed important insights into the role of mobile genetic elements in the dissemination of resistance. For example, a nationwide study in the Philippines highlighted that carbapenem resistance in *Klebsiella* spp. was predominantly driven by plasmid-mediated mechanisms, underscoring the critical role of WGS in identifying transmission pathways and informing infection control interventions (Argimón S., *et al.*, 2020). Beyond outbreak detection and AMR, WGS also provides valuable data for pathogen evolution, population structure, and host adaptation studies. The ability to retrospectively mine genomic datasets enables the monitoring of emerging lineages and the assessment of long-term trends in virulence and resistance evolution. Additionally, WGS data are increasingly being integrated into predictive models and machine learning approaches to anticipate the emergence and spread of high-risk clones (Gomes A., *et al.*, 2025). The benefits of WGS are evident: unprecedented resolution for source tracking, simultaneous characterization of virulence and AMR determinants, retrospective reusability of data, and integration across One Health surveillance systems. However, challenges remain in terms of cost, data standardization, bioinformatics infrastructure, and international data sharing. These barriers must be addressed to fully realize the potential of WGS in global public health.

1.5.5 Phylogenetic analyses

Phylogenetic methods are essential for analyzing sequence data, to elucidate molecular epidemiology, transmission dynamics, and pathogen evolution. Recent advances in phylogenetic inference and software tools enhance our capacity to unravel epidemic emergence patterns and support public health responses. In fact, through phylodynamic analyses, it is possible to examine pathogen evolutionary dynamics within populations, and tracing geographic and temporal spread (phylogeography) which are increasingly critical in this context (Baele G., *et al.*, 2018). Phylogenetic trees distill these complex data into interpretable structures that are essential to design infectious disease control strategies. The concept of phylodynamics, introduced by Grenfell in 2004, describes how host immune pressure, transmission bottlenecks, and epidemic trends jointly shape pathogen evolution (Grenfell BT., *et al.*, 2004; Ingle DJ., *et al.*, 2021; Lefrancq N., *et al.*, 2025). Commonly, phylodynamics is based on a temporal model, which reflects the accumulation of mutations during time. This model is definable as “molecular clock” and make it possible to correlate genetic distances with time, and date evolutive divergence events. Strict clock models assume uniform evolutionary rates, whereas relaxed clock models allow rate variation among lineages (Baele G, 2017; Baele G., *et al.*, 2018). Heterochronous sequence data are long time sampled sequences, which facilitate coalescent-based inferences that trace pathogen population dynamics across time and host scales. Coalescent theory links lineage ancestry to effective population size, enabling reconstruction of demographic history (Featherstone LA., *et al.*, 2022). Human demographic shifts and migration further mold pathogen phylogenies, underscoring the importance of phylogeography, whose integration of genetic and spatial data reveals infection origins and transmission pathways. Clade structure in *S. enterica* has been elucidated through core-genome phylogenetics and revealed two principal clades: A and B. They were identified using 93 core-genome loci, and *clade* A is subdivided into three *subclades*: Typhi, Agona, and a third group. In particular, *subclade* Typhi includes serotypes *S. Typhi*, *S. Paratyphi A*, *S. Missisipi* and *S. Adelaide*. *Clade* A lacks the β -glucuronidase operon, whereas Typhi subclade members possess SPI-18 and *cdtB*, which facilitate epithelial cell invasion and cytolethal distending toxin (CDT) -mediated pathogenesis. These two are present also in *Clade* B, which retains β -glucuronidase (Worley J., *et al.*, 2018; Bawn M., *et al.*, 2020; Achtman M., *et al.*, 2021). Recent studies utilizing WGS of diverse *S. enterica* isolates have

refined phylogenies, revealing new subclades and extensive horizontal gene transfer, including phage and plasmid variability (Worley J., *et al.*, 2018), and uncovering polyphyly and genetic diversity among serovars, transforming public health surveillance frameworks (Yin Z., *et al.*, 2020). Phylogeographic inference has also traced the emergence of geographically distinct *S. Enteritidis* lineages in South Korea since 2020 (Shin E., *et al.*, 2025). Molecular evolutionary studies remain indispensable for understanding pathogen biology and adaptation. Yet, since many evolutionary mechanisms remain unresolved, interpretations must be made cautiously.

1.6 *Salmonella* Choleraesuis: Characteristics, Pathogenesis and Clinical Implications

Different serovars generally display distinct antigenic formulas; however, exceptions exist. The antigenic formula 6,7:c:1,5 is shared by serovars Paratyphi C, Typhisuis, and Choleraesuis. Within *S. Choleraesuis*, three biotypes have been described, distinguishable only by biochemical characteristics, which poses significant challenges when rapid characterization is required (Longo A., *et al.*, 2019; Chiu CH., *et al.*, 2004). Specifically, biotyping subdivisions based on H₂S production and mucate/dulcitol utilization have defined three variants: Choleraesuis sensu stricto, Choleraesuis var. Kunzendorf, and Choleraesuis var. Decatur (Le Minor L., *et al.*, 1985). *S. Choleraesuis* belongs to group C1 in the White-Kauffmann classification and is a swine-adapted serovar; among the three biotypes, Choleraesuis var. Kunzendorf is the predominant type responsible for porcine infections (Pedersen K., *et al.*, 2015). This pathogen is the etiological agent of swine paratyphoid, a disease characterized by enterocolitis and often fatal systemic illness. In domestic pigs, it is frequently associated with pneumonia and occurs particularly in weaned pigs younger than five months of age (Soliani L., *et al.*, 2023). Importantly, *S. Choleraesuis* can survive and remain infectious in the environment, leading to persistent farm contamination and reinfection of newly introduced animals. Contaminated food or water may also serve as reservoirs of human infection. Experimental and field studies demonstrated that infected pigs could develop a carrier state lasting up to twelve weeks (Gray JT., *et al.*, 1996; Anderson RC., *et al.*, 1998). Moreover, secondary diseases occurring during PRRSV (Porcine Reproductive and Respiratory Syndrome Virus) outbreaks are thought to result from reactivation of quiescent *S. Choleraesuis* can cause infections or reinfections originating from subclinically infected pigs (Charerntantanakul W., 2012). Although not strictly host-restricted, *S. Choleraesuis* is capable of causing invasive disease in humans. While human infections remain poorly documented in Europe, the epidemiological situation is markedly different in Asia, where numerous cases have been reported (Bangtrakulnonth A., *et al.*, 2004; Chang YF., *et al.*, 2005; Sriyapai P., *et al.*, 2021). In Thailand, between 1988 and 1993, *S. Choleraesuis* ranked as the tenth most common serotype causing human salmonellosis (Bangtrakulnonth A., *et al.*, 2004), whereas in Taiwan it was the second most frequent in two epidemiological surveys. A transient decline between 1996 and 1998 was attributed to a foot-and-mouth disease outbreak, which led to the extensive culling

of pigs. Molecular typing studies demonstrated that most human and swine isolates shared highly similar DNA fingerprints, suggesting zoonotic transmission through contaminated food or water (Chiu CH., *et al.*, 2004). Human infections caused by *S. Choleraesuis* are frequently invasive and often progress to septicemia (Chiu CH., *et al.*, 2004; Sirichote P., *et al.*, 2010). A retrospective study of adult patients with *S. Choleraesuis* bacteremia revealed that most had underlying risk factors such as malignancies, liver cirrhosis, systemic lupus erythematosus, or corticosteroid therapy; notably, 21% developed focal infections including septic arthritis, pneumonia, peritonitis, or cutaneous abscesses (Chiu CH., *et al.*, 2004). From a clinical perspective, reviews of *Salmonella* aortitis indicated that the most common serotypes were Typhimurium, Enteritidis, and Choleraesuis, in decreasing order. More recent studies confirm and extend these observations. A surveillance study conducted in Taiwan between 2004 and 2022 documented that *S. Choleraesuis* decreased from being one of the most frequent serotypes (seventh in 2004-2005) to being virtually absent from human isolates in 2022, likely due to farm-level control measures and changes in agricultural practices (Liao YS., *et al.*, 2024). Nevertheless, both human and swine isolates continue to exhibit high levels of antimicrobial resistance, particularly against cefotaxime and ciprofloxacin (Liao YS., *et al.*, 2024). Earlier studies in Taiwan already showed that fluoroquinolone resistance in *S. Choleraesuis* rose sharply, with resistance rates to ciprofloxacin reaching 60–70% in both human and swine isolates; genomic typing revealed *gyrA* mutations and close genetic relatedness between pig and human strains (Chiu CH., *et al.*, 2002; Chiu CH., *et al.*, 2005). A clinical report highlighted the emergence of community-acquired invasive *S. Choleraesuis* infections resistant to both ciprofloxacin and ceftriaxone, mediated by CMY-2 β -lactamase production, posing a serious therapeutic challenge (Ko WC., *et al.*, 2004). More recently, a case of chest wall abscess and costochondritis due to *S. Choleraesuis* was described, underscoring that focal extracavitary manifestations remain possible in modern clinical practice (Mokraoui NR., *et al.*, 2023). These updated clinical data confirm that *S. Choleraesuis* remains an important zoonotic pathogen with a marked propensity for invasiveness, frequent progression to bacteremia, and focal complications, especially in individuals with underlying comorbidities. Rising antimicrobial resistance, particularly to fluoroquinolones and third-generation cephalosporins, now represents a major therapeutic challenge, reducing empirical treatment options and highlighting the importance of continuous surveillance in both human and animal sectors.

1.6.1 Pathogenesis and Cellular Interactions

Serotype *Choleraesuis* can survive, multiply, and cause bacteremia after ingestion by macrophages. The carrier state of serotype *Choleraesuis* in swine has been described following experimental infection. Infected pigs usually exhibit clinical signs within 36 to 48 hours post infection and shed 10^3 to 10^6 CFU of bacteria per gram of feces during peak clinical disease (Chiu CH., *et al.*, 2004). Most naturally exposed pigs, after recovery, were able to clear the pathogen between nine- and twelve-weeks post infection, indicating that a long-term carrier status is an uncommon event (Gray JT., *et al.*, 1996; Anderson RC., *et al.*, 1998). An experimental and natural infection model in weaned pigs has been developed, showing that oral inoculation with 10^8 CFU of *S. Choleraesuis* induced acute illness and the recovery of the bacterium from ileocolic lymphonodes at necropsy seven days post challenge (Anderson RC., *et al.*, 1998). This serotype colonizes and invades the intestinal epithelium, disseminates to peripheral organs and causes septicemia in pigs, similarly to what is observed with serotype Typhimurium in mice. Histologically, *S. Choleraesuis* shows a predilection for the colonic mucosa and the luminal surface of ileal M cells within Peyer's patches, and its invasive capacity has been confirmed by the presence of large numbers of labeled bacteria within macrophages in the enteric mucosa and in the associated lymphonodes (Hyland KA *et al.*, 2006; Chiu CH., *et al.*, 2004). Persistence within porcine macrophages does not appear to correlate directly with virulence (Watson R., *et al.*, 2000), as this serotype is highly virulent to pigs but persists at lower levels compared to serotype Typhimurium. The fact that *S. Choleraesuis* is much more likely to cause sepsis than *S. Typhimurium* may not be due simply to virulence and the ability to escape the intestinal barrier. For example, survival in blood cells may prolong infection, increasing the concentration of inflammatory cytokines produced by these cells and the development of the acute phase of sepsis (Ibrahim H, *et al.*, 2018). This supports the view that host specificity of serotype *S. Choleraesuis* does not correlate with invasion of the porcine intestinal mucosa. Its interaction with porcine polymorphonuclear leukocytes (PPMN) has been well characterized in vitro, showing that both virulent and avirulent strains can be killed, although virulent strains survive more effectively (Cheminay .C, *et al.*, 2004). Furthermore, PPMN functions are markedly suppressed in the presence of both virulent and avirulent strains, as both could prevent degranulation and inhibiting H_2O_2 production (Stabel TJ., *et al.*, 2002). Only a few animals and in vitro studies have investigated

the role of cellular immunity in host defense against serotype Choleraesuis, but they provide significant insights. Foss *et al.* demonstrated that both in vivo and ex vivo infection of the intestinal mucosa with this serotype led to a decrease in the amount of interleukin-18 (IL-18), consistent with cleavage of the precursor protein by caspase-1. Indeed, caspase-1-mediated activation of IL-18 (and IL-1 β) is crucial for systemic host defense against *Salmonella* infection (Raupach B., *et al.*, 2006). IL-18 is a cytokine structurally and functionally related to IL-12, it induces IFN- γ secretion and was originally identified in mice as an IFN- γ inducing factor. Caspase-1 activation of IL-18 may therefore represent a crucial step in mucosal immunity to *S. Choleraesuis*. The role of IL-15 was investigated by Hirose and colleagues using IL-15-depleted mice through anti-IL-15 antibody administration, and their results indicated that IL-15 is involved in early protection by activating NK cells at infected sites (Hirose K., *et al.*, 1999). Moreover, studies using mice deficient in T cells showed that these cells play an important role in the pathogenesis of lethal infection with serotype Choleraesuis, and mice lacking T cells were found to be resistant to lethal infection with this serotype, suggesting a complex role of T-cell subsets in disease outcome (Emoto H., *et al.*, 1995). Excessive tumor necrosis factor alpha production, usually detrimental in gram-negative infections, was not evident in T-cell-deficient mice, which may at least partly explain their resistance to lethal infection. Interestingly, most of these studies were performed using avirulent strains lacking the 50-kb virulence plasmid (pSCV) and characterized by high LD₅₀ in mice. It has been postulated that a plasmid-borne virulence factor may impair cellular immune responses, while Th1-associated cytokines appear to play a protective role against infections caused by plasmid less strains, being associated with increased NK-cell activity and IFN- γ production. In addition, Ibrahim and colleagues investigated the immune response to two host-adapted *S. Typhimurium* strains (14028 and 4/74) and three non-host-adapted *S. Choleraesuis* strains (A50, A45, and B195) in human monocytes between 2 and 24 h postinfection (p.i.) to better understand the higher prevalence of sepsis associated with *S. Choleraesuis* (Ibrahim H., *et al.*, 2018). Both serovars induced production of cytokines typically associated with acute sepsis, including TNF- α , IL-1 β , and IL-6, but *S. Choleraesuis* strains generally triggered significantly higher levels than *S. Typhimurium* ($P = 0.01$ – 0.05). Moreover, all *S. Choleraesuis* strains markedly increased IL-10 secretion by monocytes at 6 and 24 h compared with *S. Typhimurium* ($P < 0.01$). Monocyte survival was also greater after infection with *S. Choleraesuis* A50 ($\approx 80\%$ viable cells at 24 h) than with *S. Typhimurium* ($\approx 40\%$). Further qPCR microarray analysis revealed differential

expression of genes within the JAK/STAT signaling pathway between the two serovars. As elevated IL-10 levels and enhanced monocyte survival are recognized markers of sepsis progression, these findings suggest that exaggerated IL-10 induction by *S. Choleraesuis*, particularly strain A50, may explain its higher propensity to cause invasive disease in humans.

1.6.2 Evolution of the Virulence Plasmid

Serotype *Choleraesuis* typically harbors a 50-kb virulence plasmid (pSCV), but recent isolates from both pigs and humans carried larger plasmids of 125 to 140 kb. These larger plasmids were confirmed to be derived from the 50-kb pSCV through PCR analysis of deletion regions, and they also contained additional DNA fragments from other plasmids of 75 and 90 kb. Most clinical isolates harboring these plasmids were resistant to multiple antibiotics (De Lorenzi G., *et al.*, 2025). At least two resistance genes, *sul* and *bla*_{TEM-1}, conferring resistance to sulfonamides and ampicillin, respectively, were present on the enlarged plasmids, apparently acquired through recombination. This plasmid-mediated acquisition of resistance genes constitutes a clear example of plasmid evolution and poses a significant public health concern. Virulence plasmids sit at hubs of genetic exchange, and surveillance reports coupled with genomic analyses show that pSCV-like backbones can acquire integrons, transposons and resistance cassettes yielding expanded plasmids that combine *spv* carriage with multidrug resistance determinants, a trend that has been epidemiologically linked to outbreaks and to hard-to-treat invasive disease, particularly in East Asia, reinforcing the public-health importance of monitoring the content and mobility of these replicons in agricultural and clinical ecosystems (Chiu *et al.*, 2004; Alikhan *et al.*, 2018). Such plasticity complements island-level variation on the chromosome and together these layers of acquisition and loss provide a coherent genomic explanation for the swine-adapted biology of *S. Choleraesuis* and its marked propensity for extraintestinal disease in humans (Chiu CH., *et al.*, 2004; McClelland M., *et al.*, 2001). Plasmids pSC138 and pSCV50 were first found in *S. Choleraesuis* SC-B67 (Ye J., *et al.*, 2011; Yu H., *et al.*, 2006). The pSC138, a Rep₃ replicon plasmid, carries a truncated Qin prophage, transposons Tn1721, Tn6088 (Tn21-like), Tn6091, and Tn6092, and conjugative transfer/maintenance *tra* and *trb* operons. The Tn6092 is inserted at the conjugative region, and the insertion could be the reason why pSC138 is non-conjugative (Ye J., *et al.*, 2011). The pSCV50 carries two replicons, RepFIB and RepFIIA, *rglA/scv_50* genes (encoding resolvase/integrase) and a truncated conjugative transfer *tra* operon. Thus, pSCV50 is also a non-conjugative plasmid (Yu H., *et al.*, 2006; Chu C., *et al.*, 1999). Building on this, Chen and colleagues determined the complete sequence of plasmid pOU7519 from *S. Choleraesuis* strain OU7519, revealing a 127,212 bp chimeric structure derived from pSCV50 and pSC138 (Chen C., *et al.*, 2017). This plasmid is conjugative and carries multiple mobile

genetic elements, including prophages, insertion sequences, integrons, and notably a Tn6088-like transposon. Analysis of the recombination junction showed defective sequences in the *int* gene and its attachment site (*att*), likely preventing excision and stabilizing the pSCV50-pSC138 chimera. Similar chimeric structures were identified in other large plasmids. These findings highlight that irreversible, stable virulence–resistance chimeras can emerge in *S. Choleraesuis*, posing a serious risk to both animal and human health by coupling multidrug resistance with enhanced pathogenic potential.

1.6.3 Antimicrobial Resistance

Antimicrobial resistance (AMR) in non-typhoidal *Salmonella* serotypes represents a major global public health concern. Until the 1980s, conventional drugs such as ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole were widely used; however, since then, multidrug-resistant (MDR) strains have been increasingly reported (Chiu CH., *et al.*, 2004). From a One Health perspective, AMR also poses a serious threat to animal health and livestock production. In swine, *S. Choleraesuis* has been of particular concern, especially in Asia, where this serovar is more prevalent (Chang CC., *et al.*, 2005). Antimicrobial therapy is necessary for the treatment of serovar *Choleraesuis* infections, because approximately 78% of the infected individuals in Taiwan developed bacteremia with little or no intestinal involvement (Chang CC., *et al.*, 2005). In Taiwan, over 90% of clinical *S. Choleraesuis* isolates were resistant to ampicillin and trimethoprim-sulfamethoxazole, leading to recommendations for fluoroquinolones and extended-spectrum cephalosporins as the drugs of choice for treatment (Chu C., *et al.*, 2005; Chiu CH., *et al.*, 2004). Unfortunately, since the early 2000s, strains resistant to ciprofloxacin and ceftriaxone have emerged in Taiwan (Su LH., *et al.*, 2011), with resistance rates rising to 59% by 2002. The first ciprofloxacin-resistant isolate also displayed intermediate resistance to ceftriaxone (Jean SS., *et al.*, 2005; Sun HY., *et al.*, 2005), leaving imipenem as the only effective therapeutic option at that time (Sriyapai P., *et al.*, 2021). At the molecular level, fluoroquinolone resistance has been primarily associated with point mutations in *gyrA* and *parC*, while resistance to extended-spectrum cephalosporins is mediated by extended-spectrum β -lactamases (ESBLs), including CTX-M and CMY-2 enzymes (Cuypers WL., *et al.*, 2018; Gu Y., *et al.*, 2021; Li WC., *et al.*, 2005; Wang J., *et al.*, 2025). Resistance determinants are frequently plasmid-borne, as plasmids act as vehicles carrying conjugation genes and mobile genetic elements such as transposons, integrons, and prophage sequences (Frost LS., *et al.*, 2005). Plasmids can be transferred horizontally across strains and species (Harrison E., *et al.*, 2012), thus accelerating the spread of AMR. Emerging plasmids combining both virulence and resistance traits in *Salmonella* from clinical and agricultural settings represent a growing threat to public health (Tzeng JL., *et al.*, 2015). For example, in one CMY-2-producing *S. Choleraesuis* strain, ceftriaxone resistance was linked to a 140-kb F-like plasmid harboring a class 1 integron with *dfr* and *aadA2* genes (Chiu CH., *et al.*, 2004). This strain also showed resistance to ciprofloxacin, making carbapenems the last

therapeutic option (Wang J., *et al.*, 2025). Alarming, multi drug-resistant *Salmonella* strains and transfer of genes implied in AMR is well documented across strains and species (Campos J., *et al.*, 2016). In Europe, AMR in *S. Choleraesuis* isolated from pigs has also been documented, though data remain limited (De Lorenzi G., *et al.*, 2025; Gil-Molino A., *et al.*, 2020; Ernholm L., *et al.*, 2022; EFSA and ECDC, 2017). However, it's extremely important to surveillance antibiotic resistances in reservoirs. In swine, recent European data shows an high level of spreading resistant strains (Figure 8).

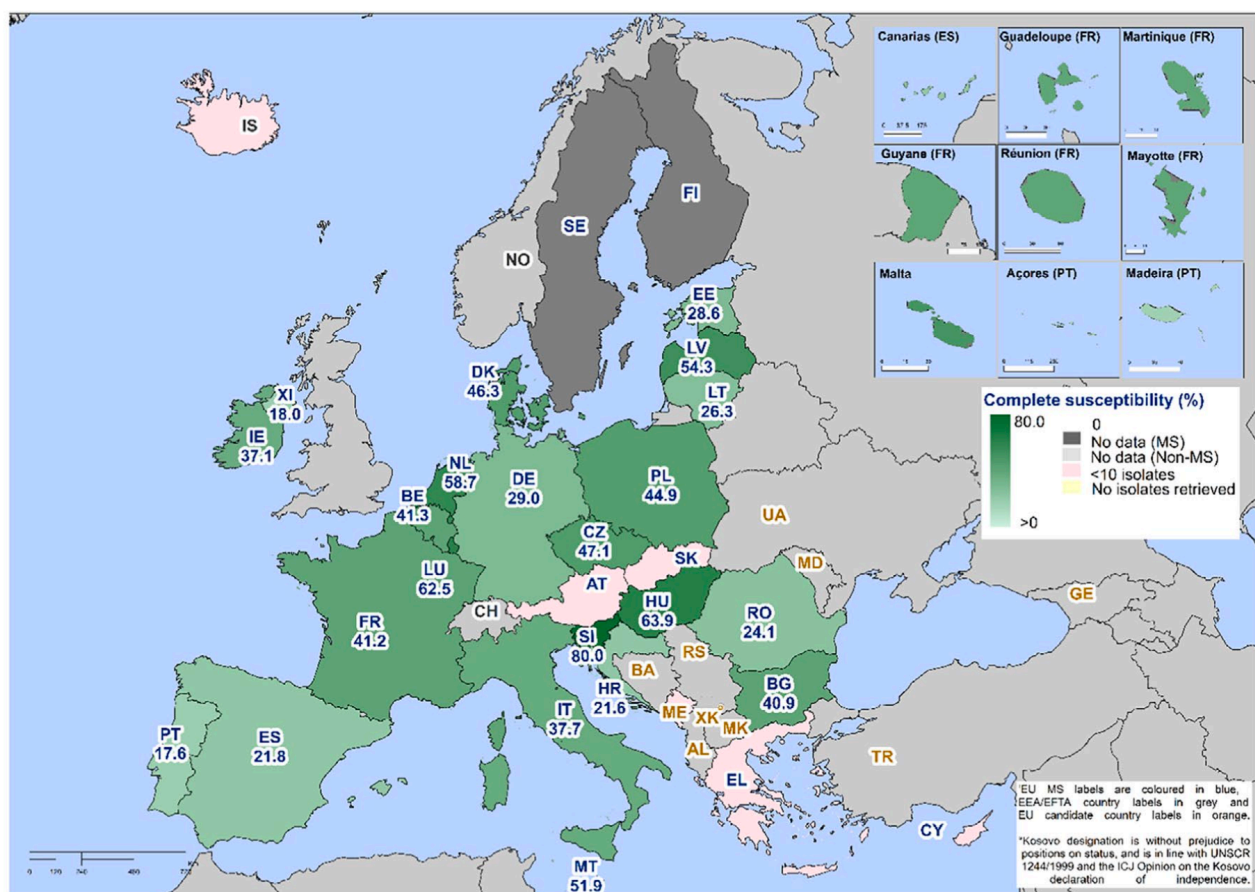


Figure 8: Spatial distribution of complete susceptibility to the selected antimicrobials tested among *Salmonella* spp in pigs. Maps are presented only when at least four Member States reported data. 'No data' refers to the absence of reported data by a MS or non-MS for a given matrix in a given reporting year; 'No isolates retrieved' refers to the MSs or non-MSs that tested for the presence of spp. but retrieved no isolates in a given matrix in a given year (ECDC/EFSA 2024).

To mitigate the spread of resistance and support rational antibiotic use, Italy recently developed national guidelines for the prudent use of antimicrobials in pigs (Bassi F., *et al.*, 2025). These guidelines recommend that antimicrobial therapy against *S. Choleraesuis*

infections be guided by in-vitro susceptibility testing. Among susceptible agents, first-line antimicrobials should be prioritized, including sulfonamides (alone or potentiated), spectinomycin, tetracyclines, and phenicols. Second-line options such as amoxicillin or amoxicillin-clavulanic acid should only be considered if first-line drugs are ineffective. Critically important antimicrobials for human health, such as third- and fourth-generation cephalosporins or fluoroquinolones, should be reserved as third-line treatments, to preserve their efficacy and minimize selective pressure (Bassi F., *et al.*, 2025).

1.6.4 Vaccination and Control Strategies

Vaccination against *S. Choleraesuis* has been widely implemented in China, where the live attenuated strain C500 has been used for decades to prevent paratyphoid fever in piglets. Although effective and immunogenic, C500 is associated with side effects, prompting the development of safer derivatives such as SC3, engineered to improve immune responses and safety (Zhu L., *et al.*, 2017; Bian X., *et al.*, 2024). In particular, the SC3 strain incorporates multiple genetic modifications, including $\Delta fur9$, $\Delta pagL7$, $\Delta pagP81::Plpp$ $lpxE$, and $\Delta lpxR9$ mutations, that reduce endotoxin activity (via monophosphorylated lipid A) while enhancing IgG and secretory IgA responses; these modifications also support its use as a delivery vector for heterologous antigens, making SC3 both safer and versatile compared with C500 (Bian X., *et al.*, 2024). More recently, new C500-derived variants such as C520 (with *asd* and *crp* deletions) and C522, expressing a fusion of FedF adhesin and Shiga toxin B domain, have shown reduced toxicity, persistent mucosal colonization, and robust immunogenicity; notably, C522 provided full protection against both *S. Choleraesuis* challenge and F18+ STEC in pig models, thus offering dual protection (Liu G., *et al.*, 2023). Innovative prime-boost strategies have also been tested, combining an oral priming dose with attenuated *S. Typhimurium* $\Delta znuABC$ followed by an intramuscular booster with inactivated *S. Choleraesuis*, which showed superior protection compared to inactivated vaccines alone, as evidenced by reduced fever, decreased tissue colonization, and stronger immune responses (Alborali GL., *et al.*, 2017). Specifically, this heterologous regimen proved more effective at limiting weight loss, reducing colonization of cecum and ileocecal lymph nodes, and decreasing shedding of the attenuated strain (which became undetectable by day 18 post-vaccination), compared to homologous inactivated vaccines alone (Alborali GL., *et al.*, 2017). In Italy, in contrast, vaccination of pigs against *S. Choleraesuis* is neither practiced nor mandated by legislation due to its limited epidemiological impact. Control strategies rely instead on biosecurity, surveillance, and hygiene, with no strict legal requirement to cull or block herds, although such measures may be applied on a case-by-case basis in response to outbreaks. Nevertheless, recent studies in northern Italy (2022-2024) have documented rising antimicrobial resistance in *S. Choleraesuis* isolates from clinically diseased pigs, revealing high resistance rates to multiple antibiotic classes (e.g., sulfisoxazole ~90%, ampicillin ~85%, enrofloxacin ~83%,

florfenicol ~74%, tetracycline ~71%), highlighting the importance of non-antibiotic control measures, including potential vaccination strategies in the future (De Lorenzi G., *et al.*, 2025).

AIM OF STUDY

This doctoral project has the aim to valorize and expand the potential of the historical *Salmonella enterica* biobank preserved at the EpiSoMI laboratory and established during the operational period of the **Centro Enterobatteri Patogeni per l'Italia Settentrionale** (CEPIS), which has collected more than 15,000 clinical isolates since 1989 across Lombardy. Samples were accompanied by datasheets reporting demographic, clinical, and epidemiological metadata. The project initially set out as an epidemiological investigation of *S. enterica* infections in Lombardy, focusing on the period 2001–2016, during which the four predominant serovars were *S. Typhimurium*, its monophasic variant, *S. Enteritidis*, and *S. Napoli*. While the primary objective was to digitize and structure the archive into a searchable database capable of enabling retrospective epidemiological analyses, particular attention progressively shifted to analysis of invasive serotypes, as *S. Napoli* and *S. Choleraesuis*. *S. Choleraesuis* is a relatively minor serovar in terms of frequency but one associated with the highest invasiveness indices and hospitalization rates in the dataset. *S. Choleraesuis* is documented to have an elevated predilection for systemic infection. To investigate the basis of this marked invasiveness, the project digitized and structured the archive and coupled retrospective epidemiology with experimental and genomic analyses. Archived strains underwent viability checks, comparative fitness and acid-survival assays, and molecular typing by multilocus sequence typing (MLST) and whole-genome sequencing (WGS). MLST supplies robust sequence-based typing that complements serotyping, while WGS affords high-resolution phylogenetic reconstruction and reliable in-silico prediction of antimicrobial resistance, permitting verification of historical susceptibility data and situating results within a One Health framework. By standardizing and enriching the biobank, the study creates a lasting resource that links patient-level metadata with genotype and phenotype—an approach central to modern biobanking and retrospective molecular epidemiology. The goal is to identify mechanistic and epidemiological determinants of *S. Choleraesuis* invasiveness and to translate these insights into improved surveillance and targeted control measures under a One Health perspective.

Specific objectives

The study was structured around the following specific objectives:

- i. To digitize and organize the historical *Salmonella enterica* biobank by integrating microbiological strains with clinical, demographic, and epidemiological data.
- ii. To describe the epidemiology of the main serotypes circulating in Lombardy in the period 2001–2016, focusing on invasive infections.
- iii. To perform phenotypic tests to assess the fitness and acid survival of invasive serotypes, particularly *S. Choleraesuis* and *S. Napoli*.
- iv. To conduct MLST and whole-genome sequencing to reconstruct phylogeny, validate antimicrobial resistance profiles, and identify genetic markers linked to invasiveness.

2 MATERIAL AND METHODS

2.1 From the datasheet: epidemiological and clinical study

2.1.1 Data set up and definitions

We retrospectively analyzed datasheets reporting information regarding demographic and clinical data of cases, the samples analyzed, the serovar of *S. enterica* isolated obtained by hospitals or clinical laboratories and collected continuously for a long period for studies on the circulation and characterization of *Salmonella* spp. The information of the serotype, performed using Kauffmann and White's classification (Kauffmann F., et al., 1966), was obtained from datasheets. The datasheets reported the eader of Enter-Net Italia (Figure 9), the network of laboratory for the surveillance of enteric pathogens. Despite this, we do not find any documents to prove that the samples and the related forms represent a complete and systematic collection organized as part of a surveillance program. Overall, we found a collection of over 15,000 datasheets and related clinical strains of *S. enterica* isolated in the Lombardy region from 1989 to 2016.

Data analysis. Here we present the analysis of data from 2001 to 2016. Several records in the Enter-net forms were incomplete, and specific variables (e.g., *Salmonella* serovar, risk factors, clinical information) were not consistently reported. Therefore, for each analysis, the denominator was adjusted according to the number of records in which the variable of interest was available.

Evaluation of invasiveness index. To analyze and investigate the severity of the different serotypes we calculated the invasiveness index (II) with the following formula:

$$\text{Invasiveness Index} = \frac{\text{Number of blood (sterile-site) isolates of the strain (or serotype)}}{\text{Total number of isolates (or total cases) of that strain (or serotype)}} \times 100\%$$

The invasiveness index of a bacterial strain or serotype is the proportion of isolates of that strain/serotype that are recovered from sterile sites (e.g. blood, cerebrospinal fluid) relative to the total isolates (or total cases) of that strain/serotype. It is a measure of how often the strain causes invasive disease, compared to how often it is isolated in non-

invasive situations. We considered an infection as invasive when the pathogen is isolated from blood.

Definition of outbreak. An outbreak was defined as the occurrence of two or more epidemiologically linked cases which have common food consumption, kinship ties, closeness in geographical area and time.

Incidence. It is defined as the number of new cases detected in the study population in one year, expressed as cases per 100,000 individuals at risk per year.

$$\text{Annual incidence rate} = \frac{\text{Number of new cases in one year}}{\text{Population at risk during the same year}} \times 100,000$$

SCHEDA DI NOTIFICA FONTE UMANA

Sorveglianza degli enterobatteri patogeni: **SCHEDA PER STIPITI ISOLATI DA FONTE UMANA**

A. Origine e caratteristiche del campione biologico

A1. Laboratorio di origine		Prov.
A2. Prelievo effettuato nel comune di (località):		
Prov.		
A3. Codice dello stipite assegnato dal laboratorio di origine:	A4. Data prelievo del campione:	
A6. Motivo di esecuzione dell'esame colturale:		
☐ Non noto ☐ Inf. acuta ☐ Inchiesta epidemiologica ☐ Controllo		

U. Informazioni sugli stipiti di provenienza umana

U1. Tipo campione: ☐ Feci ☐ Sangue ☐ Altro specificare	
U2. Cognome e nome del paziente:	
U3. Sesso: ☐ Non noto ☐ Maschio ☐ Femmina	
U4. Data di nascita:	U5. Età, anni:
U6. Comune di residenza del paziente (località):	
Prov.	
U7. Viaggi effettuati nei 30 gg prima dell'insorgenza dei sintomi:	
☐ Non noto ☐ No ☐ Si (spec. dove)	
U8. Paziente ospedalizzato: ☐ Non noto ☐ No ☐ Si	
U9. Alimenti implicati:	
☐ Non noto ☐ No ☐ Si (spec.)	
U10. Se alimenti implicati, in base a:	
☐ Sospetto ☐ Dati epidemiologici ☐ Isolamento microbiologico	

C. Identificazione microbiologica e sensibilità agli antibiotici

C1. Identificazione:			
☐ Salmonella	☐ Shigella	☐ Campylobacter	
☐ Yersinia	☐ E. coli	☐ Altro (spec.)	
C2. Tipizzazione:			
C3. Sensibilità agli antibiotici (segnare in ciascuna casella S, I o R):			
NA	AM	CTX	CIP
K	S	S3	Te
KF	SXT		
			GM
			AMC

D. Parte riservata al laboratorio di riferimento (regionale o sovranazionale)

D1. Codice laboratorio:	D2. Codice assegnato allo stipite dal lab. di riferimento:		
D3. Tipizzazione finale:	D4. Data tipizzazione:		
D5. Sensibilità agli antibiotici (segnare in ciascuna casella S, I o R):			
NA	AM	CTX	CIP
K	S	S3	Te
KF	SXT		
D6. Cognome e nome del compilatore:			
Telefono:			
D7. Data di compilazione della scheda:			
D8. Note:			

Figure 9: e.g. Datasheets reporting data on cases of salmonellosis linked with *S. enterica* strains collected in the biobank.

2.2 From the biobank to the bench: focus on invasive serotypes

2.2.1 Fitness and Survival to acid pH

S. enterica strains. We compared invasive serotypes (IS) of *S. Choleraesuis* with IS of *S. Napoli* and *S. Typhimurium*. A total number of fifteen *S. enterica* clinical invasive strains were used to conduct experiments on fitness and acid survival. As reference strain we used *S. Typhimurium* ATCC14028. To evaluate characteristics of survival at gastric pH of *S. Choleraesuis* IS, we compared them with three *S. Napoli* strains from blood and six *S. Typhimurium* strains (three strains from stools and three strains from blood).

Table 2: *S. enterica* strains. ST = *S. Typhimurium*; SN = *S. Napoli*; SC = *S. Choleraesuis*. (b) = blood samples; (s) = stool samples. (*) Branchu P., *et al.*, 2018.

S. enterica strains	Year of isolation	Body district	City	Isolation date	Serotype	
STb1	2004	Blood	Milano	04/08/04	Typhimurium	From this study
STb2	2008	Blood	Milano	26/04/08	Typhimurium	
STb3	2012	Blood	Milano	29/09/12	Typhimurium	
STs1	2006	Stool	Como	25/01/06	Typhimurium	
STs2	2010	Stool	Cremona	22/05/10	Typhimurium	
STs3	2013	Stool	Milano	19/03/13	Typhimurium	
SN1	2006	Blood	Como	04/08/06	Napoli	
SN2	2010	Blood	Milano	23/08/10	Napoli	
SN3	2012	Blood	Monza Brianza	22/10/12	Napoli	
SC23	2010	Blood	Milano	06/05/10	Choleraesuis	
SC48	2012	Blood	Milano	22/07/12	Choleraesuis	
SC57	2013	Blood	Milano	23/03/13	Choleraesuis	
SC24	2010	Stool	Lodi	14/06/10	Choleraesuis	
SC26	2010	Prosthesis	Milano	21/06/10	Choleraesuis	
SC65	2016	Stool	-	-	Choleraesuis	
ATCC 14018			-		Typhimurium	(*)

Growth curves for fitness. *S. enterica* strains used for experiment are reported in Table 3 and were stocked at -80°C in Muller Hilton Broth (MH) and 10% of glycerol. An overnight culture is prepared in static at 37°C. To start the experiment, we dilute 1/200 the culture in MH broth. Every hour, for seven timepoints we read OD₆₀₀ and titered the growth. Both the growth rate ($\mu_{\max}$) during the exponential phase and the final OD (a proxy for carrying capacity) were used to characterize strain behavior. To calculate the specific growth rate (μ):

$$\mu = \frac{\ln(OD_{t2}) - \ln(OD_{t1})}{t_2 - t_1}$$

where:

- OD_{t1} and OD_{t2} = optical density (OD₆₀₀) values at two consecutive times (exponential phase),
- $t_2 - t_1$ time interval (in minutes or hours),
- μ = specific growth rate (min⁻¹).

The $\mu_{\max}$ value is the maximum among the values calculated along the growth curve.

***S. enterica* serotypes survival to acid pH.** We tested survival of serotypes to pH which mimic gastric acidity . Strains are reported in Table 3. We choose pH which mimic the condition before (pH 2) and after digestion (pH 4). We set an experiment starting by an overnight culture (static at 37°C). Cells were diluted 1/1000 in PBS, pH 4 and pH 2. Every 30 minutes (for a total of 2 h of treatment) we titered the cultures, centrifuge 5 min at 6000 RPM and resuspend in PBS 1x, to check the effect on different strains.

2.2.2 Check from the biobank of *S. Choleraesuis* strains

Cultural methods. *S. Choleraesuis* clinical strains, belonging to the period 2006-2015 (n=85), were picked up from our collection. First, we evaluated vitality of the strains, which could be compromised by time and storage, growing bacteria on Tryptone Soy Agar (TSA) or Tryptone Soy Broth (TSB). Then serovar *S. Choleraesuis* was confirmed by PCR.

Primer design. PCR primers for the specific detection of *S. Choleraesuis* were designed by Tsai-hsin Chiu, *et al.*, 2005 on the *fliC* gene and *viaB* gene. *FliC* gene is specific to distinguish *S. Choleraesuis* and *S. Paratyphi C* from other serovars, while *viaB* gene discriminates between these two serovars. *FliC*-F and *FliC*-R are 5'-AAG GAA AAG ATC ATG GCA CAA-3' and 5'-GAA CCC ACC ATC AAT AAC TTT G-3', while *viaB* primers as R1-F (5'-GTT ATT TCA GCA TAA GGA G-3') and R1-R (5'-ACT TGT CCG TGT TTT ACT C-3').

Colony PCR amplification. Bacteria cells were grown on TSA over-night at 37°C, and then a single colony was resuspended in 50 µl of distilled water. Cells were vortexed and lysed by heating at 100°C for 10 min. 5 µl of the cell dilution was mixed with the PCR solution composed as reported in Table 3. Final volume of the mixture was adjusted to 45 µl by addition of distilled water. PCR conditions were denaturation at 94°C for 10 min, then 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 7 min. PCR conditions for primers R1-F and R1-R were denaturation at 94°C for 10 min, then 35 cycles of 96°C for 30 s, 56°C for 15 s, and 74°C for 30 s, and final extension at 72°C for 7 min. The amplicons were analyzed by electrophoresis on a 2% agarose gel.

Table 3: PCR reagents and volumes.

Reagents	Volume
5X Green GoTaq® Reaction Buffer 150 mM Tris-HCl, pH 8.0 a 25 °C, 500 mM KCl (Promega)	10 µl
dNTP Mix 10 mM (Promega)	1 µl
Primer Forward (30 µM)	1 µl
Primer Reverse (30 µM)	1 µl
GoTaq® DNA Polymerase 5 U/µl (Promega)	0,25 µl
H₂O	31,75 µl

2.2.3 Invasive *S. Choleraesuis* strains: WGS and cgMLST

Whole genome sequencing (WGS) of serovar *Choleraesuis* strains isolated from human cases in Lombardy between 2006 and 2016 was performed at the IZSLER facility in Parma.

Genomic DNA extraction. Genomic DNA was extracted using Maxwell(R) HT 96 gDNA Blood purification kit (Promega, Madison, USA) following the manufacturer's protocol, which includes four steps: sample lysis, DNA binding, washing, and elution.

Sequencing library preparation. Extracted DNA was then used for sequencing library preparation. Genomic libraries were prepared using Illumina® DNA Prep (M) Tagmentation kit (Illumina, San Diego, CA), which simultaneously fragments DNA and ligates short adapter sequences through transposase-mediated tagmentation. This process enables anchoring of DNA fragments to the sequencing platform. Library preparation involved three main steps: (i) fragmentation and size selection, (ii) adapter ligation, and (iii) library quantification.

Whole genome sequencing. Paired-end raw reads (2 x 150 bp) were produced using Illumina Nextseq (Illumina, San Diego, CA), checked for quality and for bacterial species identity and then trimmed with Trimmomatic (Bolger AM., *et al.*, 2014).

In silico detection of resistance genes and genome assembly. Antibiotic resistance genes were *in silico* determined using Resfinder database via Staramr ver. 0.7.2 (Barath A., *et al.*, 2022; Zankari E., *et al.*, 2012). Species confirmation and possible contaminations were evaluated with Kraken2 (Wood DE., *et al.*, 2019). Filtered reads were assembled using Unicycler ver. 0.4.8 (Wick RR., *et al.*, 2017), and high-quality assemblies were evaluated by QUAST ver. 4.2 (Gurevich A., *et al.*, 2013), holding assemblies with coverage >30× and contig number < 300 (Timme RE., *et al.*, 2020).

Core-genome Multi-Locus Sequence Typing (cgMLST). For molecular typing, Multi-Locus Sequence Typing (MLST) was performed in silico from genome assemblies using the Pasteur BIGSdb for *Salmonella* spp. (https://pubmlst.org/bigsdb?db=pubmlst_Salmonella_seqdef).

Core genome MLST (cgMLST) analysis was conducted with BioNumerics software v7.6.3 (Applied-Maths, bioMérieux), applying the EnteroBase cgMLST scheme (Alikhan NF., *et al.*, 2018; Acthman M., *et al.*, 2012) and single-linkage clustering. The allelic distance threshold for cluster definition was set at AD = 5, in accordance with guidelines from the European Centre for Disease Prevention and Control and the European Food Safety Authority (EFSA and ECDC 2020; EFSA and ECDC 2021).

2.3 Statistical analysis.

To compare the frequency of binary outcomes between groups, contingency table analyses were conducted. Twoxtwo table analysis was performed using the OpenEpi online tool (version 3.01; <https://www.openepi.com>). Two-tailed p-values were calculated using Fisher's exact test or the chi-square test with Yates' correction, depending on the cell frequencies, following standard epidemiological guidelines. To assess associations between more than two categories, a chi-square test of independence was conducted. Data were arranged in an n×2 contingency table, and expected counts were calculated under the assumption of independence. Categories with fewer than 10 total observations were excluded to preserve the validity of the test. The chi-square statistic, and p-value were reported. This analysis was performed using Python 3.11, with the SciPy library (version 1.11.0). The *p* Value (two-tailed) from Fisher's exact test was calculated using the on-line GraphPad QuickCalcs resource (<http://www.graphpad.com/quickcalcs/contingency1.cfm>, accessed on 15 July 2025) to evaluate statistical differences between the compared groups. *p* Values ≤ 0.05 were considered significant.

A language-editing tool (ChatGpt.com) was used for minor textual refinements, as typographical adjustments, grammar correction, and stylistic smoothing. This limited and transparent use is consistent with current guidelines on AI-assisted reported in literature (Yoo JH., *et al.*, 2025; Elsevier guidelines)

3 RESULTS

3.1 Epidemiological and clinical study

3.1.1 Data from 2001 to 2016

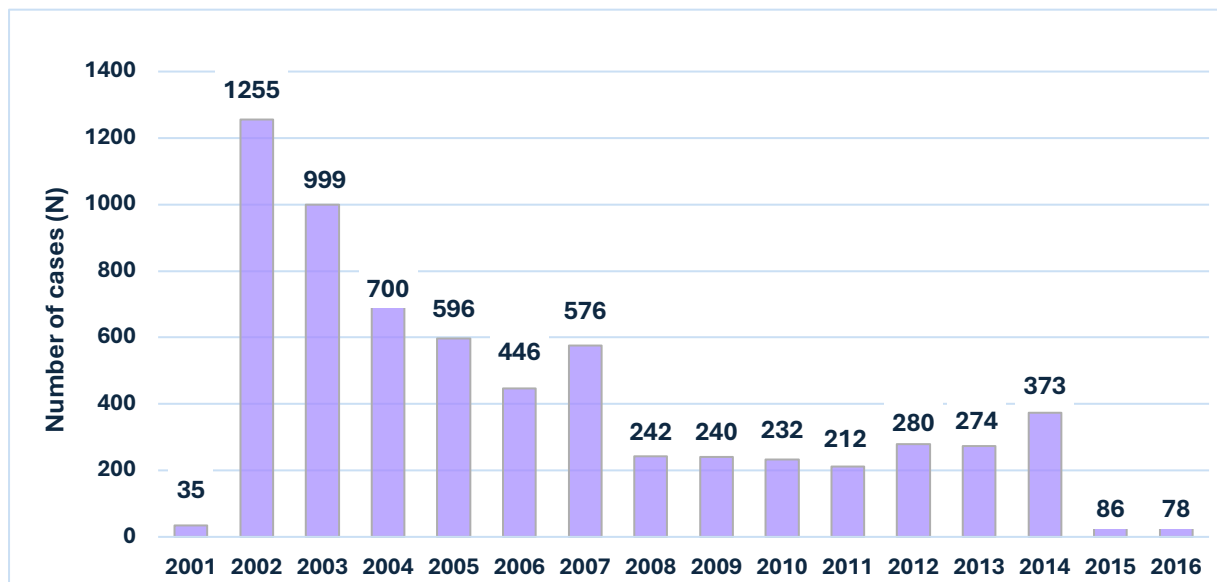


Figure 10: Number of cases of salmonellosis from 2001 to 2016 reported in the datasheets per year of isolation.

From 2001 to 2016 a total number of 6,624 cases of salmonellosis were reported in the datasheets archive and considered for this analysis. Some of the 6,624 Enter-Net datasheets were incomplete and the denominator used in each analysis varied according to the number of records in which the variable of interest was reported. Six thousand five hundred and sixty-six infections (99%, 6566/6624) were supported by 164 different non-typhoidal *Salmonella*. Four typhoidal *Salmonella* serotypes (*S. Typhi*, *S. Paratyphi A*, *B* and *C*) were reported in 58 datasheets. In Supplementary table 1 is reported the number of cases for serotype recorded during the study period. Figure 10 shows the total number of cases recorded for each year. As shown in the graph, the years 2001, 2015, and 2016 were not representative and recorded few cases compared to the rest of the period. This is because the collection of records was not systematic over the years, and our study collected this information aware of the bias. However, the large amount of data and information allowed to describe the trend of serotypes and their prevalence.

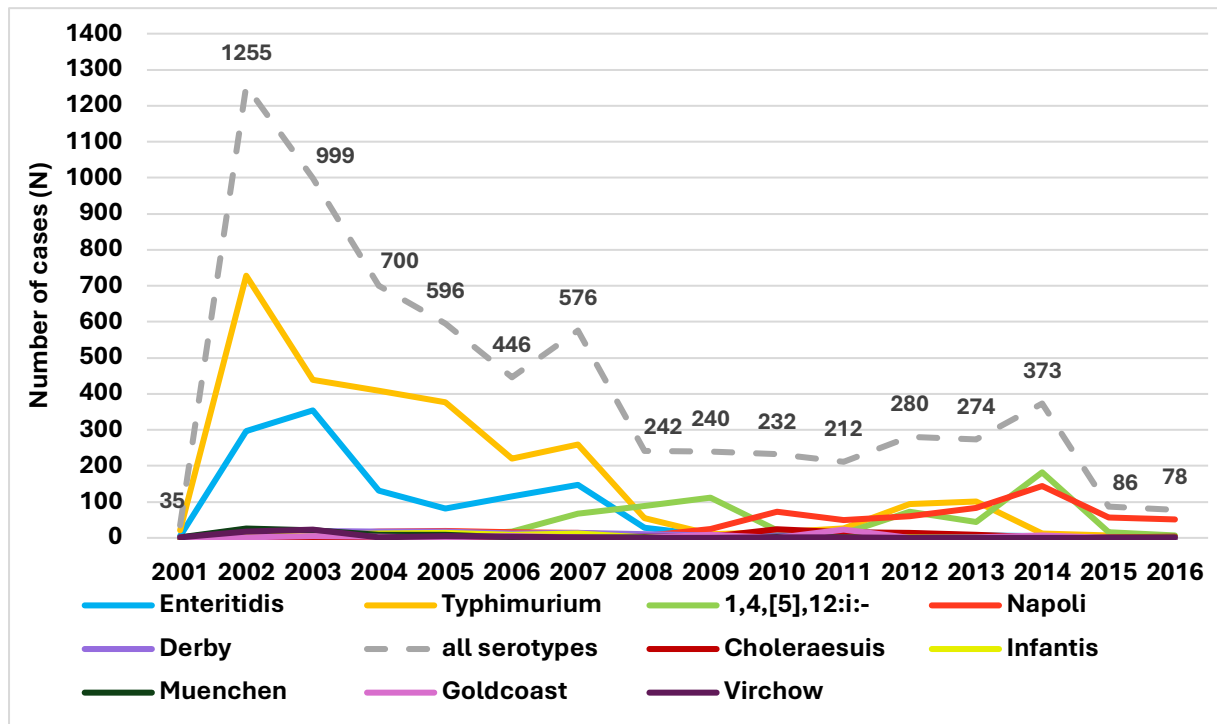


Figure 11: Number of salmonellosis cases per year from 2001 to 2016: trends of the ten most relevant serotypes circulating in that period.

In Figure 11 are reported the cases trend of the ten most isolated serotypes during the study period. Among the NTS serotypes, the four most frequently detected were *S. Typhimurium*, accounting for 41.8% (n=2,772) of cases, followed by *S. Enteritidis* with 18.5% (n=1,223), *S. 1,4,[5],12:i:-* (*S. Typhimurium* monophasic variant) with 9.8% (n=649), and *S. Napoli* with 9.6% (n=636). Considering a subset of 6,624 cases, these four serotypes collectively represented 79.7% (n=5,280) of *Salmonella* infections. During the period analyzed, there were three main peaks, in the year 2002, 2007 and 2014. The first and second peak given above all by serovars *S. Typhimurium* and *S. Enteritidis*, that were prevalent and gradually decreased since 2009, when we have a switch of these two serotypes with *S. 1,4,[5],12:i:-* and *S. Napoli*, which cases gradually increased until mainly support 2014 peak. Between 2010-2012, there was a decrease in cases supported by the four main serotypes with the introduction of minor serotypes, e.g. *S. Choleraesuis* increase 5-6 time in the number of cases in this period, compared to the decade. *S. Typhimurium* and its variant, *S. 1,4,[5],12:i:-*, tend to alternate each other's presence on the territory over the years. While serotype *S. Napoli* showed a gradual increase in number of cases starting in 2008, year corresponding to the almost total disappearance of the serovar *S. Enteritidis*, whose contribution to salmonellosis infections had been

fundamental in the first years analyzed. From the big amount of data in our possession, we obtained interesting picture of the circulation of *S. enterica* serotypes in Lombardy.

3.1.2 Cases description

Demographic data of patients was reported in 5,870 out of 6,624 (88.6%) datasheets. Salmonellosis affected male patients in 51.8% of cases. Figure 12 shows the age distribution of *S. enterica* cases, with a peak at 2 years of age (12%, 700/5,870). Children aged 0-4 years represent the highest proportion of infections, accounting for 37.7% (n=2,213) of cases (p value < 0.0001) (Table 4). Analyzing the age distribution across different serotypes, we observed variations depending on the serovar. The four most common serotypes (*S. Typhimurium*, *S. Enteritidis*, *S. 1,4,[5],12:i:-* and *S. Napoli*) primarily affected the 0-4 years age group, whereas others, such as *S. Choleraesuis*, were found in patients over 64 years old (Table 4).

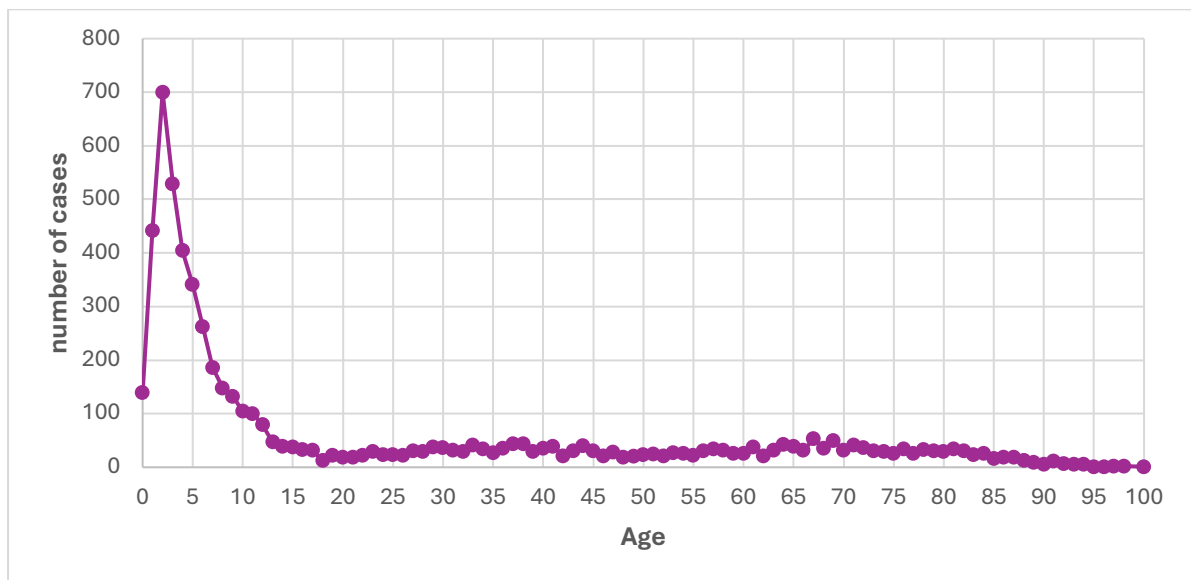


Figure 12: Age distribution of *Salmonella enterica* cases

3.1.3 Source of isolation.

The information on the source of isolation was reported in 5,853 out of 6,624 (88.4%) datasheets. The collected strains were isolated from various human samples, including stool, blood, urine, and others (bile, wound swab, pus, effusions and fluids from various body districts). *Salmonella* was detected in stool samples in 94.5% (5,529/5,853) of cases, in blood in 4.2% (244/5,853) and in other body districts in 1.4% (83/5,853) (Table 4). In the 0-4 year age group, 98.1% (2,067/2,106; $p < 0.0000001$) of infections remained localized to the gastrointestinal system, while in adults over 64 years of age a significant increase in blood isolates was observed (12.7%; 94/740; $p < 0.0000001$) (Figure 13). Among blood isolates, age was known in 206 cases (92.1%; 233/253). Most of these were isolated in individuals over 64 years old (40.3%; 94/233; $p < 0.0000001$). From stool samples, the predominant serotypes were *S. Typhimurium* (44.1%; 2,443/5,529) and *S. Enteritidis* (20.2%; 1,119/5,529). *S. Choleraesuis* was the most frequently isolated serotype from blood samples, detected in 24.6% (60/244) of cases, followed by *S. Typhimurium* (22.5%, 55/244) and *S. Napoli* (15.8%, 38/244)

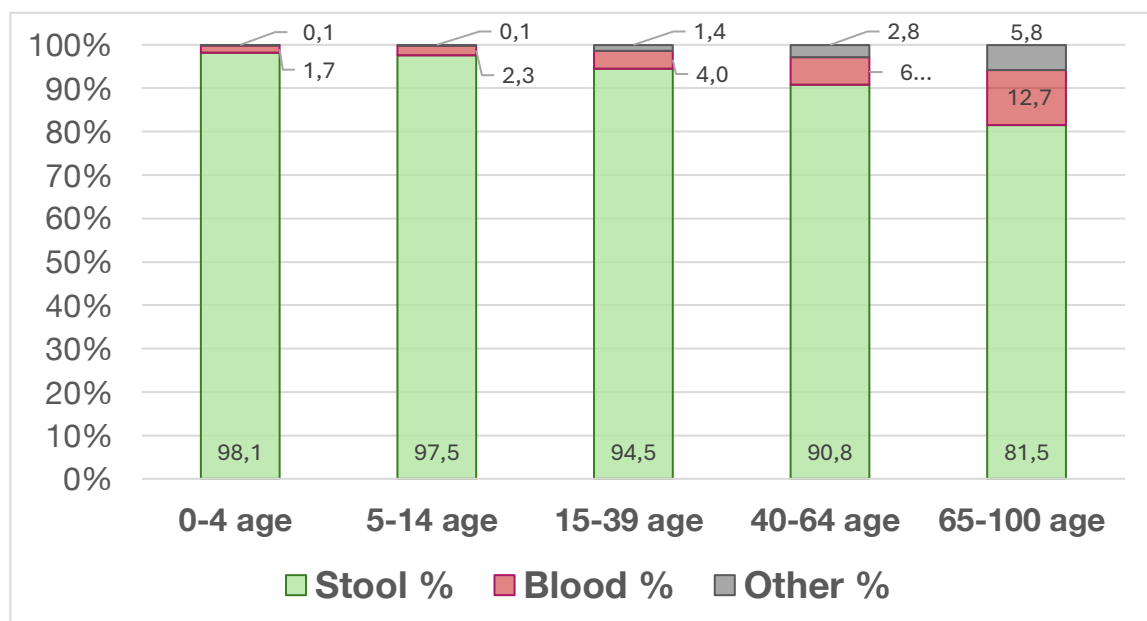


Figure 13: Distribution of sample origin type according to the age group.

3.1.4 The Invasiveness Index and hospitalization.

The Invasiveness Index (II) was calculated for invasive infections and reported in Table 4. *S. Choleraesuis* results to have the higher II (76.9% ;60/78), followed by *S. Napoli* for (8.1%; 38/470) and *S. Typhimurium* (2.2%; 55/2,518). Additionally, *S. Typhi* was identified in 9% (22/244) of bloodstream infections (imported cases with a history of travel in endemic area), with an II of 64.7% (22/34). A chi-square test of independence revealed a significant association between serotype and the proportion of invasive cases ($\chi^2 = 1244.18$, $p < 0.0001$), indicating that some serotypes (notably *S. Choleraesuis* and *S. Typhi*) are markedly more invasive than others. In Figure 14 is reported the age distribution of invasive cases isolated from blood of the two most invasive serotypes.

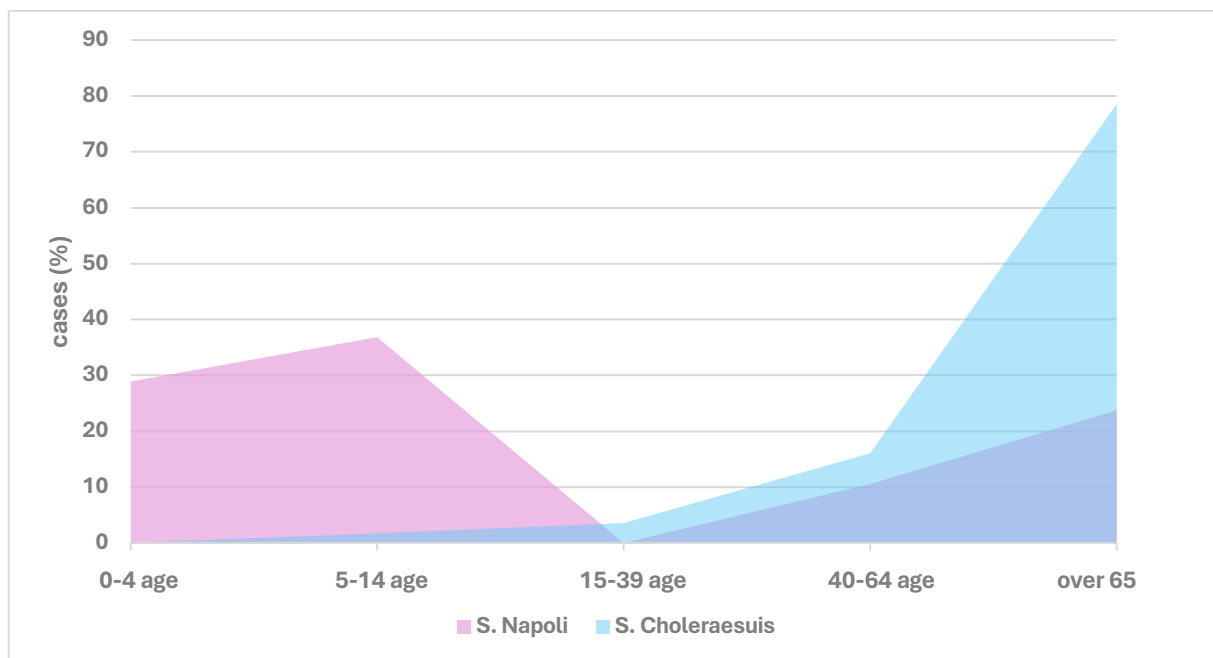


Figure 14: Age distribution of invasive cases in *S. Napoli* and *S. Choleraesuis*

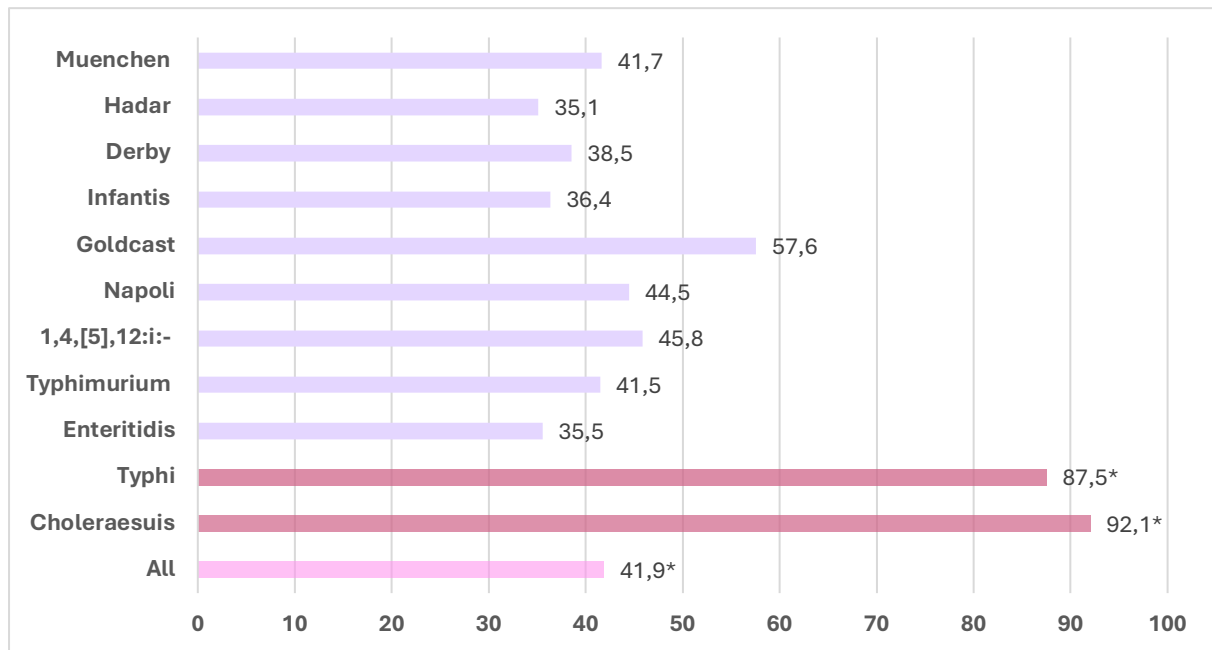


Figure 15: Hospitalization rate of 10 most representative *S. enterica* serotypes.

Hospitalization data, available for 4,345 patients, showed that 1,821 (42%) required hospital care and treatment. The four most common serotypes showed a range of hospitalization from 35.5% - 45.8% (Table 4). In Figure 15 *S. Choleraesuis* had the highest hospitalization rate (92.1% 58/63 vs 42%, 1.821/4.345; *S. Choleraesuis* vs all NTS, $p < 0.0000001$) comparable to that of *S. Typhi* (87.5%, 21/24). Analysis of the age distribution of hospitalized cases reveals, as expected, a pronounced peak in the range 0-4 years age group, consistent with the overall case distribution. However, by cross-referencing the data on hospitalized patients with the type of isolation sample (used as an indicator of the invasiveness and severity of the disease), in Figure 16 we observed a clear age-related pattern: the majority of stool isolates were from children aged 0-4 years (656/1,562; 42.0%), while the majority of blood isolates were from patients aged 64 years and older (82/167; 49.1%). This association between sample type and age group was statistically significant ($\chi^2 = 139.0$, $df = 4$, $p < 0.001$), indicating that gastrointestinal infections are more frequently detected in young children, whereas invasive infections tend to occur more often in the elderly. Follow-up visits were necessary for 387 patients (5.8%, $n=6,624$), with 6.5% ($n=25$) returning for a third visit and 0.8% ($n=3$) requiring a fourth. The age distribution of these cases did not show significant differences compared to the overall patient population, suggesting that follow-ups were not specifically associated with co-morbidities or frail patients. Cases with prolonged convalescence

were primarily associated with *S. Typhimurium* (51.2%, n=198), *S. Enteritidis* (12.9%, n=50), and *S. Napoli* (9.6%, n=37). In three cases, infections caused by the *S. Napoli*, *S. Typhimurium*, and *S. Typhi* serotypes were observed to persist for more than 2–3 months.

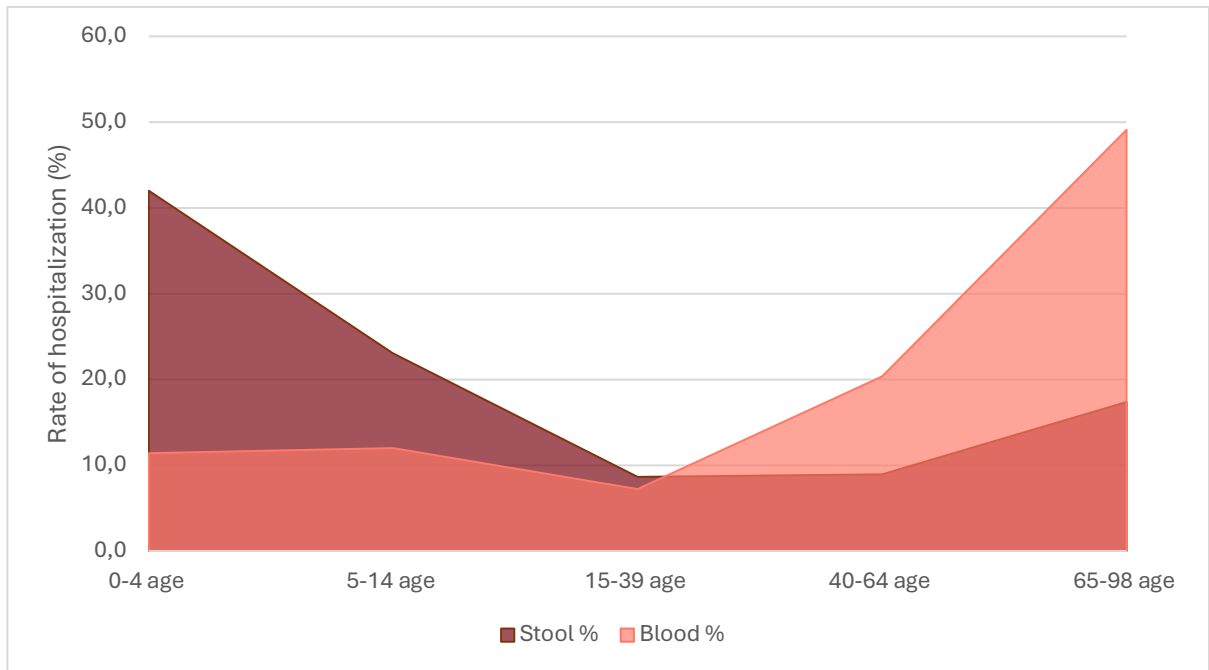


Figure 16: Stool and blood samples reveal different age distribution in hospitalized population.

3.1.5 Outbreaks, foodborne and travel-related infections

During the study period, a total of 52 foodborne outbreaks were recorded, involving 201 confirmed cases of *Salmonella* infection. The serotypes most frequently identified were *S. Enteritidis* (63.5%, 33/52) and *S. Typhimurium* (28.8%, 15/52), while other serotypes such as *S. Napoli*, *S. 1,4,[5],12:i:-*, *S. Muenchen*, and *S. Saintpaul* were responsible only for isolated, sporadic outbreaks. In 43 out of the 52 outbreaks (82.7%), the food sources could be identified. Eggs and egg-derived products represented by far the most common vehicle, being implicated in 65.1% of outbreaks (28/43). Other sources included meat (20.1%, 9/43), fish and crustaceans (4.7%, 2/43), ice cream (4.7%, 2/43), and water (2.3%, 1/43). Notably, the highest concentration of outbreaks occurred between 2002 and 2003, when 63.5% (n=33) of the total cases were reported. Parallel to outbreak investigations, additional information was collected on datasheets, regarding the possible source or food implicated in the infection. Data was available for 346 out of 6,624 cases (5.2%). Once again, *S. Enteritidis* (52.6%, 182/346) and *S. Typhimurium* (31%, 107/346) emerged as the most common serotypes linked to direct food consumption, confirming the central role of these two variants in human salmonellosis. Among the reported food vehicles, eggs were again the leading source, being implicated in 52.9% (183/346) of cases, reinforcing the consistent association between egg products and *Salmonella* infections. For few patients it was available travel history provided further depth to the epidemiological picture. Such data was available for 1,341 patients (20.2%), of whom 194 (14.3%) reported a recent travel. These cases were associated either with travel within Italy (46%, 89/194) or abroad (27.3%, 53/194). A distinction emerged in the distribution of serotypes by travel destination: *S. Typhimurium* was predominantly associated with domestic travel (54%, 48/89), whereas *S. Enteritidis* was more frequently linked to international travel (41.5%, 22/53). Interestingly, egg consumption during travel was reported as a potential source of infection in 19% of these cases (n=37), underlining that risk behaviors linked to dietary habits persist regardless of geographical setting.

Tabella 4: Data by datasheets

	all serotypes	S. Typhimurium	S. Enteritidis	S.1,4,[5],12:i:-	S. Napoli	S. Choleraesuis	S. Derby	S. Typhi
N Cases	6,624	2,772	1,223	649	636	82	148	37
Sex Distribution								
Available data	6304							
M (%)	3,265 (51.8)							
Distribution of serotypes for age								
Available data	5,870	2,566	1,146	420	499	76	135	29
0-4 age (%)	2,213 (37.7)	1,163 (45.3)	301 (26.3)	183 (43.6)	205 (41.1)	0 (0)	50 (37.0)	4 (13.8)
5-14 age (%)	1,438 (24.5)	679 (26.5)	283 (24.7)	119 (28.3)	159(31.9)	4 (5.3)	15 (11.1)	7 (24.1)
15-39 age (%)	736 (12.5)	242 (9.4)	269 (23.5)	38 (9.0)	24 (4.8)	3 (3.9)	7 (5.2)	13 (44,8)
40-64 age (%)	704 (12.0)	233 (9.1)	202 (17.6)	31 (7.4)	38 (7.6)	16 (21.1)	23 (17.0)	5 (17.2)
>64 age (%)	779 (13.3)	249 (9.7)	91 (7.9)	49 (11.7)	73 (14.6)	53 (69.7)	40 (29.6)	0 (0.0)
Type of samples and Invasiveness index								
Available data	5,853	2,518	1,150	433	470	78	135	34
Stool (%)	5,529 (94.5)	2,443 (97)	1,119 (97.3)	426 (98.4)	430 (91.5)	8 (10.3)	128 (94.8)	12 (35.4)
Blood, Invasiveness index*(%)	244 (4.2)	55 (2.2)	25 (2.2)	5 (1.2)	38 (8.1)	60 (76.9)	4 (3.0)	22 (64.7)
Others** (%)	83 (1.4)	20 (0.8)	6 (0.5)	2 (0.5)	2 (0.4)	10 (12.8)	3 (2.2)	-
Hospitalization								
Available data	4,345	1,919	889	253	353	63	96	24
Hospitalized (%)	1,821 (41.9)	796 (41.5)	316 (35.5)	116 (45.8)	157(44.5)	58 (92.1)	37 (38.5)	21 (87.5)
Not required	2,524	1,123	573	137	196	5	59	3
Travels and associated serotypes								
Available data	1,361	716	337	57	96	12	20	12
History of Travel	194 (14.3)	78 (10.9)	57 (16.9)	4 (7)	13 (13.5)	1 (8.3)	0	10 (83.3)
Distribution of serotypes (%)	194 (100)	78 (40.2)	57 (29.4)	4 (2.1)	13 (6.7)	1 (0.5)	0(0)	10 (5.2)
Compsuntion of food								
Available data	466							
Diclare a link with food	346 (100)	107 (30.9)	182 (52.6)	8 (2.3)	11 (3.2)	1 (0.3)	1 (0.3)	0 (0.0)
Implicated Food								
Water (%)			3 (0.9)					
Meat (%)			58 (16.8)					
Crustaceans and molluscs (%)			21 (6.1)					
Fish (%)			18 (5.2)					
Milk, butter and derivates (%)			12 (3.5)					
Eggs (%)			67 (19.4)					
Long-life canned foods (%)			2 (0.6)					
Fresh vegetables (%)			2 (0.6)					
Composite Food - eggs and milk (%)			101 (29.2)					
Composite Food - eggs (%)			16 (4.6)					
Egg-containing food			183 (52.9)					

3.1.6 Antimicrobial resistance.

Analyses on antimicrobial resistance were available for 5,057 isolates (76.3%), and 2,791 (55.2%) strains resulted to be resistant at least to one antibiotic. When looking at the temporal distribution of resistance, during the analyzed period, the percentage of resistant isolates ranged from 31.8% - 83.6% (Figure 17) with a marked peak particularly in 2008. However, giving a clear interpretation of the trend over the years it is complex and probably affected by incompleteness of the archive. Data analysis was complicated by the fact that every laboratory applied a different panel of antibiograms. Overall, 50 different antibiotics were applied between 2001 and 2016 and reported in the datasheets. Therefore, analyses on antimicrobial resistance were conducted by grouping the different antibiotics by drug class and reported in Table 6. Antimicrobial susceptibility testing of *S. enterica* isolates revealed heterogeneous resistance patterns across major antibiotic classes.

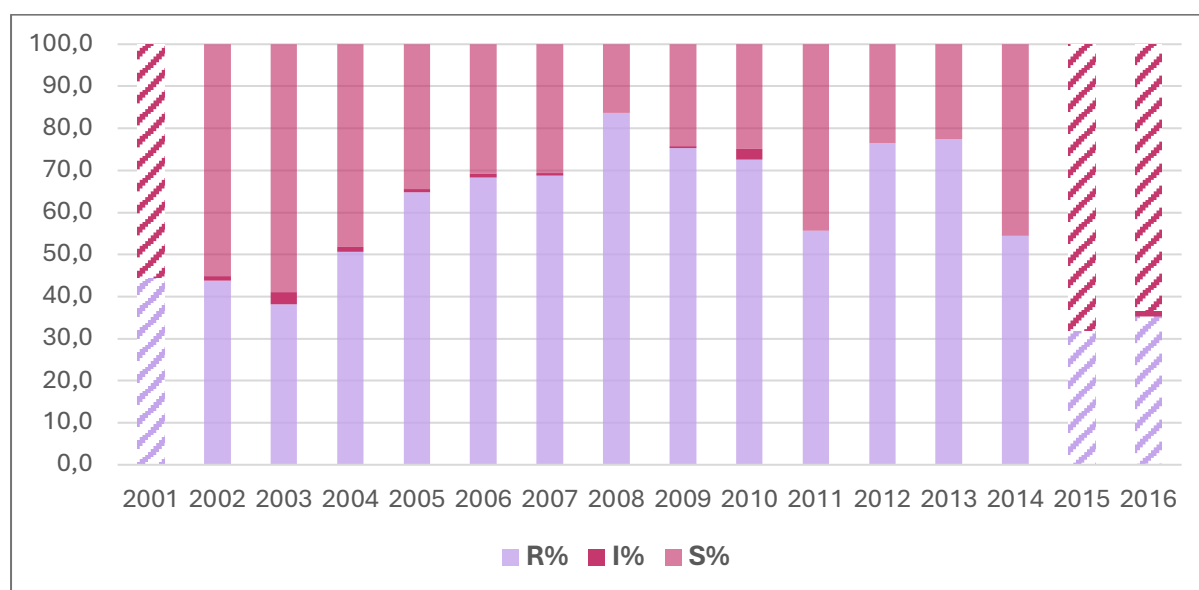


Figure 17: Percentages of R (Resistant), I (Intermediate) and S (Sensible) of *S. enterica* strains in the period 2001 – 2016. Years 2001, 2015 and 2016 were less representative in terms of numbers.

Table 5: Data on antibiotic resistance from datasheets.

Year	R % (N)	I% (N)	S % (N)	Not known (N)	Total cases (N)
2001	44.4 (12)	0.0 (0)	55.6 (15)	8	35
2002	43.7 (465)	1.1 (12)	55.1 (5896)	192	1255
2003	38.2 (344)	2.8 (25)	59.0 (531)	99	999
2004	50.6 (298)	1.2 (7)	48.2 (284)	111	700
2005	64.8 (324)	0.8 (4)	34.4 (172)	96	596
2006	68.3 (224)	0.9 (3)	30.8 (101)	118	446
2007	68.7 (349)	0.6 (3)	30.7 (156)	68	576
2008	83.6 (189)	0.0 (0)	16.4 (37)	16	242
2009	75.2 (155)	10.5 (1)	24.3 (50)	34	240
2010	72.6 (114)	2.5 (4)	24.8 (39)	75	232
2011	55.6 (74)	0.0 (0)	44.4 (59)	79	212
2012	76.5 (65)	0.0 (0)	23.5 (20)	195	280
2013	77.4 (65)	0.0 (0)	22.6 (19)	190	274
2014	54.4 (74)	0.0 (0)	45.6 (62)	237	373
2015	31.8 (14)	0.0 (0)	68.2 (30)	42	86
2016	35.2 (25)	1.4 (1)	63.4 (45)	7	78

Resistance to penicillins was the most pronounced, with 46.9% (2000/4264) of isolates showing reduced susceptibility, confirming a longstanding resistance reservoir within this class. In contrast, penicillins combined with β -lactamase inhibitors showed lower resistance (22.2%, 383/1729), suggesting partial preservation of efficacy. Resistance to tetracyclines/glycylcyclines was also high (48.0%, 651/1355), consistent with the extensive historical use of tetracyclines in both veterinary and human medicine. Aminoglycosides demonstrated moderate resistance (28.3%, 927/3272), though this class remained more active than penicillins and tetracyclines. Among β -lactams, resistance decreased with successive generations of cephalosporins: 17.0% (565/3329) for first-generation, 26.8% (251/936) for second-generation, but only 1.5% (41/2680) for third-generation and 2.2% (20/925) for fourth-generation agents. Monobactam (aztreonam) resistance was 1.4% (11/788), while carbapenems showed the lowest resistance rate (0.7%, 13/1768). Fluoroquinolone/quinolone resistance was relatively low overall (2.9%, 136/4711), though present in clinically relevant numbers. Macrolide resistance was 50% (2/4), though the small sample size limits interpretation. Polymyxin (colistin) resistance was absent (0/98), while resistance to phenicols (16.8%, 112/668) and sulfonamides/folate pathway inhibitors (10.2%, 480/4722) persisted at notable levels.

Occasional resistance to fosfomycin (2.7%, 4/150) and nitrofurantoin (14.8%, 4/27) was also observed. Overall, these results highlight the persistence of multidrug resistance traits in *S. enterica* during the study period, with particularly high resistance against penicillins and tetracyclines, contrasted by the retained effectiveness of third- and fourth-generation cephalosporins, carbapenems, and colistin.

Table 6: Antibiotic resistance of strains obtained from datasheets.

Antibiotic classes	Antibiotic tested	R (N)	Known data (N)	R%
Aminoglycosides	Amikacin, Gentamicin, Netilmicin, Tobramycin, Streptomycin, Kanamycin	927	3272	28.3
Beta - lactams				
Penicillins	Amoxicillin, Ampicillin, Cloxacillin, Oxacillin, Mezlocillin, Piperacillin, Ticarcillin.	2000	4264	46.9
Penicillins + β -lactamase inhibitor	Ampicillin/Sulbactam, Piperacillin/Tazobactam, Ticarcillin/Clavulanic acid	383	1729	22.2
First-generation cephalosporins	Cephalothin, Cefazolin	565	3329	17.0
Second-generation cephalosporins	Cefuroxime, Cefuroxime Axetil, Cefoxitin	251	936	26.8
Third-generation cephalosporins	Cefotaxime, Ceftazidime, Ceftriaxone, Cefixime, Cefpodoxime, Cefixime.	41	2680	1.5
Fourth-generation cephalosporins	Only Cefepime tested	20	925	2.2
Monobactams	Only Aztreonam tested	11	788	1.4
Carbapenems	Imipenem, Meropenem, Ertapenem	13	1768	0.7
Fluoroquinolones / Quinolones	Ciprofloxacin, Levofloxacin, Norfloxacin, Ofloxacin	136	4711	2.9
Macrolides	Only Erythromycin tested	2	4	50.0
Tetracyclines / Glycylcyclines	Tetracycline, Doxycycline, Tigecycline	651	1355	48.0
Polymyxins	Only Colistin tested	0	98	0.0
Phenicol	Only Chloramphenicol	112	668	16.8
Sulfonamides / Folate synthesis inhibitors	Cotrimoxazole, Trimethoprim, Sulfamethoxazole, Trimethoprim/Sulfamethoxazole	480	4722	10.2
Others:	Fosfomycin	4	150	2.7
	Nitrofurantoin	4	27	14.8

The antibiotic-resistance profile was calculated for *S. Choleraesuis* strains isolated from blood. A total of 46 isolates showed an antibiotic-resistance profile. The profile is shown in Table 7. Sulfonamides show a resistance rate of 90.5% (38/42), followed by tetracyclins and penicillins with a resistance rate of 90% and 87.0% (40/46). Third- and fourth generation cephalosporins show 0% resistance (0/41). In Supplementary Figure S1 there is the total AMR profile of all *S. Choleraesuis* antibiograms available (60/82).

Table 7: Classes of antibiotics tested and corresponding resistance percentages for *S. Choleraesuis* strains isolated from blood.

Antibiotic classes	Antibiotic tested	R (N)	Known data (N)	%R
Aminoglycosides	Amikacin, Gentamicin, Netylminicin, Tobramycin, Streptomycin, Kanamycin	19	25	76.0
Beta - lactams				
Penicillins	Amoxicillin, Ampicillin, Cloxacillin, Oxacillin, Mezlocillin, Piperacillin, Ticarcillin.	40	46	87.0
Penicillins + β -lactamase inhibitor	Ampicillin/Sulbactam, Piperacillin/Tazobactam, Ticarcillin/Clavulanic acid	3	15	20.0
First-generation cephalosporins	Cephalothin, Cefazolin	3	5	60.0
Second-generation cephalosporins	Cefuroxime, Cefuroxime Axetil, Cefoxitin	6	8	75.0
Third-generation cephalosporins	Cefotaxime, Ceftazidime, Ceftriaxone, Cefixime, Cefpodoxime, Ceftioxime.	0	41	0
Fourth-generation cephalosporins	Only Cefepime tested	0	11	0
Monobactams	Only Aztreonam tested	0	3	0
Carbapenems	Imipenem, Meropenem, Ertapenem	0	20	0
Fluoroquinolones / Quinolones	Ciprofloxacin, Levofloxacin, Norfloxacin, Ofloxacin	3	38	7.89
Tetracyclines / Glycylcyclines	Tetracycline, Doxycycline, Tigecycline	9	10	90.0
Phenicols	Only Chloramphenicol	0	9	0
Sulfonamides / Folate synthesis inhibitors	Cotrimoxazole, Trimethoprim, Sulfamethoxazole, Trimethoprim/Sulfamethoxazole	38	42	90.5

3.2 Focus on Invasive serotypes

Based on the analysis of available datasheets, we identified relevant information regarding strain invasiveness and serotype distribution. The serotypes most frequently associated with invasive infections were *S. Choleraesuis*, *S. Napoli*, and *S. Typhimurium*. Given their clinical relevance, we decided to further investigate these strains. Two complementary approaches were undertaken. First, we compared strains of the three selected serotypes with the reference strain ATCC 14018 to assess their relative fitness and tolerance to acidic conditions. For a pathogen such as *Salmonella*, able to adapt to multiple environments, including animal and human hosts as well as extra-host settings, such differences may confer a selective advantage in survival and transmission. In addition, we aimed to explore the propensity of *S. Choleraesuis* to disproportionately affect individuals over 65 years of age. We hypothesized that reduced gastric functionality in elderly patients may facilitate enhanced bacterial survival upon ingestion, thereby increasing infection risk. Subsequently, our focus shifted to evaluating the antibiotic resistance profiles of *S. Choleraesuis* isolates. Considering the current global concern over antimicrobial resistance, this aspect is of particular importance, especially in the context of infections affecting vulnerable populations. From the reference database, we identified eighty-five documented cases of salmonellosis attributed to *S. Choleraesuis* infection. Of these, seventy-three isolates were retrieved from the biobank. Sixty-nine strains (94.5%) were viable, and sixty-seven (97.1%) tested positive for the *fliC* gene, qualifying them for whole-genome sequencing (WGS). WGS was successfully completed for sixty-five isolates, and the resulting genomic data were integrated with physiological assays assessing bacterial fitness and survival under acidic conditions.

3.2.1 Growth curves and fitness of *S. enterica* strains

For this experiment, we tested and compared 13 strains:

- ◆ three *S. Typhimurium* strains from blood (STb)
- ◆ three *S. Typhimurium* strains from stool (STs)
- ◆ three *S. Choleraesuis* strains from blood (SCb)
- ◆ three *S. Choleraesuis* strains from stool (SCs)
- ◆ three *S. Napoli* strains from blood (SN)
- ◆ Reference strain *S. Typhimurium* ATCC 14028

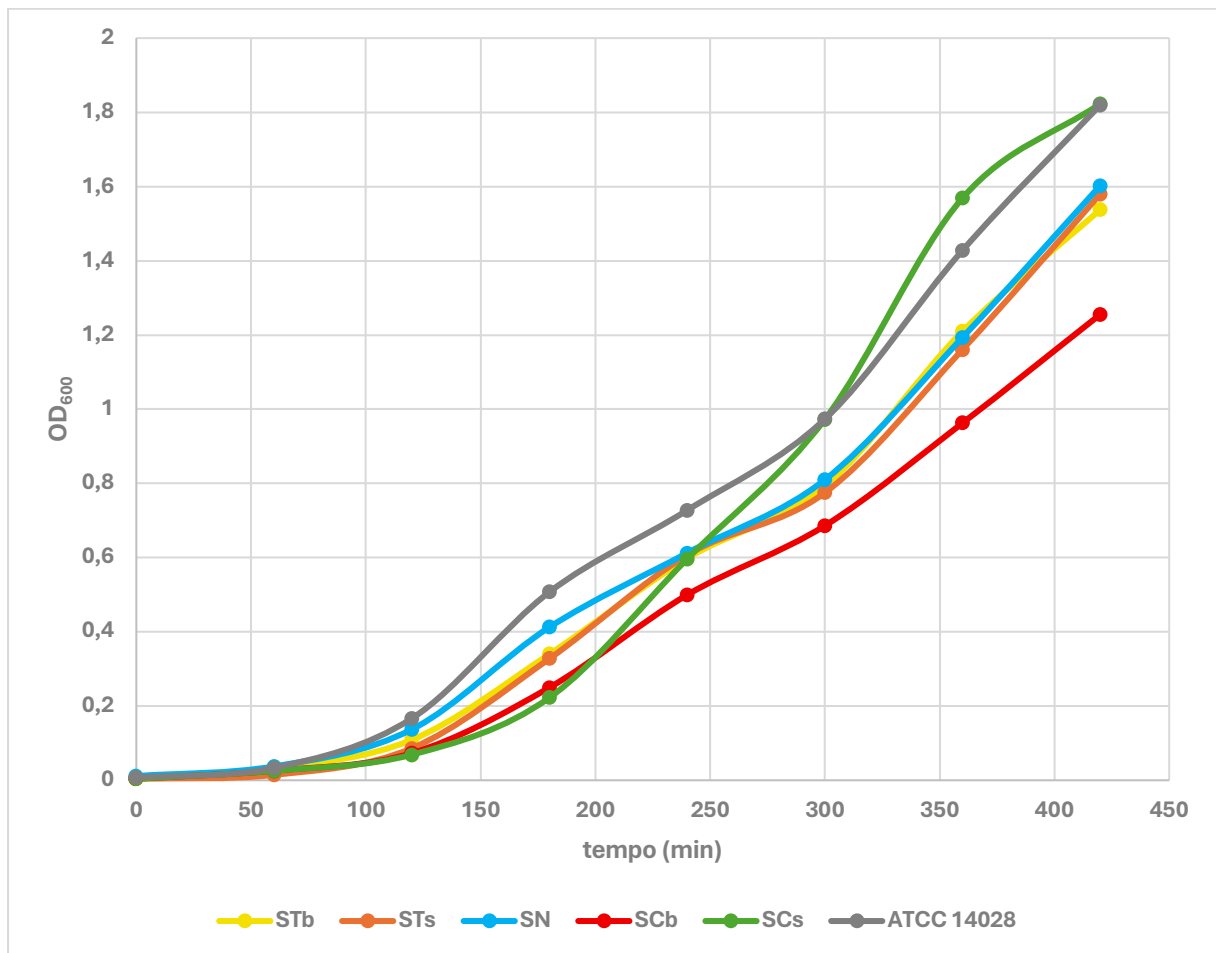


Figura 18: Fitness analysis of *S. enterica* strains reported in Table 2. *S. Typhimurium* ATCC 14028 is used as reference *S. enterica* strains. ST = *S. Typhimurium*; SN = *S. Napoli*; SC = *S. Choleraesuis*. (b) = blood samples; (s) = stool samples.

The analysis of the growth rate ($\mu_{\max}$) reveals considerable heterogeneity in the fitness of the *S. enterica* strains under optimal growth conditions, although no statistically significant differences were observed among the strains. The analysis of the growth rate ($\mu_{\max}$) showed values ranging from 0.0201 min⁻¹ (strain SN3) to 0.0409 min⁻¹ (strain STb2). Strain STb2 exhibited a growth rate of 0.0409 min⁻¹, followed by SN1 ($\mu_{\max}$ = 0.0322 min⁻¹) and SCb57 ($\mu_{\max}$ = 0.0321 min⁻¹). The *S. Typhimurium* strains isolated from blood (STb) reached a mean growth rate of 0.0348 min⁻¹. These strains exhibited rapid proliferation during the early exponential phase. By contrast, *S. Napoli* strains (SN tot) showed a $\mu_{\max}$ medium values of 0.0214 min⁻¹, indicating a slower replication rate under the tested conditions. The SC strains isolated from blood (SCb) displayed growth rates comparable to the ATCC14028 strain, while the SCs strains achieved growth rates like those of the STs strains. Interestingly, the reference strain ATCC 14028 exhibit a $\mu_{\max}$ of 0.0265 min⁻¹ and final OD₆₀₀ of 1.820, comparable to the OD₆₀₀ of SN2 (1.995) and SCs65 (1.980) (Table 8). On average, the SCs strains showed biomass values equivalent to the reference strain. The lowest values were observed in the SCb strains. On average, the clinical ST strains reached a final biomass lower, even if not statistically significant, than that of the reference strain.

Table 8: OD₆₀₀ value obtained during growth.

	OD ₆₀₀								
	T0	T1	T2	T3	T4	T5	T6	T7	$\mu_{\max}$ (min ⁻¹)
STb1	0,0026	0,0113	0,0467	0,1783	0,4407	0,693	0,961	1,27	0.0245
STb2	0,0061	0,071	0,181	0,4757	0,715	0,8987	1,3367	1,74	0.0409
STb3	0,0034	0,0188	0,1008	0,3605	0,6273	0,7888	1,3138	1,5883	0.0285
STb tot	0,004	0,0322	0,1086	0,3404	0,5976	0,793	1,2148	1,5383	0.0348
STs1	0,0034	0,0122	0,0824	0,2995	0,5798	0,7793	1,19	1,512	0.0318
STs2	0,0043	0,0137	0,084	0,375	0,6717	0,857	1,24	1,6133	0.0302
STs3	0,0039	0,0142	0,0688	0,292	0,5858	0,715	1,1183	1,53	0.0263
STs tot	0,004	0,014	0,083	0,326	0,605	0,788	1,176	1,533	0.0297
SC23	0,0046	0,018	0,0457	0,1613	0,3923	0,6373	0,8487	1,1783	0.0227
SC48	0,0066	0,0283	0,0966	0,3374	0,5821	0,7741	1,0638	1,2888	0.0243
SC57	0,0039	0,0268	0,061	0,2065	0,452	0,6033	0,9815	1,2925	0.0321
SCbtot	0,005	0,025	0,076	0,249	0,492	0,679	0,976	1,260	0.0263
SCs24	0,005	0,031	0,080	0,265	0,601	0,956	1,643	1,800	0.0299
SCs26	0,004	0,023	0,068	0,176	0,541	0,910	1,401	1,690	0.0283
SCs65	0,004	0,022	0,059	0,228	0,648	1,051	1,663	1,980	0.0306
SCs tot	0,0043	0,0254	0,0690	0,2229	0,5967	0,9725	1,5692	1,8233	0.0296
SN1	0,006	0,0413	0,152	0,4667	0,6717	0,8553	1,26	1,5733	0.0322
SN2	0,0173	0,0397	0,1557	0,477	0,664	0,916	1,4267	1,995	0.0228
SN3	0,011	0,031	0,1033	0,2943	0,5017	0,6583	0,8943	1,24	0.0201
SN tot	0,0121	0,0385	0,1393	0,4158	0,6109	0,8145	1,1904	1,6206	0.0214
ATCC 14028	0,007	0,034	0,167	0,508	0,728	0,973	1,428	1,820	0.0265

3.2.2 Study of pH survival of *S. enterica* serotypes.

For this experiment, we tested and compared 16 strains:

- ◆ three *S. Typhimurium* strains from blood (STb)
- ◆ three *S. Typhimurium* strains from stool (STs)
- ◆ three *S. Choleraesuis* strains (SC)
- ◆ three *S. Napoli* strains from blood (SN)
- ◆ Reference strain *S. Typhimurium* ATCC 14028

We sought to compare the response and survival of different *Salmonella enterica* serotypes under acidic conditions to evaluate potential advantages in survival following ingestion of contaminated food and subsequent passage through the acidic gastric environment. All experiments were conducted in triplicate, and the strains tested are listed in Table 2. Results of bacterial exposure to PBS (control), pH 2.0, and pH 4.0 for 120 minutes are presented in Figure 19. Exposure to highly acidic conditions (pH 2.0) resulted in an almost complete loss of viability across all tested serotypes. As early as the first time point (T1), bacterial counts had decreased by more than 99.9% in all isolates, with no significant differences detected between *S. Typhimurium* (from blood and stool), *S. Napoli*, *S. Choleraesuis*, and the reference strain ATCC 14028. At pH 4.0, no statistically significant differences were observed between isolates of the same serotype; however, *S. Typhimurium* strains derived from stool samples displayed a trend toward higher acid resistance compared to those from blood, although this difference did not reach statistical significance. Notably, *S. Choleraesuis* exhibited the highest survival rates at pH 4.0 when compared with *S. Typhimurium* strains. After 30 minutes of exposure, *S. Choleraesuis* showed only a 48–82% reduction in viable counts (66% on average), followed by a relatively stable trend for the remainder of the experiment. The response of *S. Choleraesuis* to acidic conditions was statistically different from that of *S. Typhimurium* clinical strains and the *S. Typhimurium* ATCC 14018 reference, but not from *S. Napoli*. The survival profile of *S. Napoli* closely resembled that of ATCC 14018.

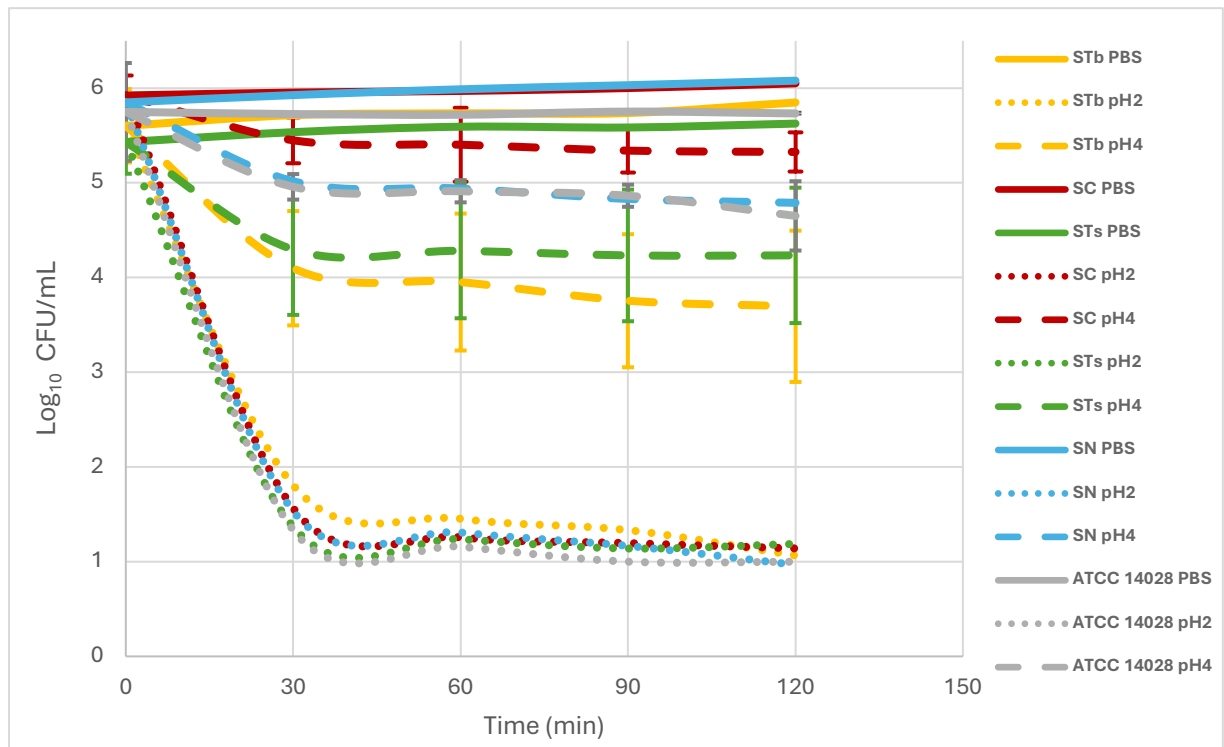


Figure 19: Comparative survival of *S. Typhimurium*; *S. Napoli* and *S. Choleraesuis* under acidic stress conditions. The graph shows changes in bacterial load ($\text{log}_{10} \text{ CFU/mL}$) over time (up to 120 minutes) for strains isolated from blood or stool, incubated in PBS (neutral pH), pH 4, or pH 2. Error bars represent standard deviation from summary of three independent replicates of three strains for serotype. *S. enterica* strains. ST = *S. Typhimurium* (STb1, STb2, STb3, STs1, STs2, STs3); SN = *S. Napoli* (SN1, SN2, SN3); SC = *S. Choleraesuis* (SC23, SC24, SC26, SC48, SC57, SC65). (b) = blood samples; (s) = stool samples.

Table 9: Evaluation of abatement of *S. enterica* serotypes to pH acid conditions.

Bacteria killing													
		t pH2						pH4					
<i>S. enterica</i> strains	T0	T1 pH2	T4 pH2	T1-T0 pH2	T4-T0 Ph2	% kill pH2 T1	%kill pH2 T4	T1 pH4	T4 pH4	T1-T0 pH4	T4-T0 pH4	% kill pH4 T1	% kill pH4 T4
STb1	3,77E+05	3,40E+02	1,00E+01	3,76E+05	3,77E+05	99,910	>99,999	2,20E+04	7,30E+03	3,55E+05	3,69E+05	94,159	98,062
STb2	7,93E+05	3,63E+02	2,00E+01	7,93E+05	7,93E+05	99,954	>99,999	4,30E+04	3,02E+04	7,50E+05	7,63E+05	94,580	96,189
STb3	3,83E+05	3,40E+02	1,00E+01	3,83E+05	3,83E+05	99,911	>99,999	4,30E+03	2,03E+03	3,79E+05	3,81E+05	98,878	99,470
STb tot	5,18E+05	3,48E+02	1,33E+01	5,17E+05	5,18E+05	99,933	> 99,999	2,31E+04	1,32E+04	4,95E+05	5,05E+05	95,539	97,453
STs1	3,27E+05	1,33E+01	1,00E+01	3,27E+05	3,27E+05	99,996	99,997	2,02E+04	1,99E+04	3,07E+05	3,07E+05	93,827	93,898
STs2	5,17E+05	6,67E+01	5,00E+01	5,17E+05	5,17E+05	99,987	99,990	1,08E+05	1,04E+05	4,09E+05	4,12E+05	79,097	79,806
STs3	1,77E+05	2,00E+01	1,00E+01	1,77E+05	1,77E+05	99,989	99,994	7,63E+03	5,27E+03	1,69E+05	1,71E+05	95,679	97,019
STs tot	3,40E+05	3,33E+01	2,33E+01	3,40E+05	3,40E+05	99,990	99,993	4,53E+04	4,32E+04	2,95E+05	2,97E+05	86,686	87,294
SN1	7,13E+05	1,27E+02	7,00E+01	7,13E+05	7,13E+05	99,982	99,990	2,57E+05	1,63E+05	4,57E+05	5,51E+05	64,019	77,196
SN2	1,05E+06	1,73E+02	2,33E+01	1,05E+06	1,05E+06	99,984	99,998	2,60E+05	2,13E+05	7,90E+05	8,37E+05	75,238	79,683
SN3	5,43E+05	1,00E+01	1,00E+01	5,43E+05	5,43E+05	99,998	99,998	4,81E+04	1,83E+04	4,95E+05	5,25E+05	91,141	96,626
SN tot	7,69E+05	1,03E+02	1,34E+01	7,69E+05	7,69E+05	99,987	99,998	1,88E+05	1,31E+05	5,81E+05	6,37E+05	75,514	82,905
ATCC 14028	8,13E+05	4,00E+01	1,00E+01	8,13E+05	8,13E+05	99,995	99,999	9,40E+04	5,43E+04	7,19E+05	7,59E+05	88,443	93,320
SC23	8,38E+05	1,00E+01	1,00E+01	8,37E+05	8,37E+05	99,999	99,999	3,80E+05	2,25E+05	4,58E+05	6,13E+05	54,627	73,134
SC48	1,50E+06	1,00E+01	1,00E+01	1,50E+06	1,50E+06	99,999	99,999	3,60E+05	2,50E+05	1,14E+06	1,25E+06	76,000	83,333
SC57	1,00E+06	7,75E+01	2,50E+01	1,00E+06	1,00E+06	99,992	99,998	3,68E+05	3,35E+05	6,33E+05	6,65E+05	63,250	66,500
SC24	3,90E+05	9,67E+01	1,67E+01	3,90E+05	3,90E+05	99,975	99,996	2,00E+05	1,57E+05	1,90E+05	2,33E+05	48,718	59,829
SC26	7,40E+05	1,00E+01	1,00E+01	7,40E+05	7,40E+05	99,999	99,999	1,31E+05	1,24E+05	6,09E+05	6,16E+05	82,342	83,198
SC65	5,88E+05	1,75E+01	1,00E+01	5,87E+05	5,87E+05	99,997	99,998	2,13E+05	1,56E+05	3,75E+05	4,32E+05	63,830	73,532
SC tot	8,38E+05	3,67E+01	1,38E+01	8,38E+05	8,38E+05	99,996	99,998	2,89E+05	2,12E+05	5,56E+05	6,25E+05	66,390	74,673

3.2.3 Analysis of Whole genome sequencing of *S. Choleraesuis* strains.

Here we present Whole-genome sequencing (WGS) analysis of *Salmonella enterica* subsp. *enterica* serovar Choleraesuis isolates collected between 2007 and 2016 in Lombardy. WGS was successfully performed on 65 strains (97.0% of the total). All isolates resulted to belong to the same sequence type (ST145), confirming the high clonality of this serovar in the studied area. The isolates were obtained from a variety of clinical matrices, predominantly blood (n=47; 77%), but also feces (n=5), pus (n=2), urine (n=2), ascitic fluid (n=1), bile (n=1), prosthetic material (n=1), and peri-aortic or tissue samples (n=2). Genome assemblies showed a mean genome length of ~4.90 Mb (range: 4.71–5.02 Mb), with an average N50 of 171,000 bp and a median of 92 contigs $\geq$ 300 bp. Despite being sequenced over nearly a decade, the genomes displayed a high degree of homogeneity both in size and in assembly statistics, consistent with the clonal structure of this serovar. In addition to the genomic features, the resistome analysis revealed a high prevalence of antimicrobial resistance determinants among the 65 *S. Choleraesuis* isolates. Regarding resistance profiles, 62 out of 65 sequenced isolates (95.4%) carried acquired antimicrobial resistance determinants, while only three (4.6%) were found to be fully susceptible. The majority (n=60; 92.3%) harbored a common resistance gene profile, including *aadA1*, *aph(3'')-Ib*, *aph(6)-Id*, *blaTEM-1B*, *dfrA1*, *sul1*, *sul2*, and *tet(B)*. This genotype corresponded to predicted resistance against aminoglycosides (streptomycin, kanamycin), β -lactams (ampicillin), trimethoprim, sulfonamides, and tetracyclines. Two isolates additionally carried *aph(3')-Ia*, broadening aminoglycoside resistance (2/65; 3.1%), and two carried *aac(3)-IV* and *aph(4)-Ia*, conferring resistance to gentamicin, tobramycin, and hygromycin (2/65; 3.1%). Sporadically, variants of *blaTEM* genes were detected (*blaTEM-34*, *blaTEM-57*) with one isolate also carrying *floR* (1/65; 1.5%), associated with chloramphenicol/florfenicol resistance. Overall, the data indicates a dominant multidrug-resistant clone of *S. Choleraesuis* circulating in Lombardy during the study period, with a stable resistance profile. Resistance was most widespread against tetracyclines, sulfonamides, trimethoprim, and β -lactams, each present in more than 90% of isolates. Notably, no plasmid-mediated quinolone resistance (PMQR) determinants and no colistin resistance genes (e.g., *mcr* variants) were identified, which is of clinical relevance since these drugs represent last-resort therapeutic options. In

Supplementary Table 2 is reported the antibiotic resistance profile of *S. Choleraesuis* strains comparing WGS data and datasheets.

Table 10: WGS data of 67 *S. Choleraesuis* strains (2007 – 2016).

Code	Year	Isolation	City	Sequence Type	Genome Length	N50 value	Contigs	Genotype	CGE Predicted Phenotype
SC1	2007	Blood	Milano	145	4897710	153575	96	aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC2	2008	Stool	Milano	145	4922200	188717	86	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC3	2008	Splenic cyst drainage fluid	Milano	145	4932784	153574	100	aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC4	2008	Blood	Milano	145	4938545	169037	95	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC5	2008	-	-	145	4936991	143382	91	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC6	2009	Blood	Milano	145	4909672	177811	95	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC7	2009	Blood	Milano	145	4935360	139475	101	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC8	2009	Blood	Brescia	145	4889876	156871	97	aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline

SC9	2010	Blood	Milano	145	4928668	161942	96	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC10	2010	Blood	Milano	145	4917094	153574	99	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC11	2010	Bile	Brescia	145	4938356	159666	96	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC12	2010	Blood	Milano	145	4938336	159666	98	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC13	2010	Blood	Milano	145	4845364	188717	89	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC14	2010	Blood	Milano	145	4889049	159666	91	aadA1, aph(3'')-Ib, aph(6)-Id, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC15	2010	Blood	Milano	145	4926989	156852	96	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC16	2010	Blood	Milano	145	4929670	161941	100	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC17	2010	Pus	Pavia	145	4797068	159666	105	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline

SC18	2010	Blood	-	145	4948095	159666	105	aac(3)-IV, aadA1, aph(3'')-Ib, aph(3')-Ia, aph(4)-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Gentamicin, Tobramycin, Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Hygromycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC19	2010	Stool	Milano	145	4919611	153574	95	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC20	2010	Stool	Brescia	145	4890593	161942	91	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC21	2010	Blood	Milano	145	4935248	159666	101	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC22	2010	Blood	Milano	145	4883752	138333	103	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC23	2010	Blood	Milano	145	4926083	170026	98	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC24	2010	Stool	Lodi	145	4714174	188715	76	sensible	sensible
SC25	2010	Blood	Milano	145	4921816	159666	91	aph(3')-Ia, blaTEM-1B, qacE, sul1, tet(B)	Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Benzylkonium Chloride, Ethidium Bromide, Chlorhexidine, Cetylpyridinium Chloride, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC26	2010	Prosthesis	Milano	145	4715207	159666	76	Sensible	Sensible
SC27	2010	Blood	Monza Brianza	145	4921806	153734	103	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id,	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin,

								blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC28	2011	Blood	Milano	145	4936722	161940	94	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC29	2011	Stool	Milano	145	4933504	156852	113	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC30	2011	Blood	Milano	145	NA (*)				
SC31	2011	Blood	Milano	145	NA (*)				
SC32	2011	Blood	Milano	145	4863139	190548	87	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC33	2011	Pus	Milano	145	4798948	159666	96	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul2	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole
SC34	2011	Periaortic liquid	Milano	145	4739833	159666	84	aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, sul2	Spectinomycin, Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole
SC35	2011	Tissue	Milano	145	4927171	188716	94	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC36	2011	Blood	Milano	145	4922126	170972	96	aadA1, aph(3')-Ia, sul1, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC37	2011	Blood	Milano	145	4884725	198540	81	aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline

SC38	2011	Blood	Milano	145	4881753	188717	85	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC39	2011	Blood	Monza Brianza	145	4881805	188716	81	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC40	2012	Blood	Milano	145	4936347	159666	97	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC41	2012	Blood	Pavia	145	4895261	150005	101	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC42	2012	Urine	Pavia	145	4896300	190548	93	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC43	2012	Blood	Pavia	145	4874420	197307	85	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC44	2012	Blood	Monza Brianza	145	4877257	188009	89	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC45	2012	Blood	Milano	145	4775299	190548	84	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC46	2012	Blood	Pavia	145	4873140	188716	89	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id,	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin,

								blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC47	2012	Blood	Pavia	145	4928696	177812	100	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC48	2012	Blood	Milano	145	5022008	130000	125	aac(3)-IV, aadA1, aph(3'')-Ib, aph(3')-Ia, aph(4)-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2	Gentamicin, Tobramycin, Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Hygromycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole
SC49	2012	Blood	Milano	145	4737095	198544	65	aph(3'')-Ib, aph(6)-Id, sul2	Streptomycin, Sulfamethoxazole
SC50	2012	Blood	Milano	145	4923332	188716	92	aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, sul2	Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Sulfamethoxazole
SC51	2012	Blood	Milano	145	4908769	197307	82	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC52	2012	Blood	Milano	145	4924834	188717	89	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC53	2012	Blood	Milano	145	4934710	177927	95	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC54	2013	Blood	Milano	145	4935778	190548	89	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC55	2013	Blood	Pavia	145	4920266	159666	99	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline

SC56	2013	Blood	Milano	145	4930621	177926	101	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC57	2013	Blood	Milano	145	4874107	197307	84	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC58	2013	Blood	Milano	145	4902117	180800	94	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole
SC59	2013	Urine	Milano	145	4912491	197304	85	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-1B, dfrA1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC60	2013	Blood	Milano	145	4751287	198682	63	aph(3'')-Ib, aph(6)-Id, sul2	Streptomycin, Sulfamethoxazole
SC61	2013	Ascetic Liquid	Milano	145	4811205	154790	97	aph(3'')-Ib, aph(6)-Id, blaTEM-1B, sul2	Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Sulfamethoxazole
SC62	2014	Blood	Milano	145	4887532	190548	82	aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC63	2015	Blood	Milano	145	4898148	190548	95	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-57, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC64	2015	Blood	Monza Brianza	145	4911118	170121	87	aadA1, aph(3'')-Ib, aph(6)-Id, blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Spectinomycin, Streptomycin, Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC65	2016	Stool	-	145	4717999	216492	64	sensible	-
SC66	2016	-	-	145	4869548	188717	85	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id,	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin,

								blaTEM-1B, dfrA1, sul1, sul2, tet(B)	Ampicillin, Cephalothin, Piperacillin, Ticarcillin, Trimethoprim, Sulfamethoxazole, Doxycycline, Tetracycline, Minocycline
SC67	2016	-	-	145	4963233	100240	165	aadA1, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, blaTEM-34, dfrA1, floR, sul1, sul2	Spectinomycin, Streptomycin, Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin, Amoxicillin, Amoxicillin+Clavulanic acid, Ampicillin, Ampicillin+Clavulanic acid, Piperacillin, Piperacillin+Tazobactam, Ticarcillin, Ticarcillin+Clavulanic acid, Trimethoprim, Chloramphenicol, Florfenicol, Sulfamethoxazole

3.2.4 cgMLST to assess clonality of *S. Choleraesuis* strains.

The cgMLST-based minimum spanning tree analysis of the 65 *Salmonella* *Choleraesuis* strains, all belonging to ST145, revealed a complex but structured population (Figure 20). Despite being part of the same sequence type, the strains displayed considerable allelic diversity, with the formation of seven main clusters. Within each cluster, strains were separated by fewer or equal to five allelic differences, indicating a high degree of clonality and the persistence of stable lineages. Most of isolates were concentrated in a dense central network, with very short genetic distances between nodes. This pattern indicates limited diversification within the dominant ST145 population. However, several peripheral nodes were observed at larger genetic distances (≥ 11 allelic differences), and one lineage was separated from the central group by over 120 allelic differences, demonstrating deeper divergence within ST145. A notable feature of the network was the position of antimicrobial-sensitive strains, which consistently appeared among the most divergent nodes. These isolates, lacking resistance genes, were genetically more distant from the central clusters dominated by resistant strains. This distribution suggests that the acquisition and maintenance of resistance determinants may have contributed to the recent evolution and clonal expansion of certain lineages within ST145. In this context, the sensitive strains could represent remnants of ancestral lineages that did not acquire resistance, while the resistant clusters reflect successful adaptation and dissemination under selective pressure. Overall, the data indicates that while ST145 represents a single clonal complex, its population structure is shaped by both microevolutionary diversification and differential acquisition of resistance traits. The central, tightly connected clusters likely represent the dominant resistant lineages, whereas the more divergent sensitive strains highlight the evolutionary transitions occurring within this population.

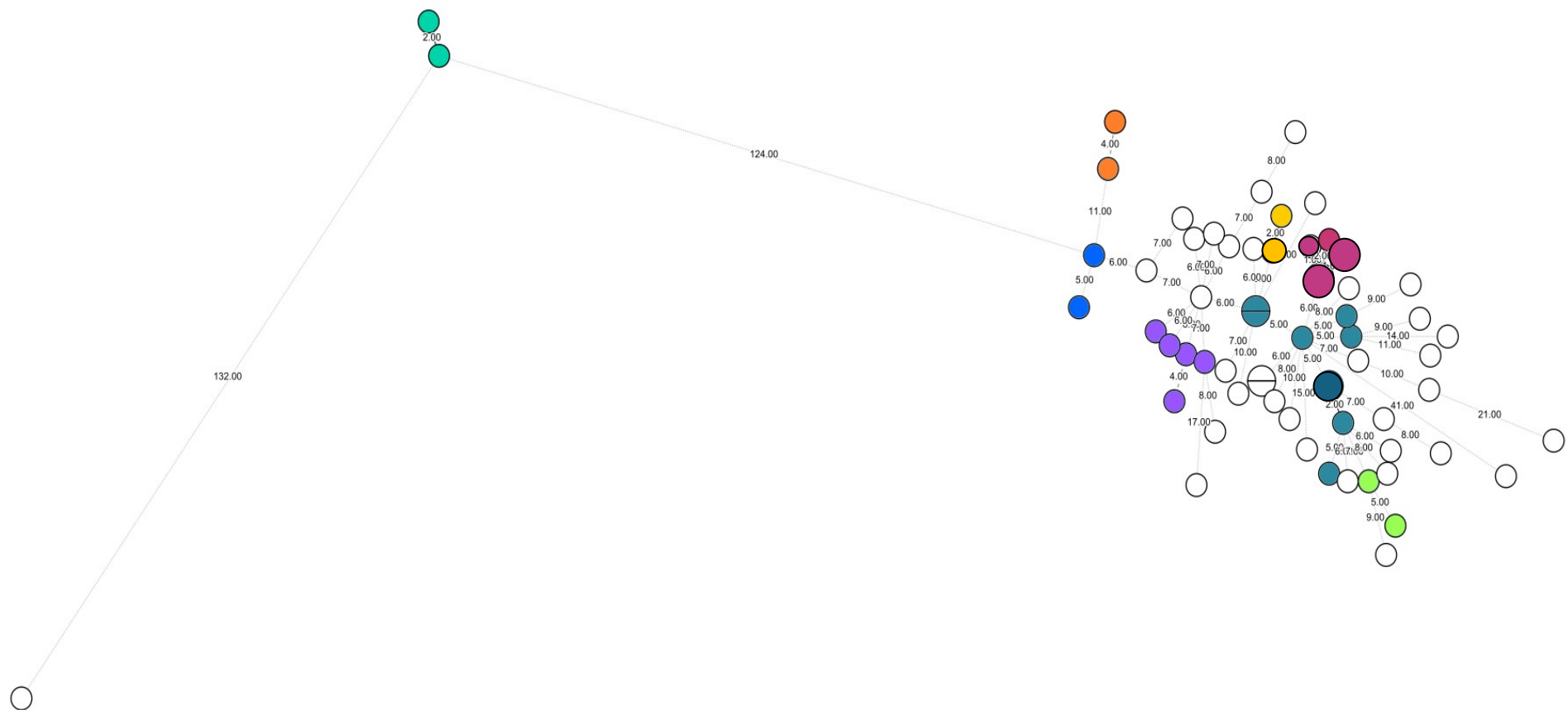


Figure 20: Minimum Spanning Tree (MST) obtained from cgMLST data for the *S. Choleraesuis* isolates belonging to the cluster. The numeric labels on the edges represent the pairwise distances between isolates computed as number of allelic differences in cgMLST. Strains with the same colour has $AD \leq 5$ and are considered belonging to the same genomic cluster.

The phylogenetic analysis of 65 *S. Choleraesuis* strains, based on cgMLST (Figure 21) revealed a clear structuring into distinct clades. The strains were distributed along branches reflecting considerable genetic heterogeneity, with some clusters showing very close relationships, while others were separated by longer distances, suggesting more pronounced evolutionary divergence. The six strains selected for experimental work (SC23, SC24, SC26, SC48, SC57, SC65) were distributed across different regions of the tree. SC23 clustered in close proximity to SC27 and SC34, whereas SC24 and SC26 were more closely related to each other, forming a small cluster. In contrast, SC48 belonged to a distant branch, grouping with more recent isolates, indicating a distinct genetic background compared to the other experimental strains. SC65 was positioned in a clearly separate clade, together with SC24 and SC26, highlighting marked divergence that makes it particularly relevant for comparative analyses. SC57 occupied a distinct but not too distant from the major clusters, suggesting it may share features with both older and more recent isolates. The first group, comprising SC23, SC48, and SC57, clustered within the main genomic group containing all reference strains. SC48 and SC57 were positioned on peripheral nodes but showed relatively low allelic distances from adjacent branches. The second group consisted of the three antibiotic-susceptible strains (SC24, SC26, and SC65), all positioned peripherally and at substantial genomic distances from the central clusters dominated by resistant isolates. Allelic distances between these isolates were high (124 AD and 132 AD), although SC24 and SC26 differed by only 2 AD. The color code, corresponding to the year of isolation (2007–2016), showed that temporal distribution did not follow a strictly linear pattern. Isolates from distant years occasionally coexisted within the same cluster indicating that genetic variation did not accumulate in a uniform temporal way but was instead shaped by episodic divergence and recombination. Some coherence was observed: for instance, several isolates from 2012–2014 tended to cluster together, suggesting the persistence of clonal lineages during that period.

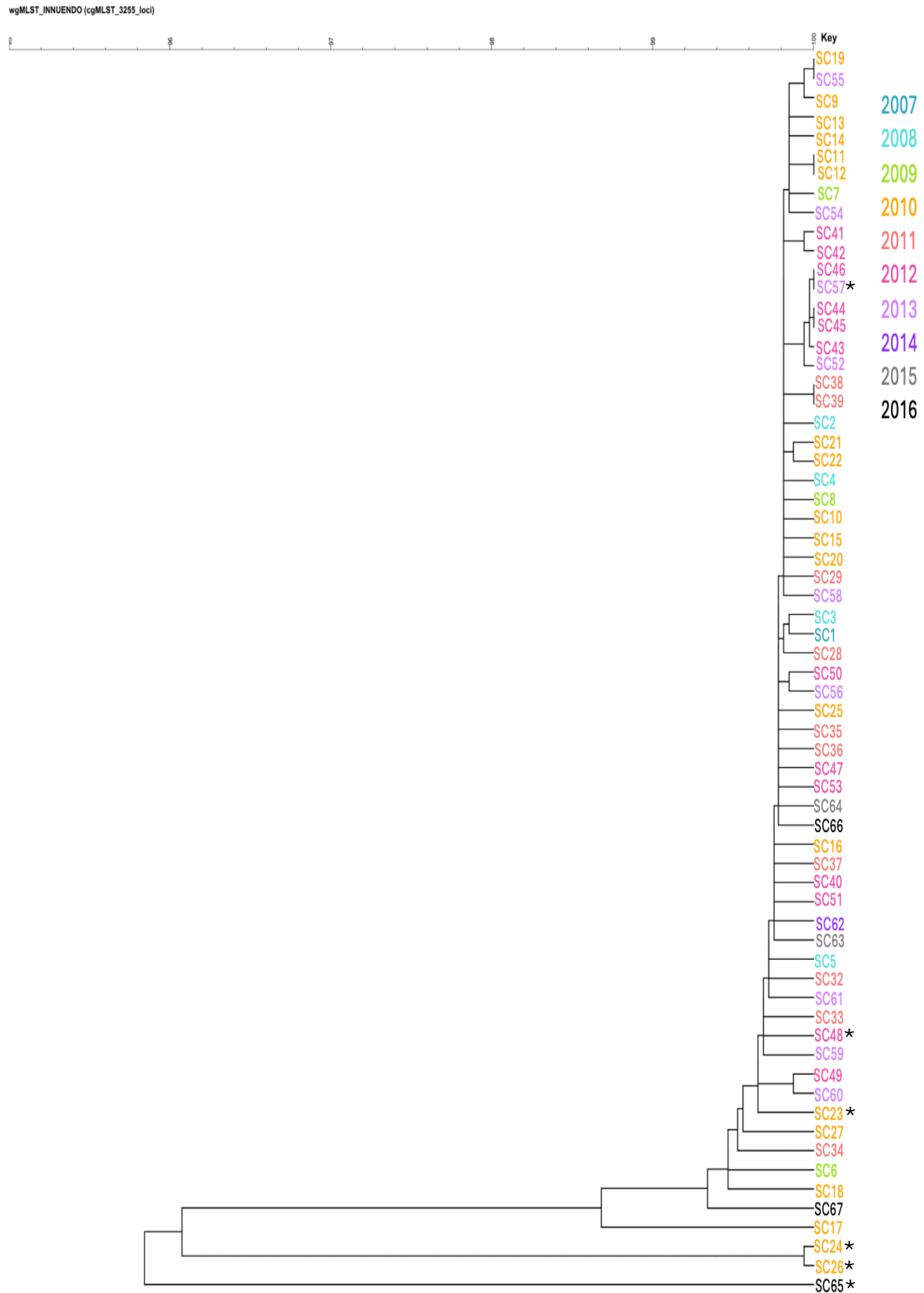


Figure 21: Phylogenetic dendrogram of 65 *Salmonella* Choleraesuis strains based on core-genome MLST. Strains are labeled with their identifiers, and colors indicate the year of isolation (2007–2016). The six strains selected for experimental assays (SC23, SC24, SC26, SC48, SC57, and SC65) are highlighted with the symbol (*).

4 DISCUSSION

Although our biobank data consists of *Salmonella enterica* isolates collected over several years without any clear information as to how these samples and data were collected, it provides a realistic snapshot of the serotypes currently circulating.

According to our analysis, the three most prevalent serovars, *S. Typhimurium*, *S. Enteritidis*, and *S. 1,4,[5],12:i:-* (monophasic variant), align with national and European data reported by the Istituto Superiore di Sanità (ISS), the European Food Safety Authority (EFSA), and the European Centre for Disease Prevention and Control (EFSA and ECDC, Zoonoses report 2023). To date, the top five European Union-acquired *Salmonella* serovars involved in human infections were: *S. Enteritidis* (70.8%), *S. Typhimurium* (8.9%), monophasic *S. Typhimurium* variant (1,4,[5],12:i:-) (5.1%), *S. Infantis* (2.0%) and *S. Coeln* (0.77%) (EFSA and ECDC, Zoonoses report 2023).

Globally, and unsurprisingly, *S. Typhimurium* and *S. Enteritidis* are the two most prevalent serotypes of *S. enterica* subsp. *enterica*, typically transmitted to humans through contaminated food products, and responsible for most of clinical cases of salmonellosis worldwide (WHO, “Salmonella (Non-Typhoidal)” 2025). The widespread distribution of these serovars can be attributed to their multiple reservoirs and ability to infect a broad range of warm-blooded animals (Feasey NA., *et al.*, 2012). Analysis of trends related to the first four serotypes reveals that *S. Typhimurium* and *S. Enteritidis* were dominant in the early years but showed a marked decline after 2007. Conversely, *S. 1,4,[5],12:i:-* and *S. Napoli* increased in prevalence after 2010, peaking around 2014. In Europe, *S. 1,4,[5],12:i:-* accounted for 4.6%, 7.2% and 8.6% of total cases of salmonellosis reported to the European Surveillance System (TESSy) in 2011, 2012 and 2013 respectively (EFSA and ECDC report zoonoses 2014 and 2015), and ranked third among the *Salmonella* serovars identified in humans in the European Union (EU) during the period.

In Italy, *S. 1,4,[5],12:i:-* was already isolated by human cases between 2004 and 2008, being the third most relevant serotype in the period, and according to our regional data, on 2014, it was recorded a pick of cases (outbreak) in another region of Italy (Abruzzo) (Cito F., *et al.*, 2014). Regarding *S. Napoli*, it was the fourth most isolated serovar in

Lombardy (our data), a serotype that emerged in Italy at least in the 1980s, with cases documented in both the UK and Italy (Gill ON., *et al.*, 1983). In subsequent years, it emerged as a significant serotype in Italy, with significant clinical and epidemiological implications.

Notably, in 2014, *S. Napoli* was responsible for an outbreak in Milan that affected several primary schools (Huedo P., *et al.*, 2016). The outbreak was linked to the consumption of ham, and primarily affected children, resulting in a high rate of hospitalization (Huedo P., *et al.*, 2016). Across Europe, from 2000 to 2013, the incidence of *S. Napoli* increased by 256%, making it the 13th most frequently isolated serovar from human cases (Gori M., *et al.*, 2018; Graziani C., *et al.*, 2015). In the period 2010-2014, *S. Napoli* emerged as the main cause of invasive non-typhoidal salmonellosis in Lombardy, accounting for 5.8% of invasive cases. Genomic and phylogenetic analyses indicated that *S. Napoli* belongs to the *S. Typhi* subclade of clade A, showing a close relationship with the *S. Paratyphi* serotype A and sharing genes associated with the typical typhoid fever virulence (Huedo P., *et al.*, 2016).

We hypothesize that on our territory, *S. Enteritidis*, which played a key role in salmonellosis cases in the early years, may have been replaced by *S. Napoli*, which subsequently occupied its ecological niche. The decline of *S. Enteritidis* circulation can be explained in part by significant changes introduced in Italian food safety legislation in 2007. Specifically, Legislative Decree No. 193 of November 6, 2007, transposed Directive 2004/41/EC on official controls related to food safety, repealing and replacing earlier national regulations. This decree imposed stricter hygiene requirements in food production, with direct effects on the poultry and egg supply chains. *S. Enteritidis* is closely linked to the consumption of eggs and poultry, while other serotypes like *S. Typhimurium* is more frequently associated with various meats (including poultry, pork, and beef) as well as dairy products (Busani L., *et al.*, 2004; Gast RK. *et al.*, 2024). *S. Enteritidis* is notable for its ability to colonize the ovarian cortex of hens and contaminate eggs, one of the primary vehicles for foodborne transmission (Gantois I., *et al.*, 2009). We found feedback on this association in our data relating to outbreaks. Before the introduction of the Legislative Decree of 2007 (period 2002-2003), we recorded many outbreaks linked to egg consumption and supported by *S. Enteritidis*. Following the new

regulations, food producers were required to adopt higher hygiene and safety standards, reducing the risk of contamination by pathogens such as *S. Enteritidis*. Enhanced official controls, including more frequent inspections by competent authorities such as the Ministry of Health and Local Health Units, further incentivized improvements in production and management practices, particularly in relation to traceability and the safety of poultry and eggs.

Although a decrease in the circulation of *S. Enteritidis* has been observed in Lombardy, the data related to travel remains noteworthy. In fact, according to our findings, *S. Enteritidis* is the serotype most frequently associated with international travel, whereas *S. Typhimurium* is commonly linked to domestic travel. This may be explained by the fact that many recorded trips were to North African countries, where *S. Enteritidis* is the predominant serotype (Al-Rifai RH., *et al.*, 2019). These findings highlight not only the importance of eggs as a recurrent vehicle but also the nuanced differences in serotype distribution depending on whether exposure occurred within national borders or abroad.

Together, the outbreak investigations and the analysis of sporadic and travel-related cases paint a coherent epidemiological landscape: *S. Enteritidis* and *S. Typhimurium* remain the dominant serotypes, with eggs consistently identified as the primary food vehicle. However, variations linked to travel and temporal clusters suggest that both local food production systems and international travel practices play a key role in shaping the risk of *Salmonella* infections.

It is also important to highlight that the cases of *S. Typhi* recorded during the study period were all associated with travel and importation from non-European countries. These patterns of exposure and infection routes are particularly concerning when considering the most affected population groups. Our data highlighted a higher number of cases in the 0–4 age group, confirming salmonellosis as a pathology mainly pediatric. This finding is consistent with the European Centre for Disease Prevention and Control (ECDC) annual report for 2022 which reported the highest notification rate in children aged 0–4 years, with 81.5 cases per 100,000 inhabitants, ten times higher than in adults aged 25–64 years (ECDC - Annual Epidemiological Report for 2022). Similar trends were observed in previous years. In 2020, the notification rate for the 0–4 age group was 76.3 cases per

100,000, and in 2018 it was even higher, at 94.9 per 100,000, both representing rates up to ten times greater than those reported for adults (ECDC - Annual Epidemiological Report for 2020; ECDC - Annual Epidemiological Report for 2018).

The analysis of isolation matrix revealed that in pediatric population *Salmonella* was isolated from stool, indicating a prevalent gastrointestinal symptomatology, while in elderly (over 64> age) we observed a more frequent systemic infection linked with invasive forms. This is coherent with what observed from the literature. Some studies highlighted how young children have a higher incidence of *Salmonella* infections, while older adults are more susceptible to invasive and severe forms of the disease, with higher mortality rates (GBD 2017; Marchello CS., *et al.*, 2022; Turgeon P., *et al.*, 2017; Bula-Rudas FJ., *et al.*, 2015).

From the 2023 One Health report on zoonoses in the European Union, the proportion of hospitalized cases was 41%, with a European Union case fatality rate of 0.2% (EFSA and ECDC, Zoonoses report 2023). From our results, 46.2% (721/1,559) of NTS salmonellosis cases involved hospitalization or occurred in hospitalized patients (known data 55.9%; 1,559/2,952). Unlikely, the period of hospitalization and reason for admission were not reported on datasheets at our disposal, that could give more information related to susceptibility and co-morbidity of patients. Analysis of the age distribution of hospitalized cases reveals, as expected, a pronounced peak in the range 0-4 years age group, consistent with the overall case distribution. By classifying hospitalized cases according to the type of biological sample (stool or blood), it is possible to differentiate between gastrointestinal and invasive/systemic forms of the infection, and a higher prevalence of invasive cases was observed in the over 64 age group, supporting the evidence of increased susceptibility among the elderly to more severe forms of the disease. By analyzing the trends of each serotype, they showed that they affect different age groups. Some serotypes disproportionately affect young children (e.g.: main serotypes *S. Typhimurium*, *S. Enteritidis*, and *S. Napoli*) while others are more common among the elderly. This finding was clear comprising age distribution of invasive disease (blood isolation) in *S. Choleraesuis* and *S. Napoli*. In fact, *S. Choleraesuis* affected primarily older adults and the markedly high invasiveness index observed in our dataset is consistent with previous findings that already highlighted its strong invasive potential in

humans (Chiu CH., *et al.*, 2004; Chiu C.H., *et al.*, 2005). Beyond the clear predisposition of elderly adults to develop severe disease, this trend may also be explained by the serotype's preferred route of transmission, its ability to invade the host (mechanisms of invasiveness), and its capacity to persist and survive in the external environment.

Notably, our data show that *S. Choleraesuis* exhibits the highest invasiveness index among the analyzed serotypes (76.9%), even comparable to *S. Typhi* (64.7%), which is classically considered the most invasive *Salmonella* serotype associated with systemic disease (Liao YS., *et al.*, 2024). This observation is particularly relevant, as *S. Typhi* is a highly host-adapted, specialized in causing enteric fever, while *S. Choleraesuis* is traditionally regarded as a swine-adapted serotype with zoonotic potential. The unexpectedly high invasiveness of *S. Choleraesuis* in our human cases underscores its potential public health relevance and raises concerns about its capacity to cause severe systemic infections in humans. A possible explanation for this elevated invasiveness lies in the evolutionary history of *S. Choleraesuis*. Previous phylogenetic analyses have shown that *S. Choleraesuis* shares a recent common ancestor with *S. Paratyphi C*, another human-adapted invasive serotype responsible for enteric fever-like illness (Alihan NF., *et al.*, 2018; Liu W.Q., *et al.*, 2009; Zhou Z., *et al.*, 2018).

This close evolutionary relationship may contribute to the enhanced ability of *S. Choleraesuis* to invade the bloodstream and disseminate systemically. Analysis of antimicrobial resistance (AMR) in *Salmonella enterica* strains, based on available datasheets, revealed high resistance to several widely used antibiotic classes. Resistance to penicillins (46.9%) and tetracyclines (48.0%) exceeded 45%, consistent with intensive use since the mid-20th century, which established them as the earliest drug classes with widespread resistance in *Salmonella*. These figures align with current European estimates of 40–50% in both human and animal isolates (EFSA and ECDC, 2025) and confirm their historical dominance in multidrug-resistant (MDR) phenotypes reported in Italian and European surveillance during the early 2000s (Listorti V., *et al.*, 2022; Pitti M., *et al.*, 2023). Resistance to aminoglycosides reached 28.3%, notably higher than current EU levels (<20%), possibly reflecting differences in tested agents (e.g., streptomycin versus gentamicin) or historical usage patterns, particularly in Northern Italy during the early 2000s. By contrast, sulfonamides and trimethoprim exhibited

relatively low resistance (10.2%), a finding that diverges from European data reporting >40% in animal-derived isolates (EFSA and ECDC, 2025), suggesting regional differences in antimicrobial usage or shifts in prescribing practices. Resistance to first- and second generation cephalosporins was moderate, while third generation (1.5%) and fourth generation (2.2%) cephalosporins showed low resistance rates, consistent with European averages (0.6-2.2%) and reflecting the success of regulatory efforts aimed at preserving these critically important drugs (EMA, 2023). This relatively preserved activity indicates that the 2001–2016 dataset may represent a pre-expansion phase, preceding the accelerated dissemination of plasmid-mediated blaCTX-M and other ESBL genes documented after 2010 (ECDC 2025; Wang J., *et al.*, 2025; EFSA/ECDC 2024). Similarly, carbapenem resistance remained very low (0.7%), while colistin resistance was absent, in line with global observations that *mcr*-mediated resistance only emerged in the mid-2010s, often associated with plasmid transfer from *E. coli* in livestock (Lima T., *et al.*, 2019; Wang J., *et al.*, 2025). These findings underscore the value of retrospective surveillance: colistin remained fully effective in the pre-*mcr* era, but recent reports confirm its erosion as a last-resort option in some regions. The most striking difference emerged with fluoroquinolones.

Resistance in Lombardy was just 2.9%, contrasting sharply with European data indicating rates as high as 18% in human isolates and up to 72% in zoonotic strains, particularly in *S. Kentucky* (EFSA and ECDC, 2025). This discrepancy is likely linked to the extensive use of fluoroquinolones in poultry and the global spread of resistant clones such as *S. Kentucky* ST198 (Mashe T., *et al.*, 2023). Nonetheless, the low levels observed in the 2001–2016 dataset provide a valuable baseline for tracking the subsequent escalation of fluoroquinolone resistance documented in later European surveillance (ECDC 2025). Overall, these findings confirm that older antibiotic classes (penicillins, tetracyclines, aminoglycosides) exhibit the highest resistance, consistent with global reports linking their overuse in both human and veterinary medicine to widespread MDR phenotypes.

Conversely, the relatively preserved efficacy of extended spectrum cephalosporins, carbapenems, colistin, and fluoroquinolones during this period highlights the success of stewardship and regulatory interventions, though even low levels of resistance in these critically important drugs warrant careful surveillance. From a One Health perspective,

the persistence of resistance to “traditional” antibiotics likely reflects long-term selective pressure across clinical and agricultural contexts, coupled with ecological stability of plasmid-borne resistance genes, which may persist even in the absence of direct antimicrobial exposure (Cheng M., *et al.*, 2024; Mattock J., *et al.*, 2024).

In parallel with these AMR findings, physiological studies conducted on isolates from the biobank provided complementary insights into the adaptive strategies of different serotypes. The results indicated that the analyzed *S. enterica* strains exhibited overall similar growth dynamics, with no statistically significant differences in maximum growth rates (μ_{max}). Nonetheless, several biologically relevant trends could be highlighted. Clear physiological differences emerged between *S. Choleraesuis* strains isolated from stool and those isolated from blood, in contrast with *S. Typhimurium*, whose stool- and blood-derived isolates showed highly similar growth behaviors. This can be explained from a genetic and physiological point of view. In fact, strain isolated from stools were also sensible to antibiotics and more efficient in grow rate. Specifically, SCs strains exhibited growth rates comparable to those of STs isolates and reached final biomass levels similar to the reference strain ATCC 14028. In contrast, SCb isolates reached the lowest final biomass values despite achieving growth rates comparable to ATCC 14028. SCb strains showed to carry a high multi drug resistance to different antibiotics, which implicate expression of resistance genes making genome longer and heavier to replicate. In fact, SCb strains harbor genomic and plasmid-borne resistance determinants impose a metabolic burden and slow replication. (San Millan A., *et al.*, 2017; Rajer F., *et al.*, 2022).

S. Napoli isolates exhibited final biomass values (T7) comparable to those of *S. Typhimurium* strains recovered from both stool and blood. This finding may help explain why *S. Napoli*, an emerging serotype in Europe, appears to possess epidemiological advantages. Although *S. Napoli* displayed the lowest growth rate among the tested serotypes, it nonetheless reached an infectivity index of 8.1%, underscoring its clinical relevance. Furthermore, as previously reported by Gori *et al.* (2018), this serotype requires a lower infectious dose, a feature that may further facilitate its capacity to cause outbreaks.

Subsequently, *S. enterica* strains were subjected to survival assays under simulated gastric acidity conditions (pH 2.0 and pH 4.0) to assess potential differences in acid tolerance among the different serotypes. More interesting differences emerged at pH 4.0. *S. Choleraesuis* displayed a statistical higher survival compared to the other *S. Typhimurium* strains but not *S. Napoli*, suggesting that this serotype (or at least the studied isolates) may possess acid-tolerance traits that allow a larger fraction of the inoculum to persist under sub-acidic conditions. It was well established that acid tolerance in *Salmonella* was both strain-dependent and inducible (acid tolerance response, ATR), and that different serotypes and strains could exhibit substantial variation in survival under simulated gastric conditions. Mechanisms described in the literature included decarboxylase systems, chaperones and acid-shock proteins, two-component regulatory networks, and outer membrane components capable of modulating proton flux and stress resistance; these mechanisms were characterized in *Salmonella* and extensively reviewed (Viala JP., *et al.*, 2011; Hong W., *et al.*, 2012; Mao M., *et al.*, 2025; Wang W., *et al.*, 2022). Specific studies reported serotype-variability in acid resistance, with genetic determinants such as *TolC* and other cell envelope components implicated in acid survival in certain *S. Choleraesuis* isolates (Lee JJ., *et al.*, 2016; Buckley AM., *et al.*, 2006). From an epidemiological perspective, *S. Choleraesuis* was frequently associated with invasive forms of disease and classical interpretations tended to describe invasive serotypes as less adapted to prolonged gastric survival compared to enteric serotypes. However, the relationship between acid tolerance and invasive potential was not straightforward and can be modulated by strain-specific genetic features and reflected clade-specific traits (Álvarez-Ordóñez A., *et al.*, 2011). Generally, ambient strains exhibited slightly higher tolerance to pH fluctuations, likely reflecting adaptation to variable external conditions. However, this does not account for the markedly higher acid resistance of *S. Choleraesuis*, which is rarely recovered from the environment despite its high gastric survival capacity.

Overall, these results suggested that distinct survival mechanisms may underline the differences observed between well-adapted environmental strains and *S. Choleraesuis*. Therefore, the apparent superior survival of *S. Choleraesuis* at pH 4.0 should be considered suggestive and biologically plausible but not definitively demonstrated. Further experiments, including larger numbers of isolates per serotype, multiple

biological replicates, and complementary assays (acid pre-adaptation treatments, intracellular survival assays), would be required to confirm these observations and identify the underlying genetic determinants.

From our genomic analysis data, we provide new insights into the population structure and antimicrobial resistance of *Salmonella enterica* serovar Choleraesuis circulating in Lombardy between 2007 and 2016. Despite being collected over nearly a decade and across several provinces, all isolates belonged to ST145, confirming the high degree of clonality of this serovar. The uniformity of genome size and assembly statistics further supported the limited genetic diversification within this lineage, in line with previous observations that *S. Choleraesuis* tends to circulate as a single dominant clonal complex (Papić B., *et al.*, 2021). A striking feature of our dataset was the widespread presence of antimicrobial resistance determinants: 95.4% of isolates carried acquired resistance genes, with a highly conserved multidrug-resistant profile (*aadA1*, *aph(3'')-Ib*, *aph(6)-Id*, *blaTEM-1B*, *dfrA1*, *sul1*, *sul2*, and *tet(B)*). This uniform resistance signature suggests the long-term dominance of a successful MDR clone. Sporadic acquisition of additional aminoglycoside resistance genes or variants of *blaTEM* further illustrated the capacity of ST145 to accumulate resistance determinants, although such events appeared rare. The presence of *floR* in one isolate points to occasional acquisition of florfenicol/chloramphenicol resistance, which may be linked to selective pressures in the veterinary sector (De Lorenzi G., *et al.*, 2025).

Of particular interest was the identification of three susceptible isolates, used for experiments (SCs), that, in phylogenetic analyses, clustered as the most divergent lineages. This finding suggested that susceptible strains persist alongside MDR clones, potentially representing either ancestral lineages or reintroductions from reservoirs where antimicrobial use is limited (e.g., wildlife or small-scale farms). Their coexistence with resistant clones reflected a dynamic population structure, in which resistance facilitates clonal expansion under selective pressure but was not indispensable for persistence in all ecological niches (Uelze L., *et al.*, 2021).

Nonetheless, the stability of the MDR clone over nearly a decade underscores the capacity of this pathogen to persist despite antimicrobial stewardship efforts (De Lorenzi

G., *et al.*, 2025). The cgMLST-based network shows that beneath all 65 *S. Choleraesuis* isolates belonging to ST145, were structured into seven main clusters, indicating microevolution within a single clonal complex. The presence of tightly connected clusters (allelic distance ≤ 5) alongside more distant peripheral nodes demonstrated that substantial diversification could occur without ST switching, consistent with within-clone genomic drift, local selective sweeps and acquisition/loss of mobile genetic elements.

Whole genome approaches such as cgMLST are therefore essential to resolve this fine-scale population structure (Alikhan NF., *et al.*, 2018). A key epidemiological signal from our data was that isolates lacking resistance genes (phenotypically sensitive) occupy the most genetically distant positions from the central clusters dominated by resistant strains. Recent reports from northern Italy document the re-emergence and characterization of *S. Choleraesuis* in pigs and wild boar, frequently involving ST145 isolates and demonstrating variable AMR (Anti Microbial Resistance) profiles, indicating local circulation and occasional spill-over between wild and domestic populations (De Lorenzi G., *et al.*, 2025). These studies support the hypothesis that the observed clusters correspond to locally circulating lineages likely shaped by animal husbandry practices, antimicrobial use, and wildlife reservoirs. Continued WGS surveillance in Lombardy and neighboring regions is therefore warranted to monitor lineage dynamics and the spread of resistance determinants (De Lorenzi G., *et al.*, 2025). Finally, our results emphasize the One Health dimension: ST145 *S. Choleraesuis* is invasive and can infect humans, and regional circulation in pigs and wild boar represents a zoonotic risk. Integration of veterinary, wildlife and public-health sequencing data will be essential to detect cross-sector transmission and to design control measures (biosecurity, prudent antimicrobial use, targeted surveillance).

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5 CONCLUSIONS

This study demonstrates the value of combining long-term biobank resources with clinical and demographic data to capture shifts in serovar distribution, antimicrobial resistance, and pathogenic potential over time.

Our findings indicate that the epidemiology of *Salmonella enterica* in Lombardy mirrors both European and global trends, while also exhibiting distinctive regional features. Historically, *S. Typhimurium* and *S. Enteritidis* have predominated, but their relative prevalence declined over time, with *S. Napoli* and the monophasic variant of *S. Typhimurium* emerging as major contributors. Notably, the decline in *S. Enteritidis* coincided with the implementation of stricter hygiene measures in poultry and egg production, underscoring the effectiveness of targeted food safety policies.

Invasive forms of salmonellosis remain a major concern for public and animal health, requiring ongoing efforts to refine epidemiological understanding and strengthen preventive strategies. Furthermore, older adults were disproportionately affected by invasive and systemic infections, carrying MDR profiles that raise important questions about the public health implications. Although our dataset likely reflects only the “tip of the iceberg,” the historical archive provided valuable insights into epidemiological trends and reinforced the importance of sustained surveillance.

Phenotypic assays on representative isolates yielded additional insights into biological differences among serotypes. Although preliminary, these findings underscore the importance of integrating physiological data with epidemiological evidence, as subtle differences in growth dynamics and acid tolerance may help explain variability in transmission and pathogenicity. The relatively slow growth rate of *S. Choleraesuis* strains implicated in invasive infections (SCb) may explain by the presence of larger genomes or virulent plasmids, conferring enhanced invasiveness and resistance traits.

Genomic analyses of *S. Choleraesuis* isolates revealed a striking degree of clonality (ST145), with the circulation of a successful multidrug-resistant (MDR) lineage persisting for nearly a decade. Despite this overall uniformity, phylogenetic analyses suggested the coexistence of resistant and phenotypically susceptible lineages. The peripheral

positioning of susceptible isolates within the phylogeny points to possible reintroductions from external reservoirs, such as wildlife or small-scale farms. The antimicrobial resistance data generated through this retrospective analysis thus provides both a valuable historical baseline and a critical tool for monitoring the spread of AMR.

Overall, these findings highlight the importance of adopting a One Health perspective. The circulation of ST145 in pigs and wild boars, combined with its demonstrated invasiveness in humans, represents a significant zoonotic threat. Continuous genomic surveillance that integrates human, veterinary, and environmental isolates is therefore essential to track lineage diversification, detect interspecies transmission, and inform targeted control strategies. Our data further emphasize the need to maintain strict monitoring and control measures along the food production chain, particularly for pork-derived products, which remain a key reservoir for *S. Choleraesuis* and a potential source of human infection.

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7 APPENDIX

7.1.1 Strain collection and Ethical Considerations

All strains included in this study were obtained from an established biobank preserved at the EpiSoMI laboratory (*Center of Coordinate Research – “**E**pidemiologia e **S**orveglianza **M**olecolare delle **I**nfezioni”*) and established during the operational period of the Pathogenic Enterobacteria Center for Northern Italy (CEPIS). The retrospective analysis was conducted using fully anonymized data, in accordance with applicable ethical and regulatory standards.

7.1.2 Supplementary data

Supplementary Table 1: Number of cases for serotypes recorded in the period 2001-2016.

serotypes		Number of cases (N)
1	Typhimurium	2772
2	Enteritidis	1223
3	Monofasica	649
4	Napoli	636
5	Derby	148
6	Infantis	86
7	Choleraesuis	82
8	Muenchen	76
9	Goldcoast	60
10	Virchow	59
11	Hadar	57
12	Bovismorbificans	46
13	Brandenburg	43
14	London	40
15	Panama	39
16	Rissen	39
17	Typhi	37
18	Heidelberg	34
19	Anatum	28
20	Agona	26
21	Bredeney	25
22	Blockley	20
23	Give	20
24	Thompson	20
25	Kottbus	18
26	Livingstone	18
27	Saintpaul	15
28	Stanley	12
29	Irchel	11
30	Paratyphi A	10
31	Zaiman	10
32	Corvallis	9
33	Montevideo	9
34	Paratyphi B	9
35	Coeln	8
36	Braenderup	7
37	Kapemba	7
38	Arizonae	6
39	Haifa	6
40	Kimuenza	6
41	Manhattan	6

42	Salamae	6
43	Kentucky	5
44	Newport	5
45	Parkroyal	5
46	Veneziana	5
47	Othmarschen	4
48	Toronto	4
49	Diarizonae	3
50	Indiana	3
51	Kedougou	3
52	Tsevie	3
53	Afula	2
54	Agbeni	2
55	Akanji	2
56	Baildon	2
57	Berta	2
58	Bispebjerg	2
59	Colorado	2
60	Fyris	2
61	Goettingen	2
62	Isangi	2
63	Jerusalem	2
64	Kasenyi	2
65	kitenge	2
66	Lagos	2
67	Larochelle	2
68	Lomalinda	2
69	Mathura	2
70	Mbandaka	2
71	Mendoza	2
72	Paratyphi C	2
73	Poona	2
74	Sangalkam	2
75	Senftenberg	2
76	Sinstorf	2
77	Soerenga	2
78	Tallahassee	2
79	Uppsala	2
80	Warb	2
81	Wippra	2
82	Zanzibar	2
83	Aberdeen	1
84	Abony	1
85	Admstua	1
86	Ahuza	1
87	Ajiombo	1

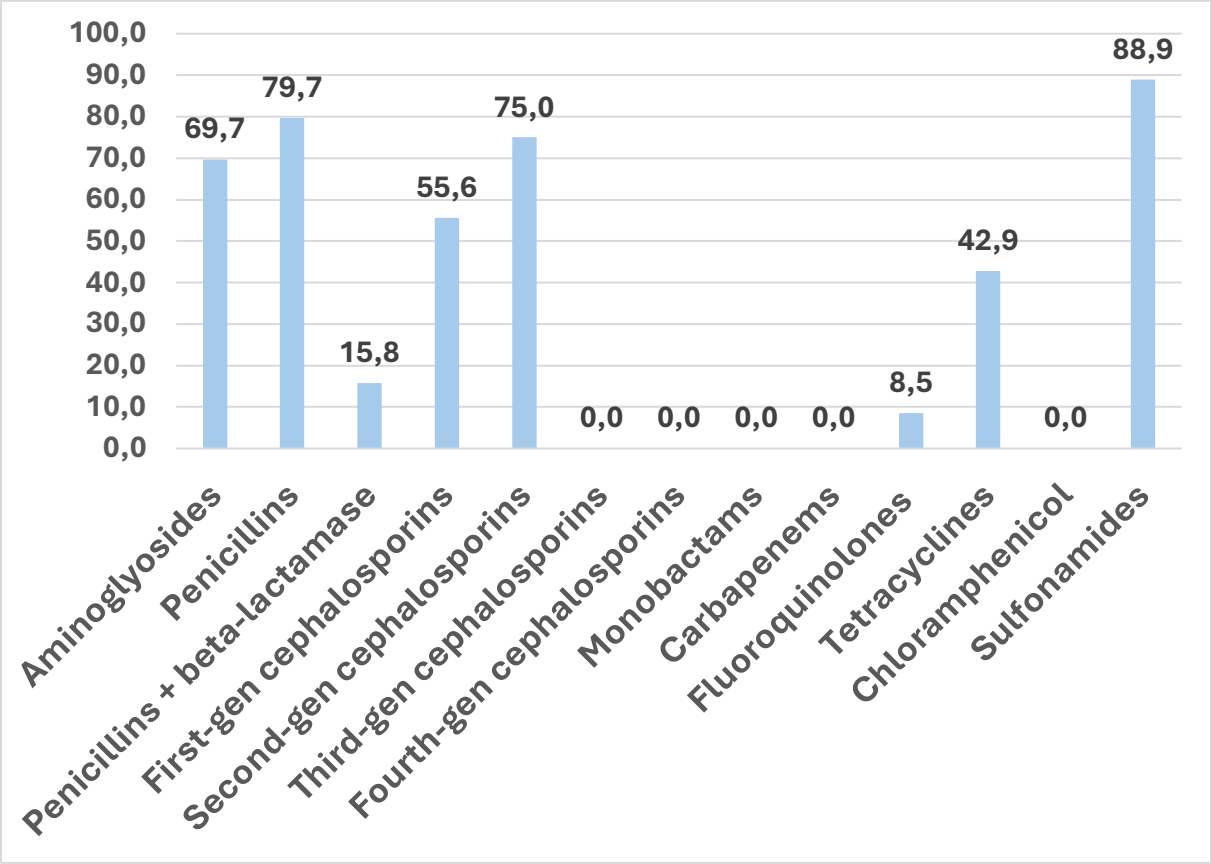
88	Albert	1
89	Api	1
90	Assinie	1
91	Ball	1
92	Bochum	1
93	Bongori	1
94	Breukelen	1
95	Brikama	1
96	Bristock	1
97	Bukuru	1
98	Calabar	1
99	Chailey	1
100	Chenna	1
101	Chester	1
102	Chudleigh	1
103	Claibornei	1
104	Clerkenwell	1
105	Cyprus	1
106	Dabou	1
107	Dar-es-Salaam	1
108	Daula	1
109	Eastbourne	1
110	EKO	1
111	Escanaba	1
112	Essen	1
113	Farmsen	1
114	Galiema	1
115	Gallinarum	1
116	Glostrup	1
117	Goeteborg	1
118	Grampian	1
119	Herzliya	1
120	Hiduddify	1
121	Hillingdon	1
122	Houston	1
123	Inglis	1
124	Israel	1
125	Kande	1
126	Kingston	1
127	Kinondoni	1
128	Kisangani	1
129	Kisii	1
130	Kokomlemle	1
131	Kuilsriver	1
132	Lerum	1
133	Litchfield	1

134	Lomita	1
135	Marmande	1
136	Meleagridis	1
137	Merston	1
138	Muenster	1
139	Ndolo	1
140	Ohio	1
141	Orion	1
142	Oritamerin	1
143	Pensacola	1
144	Pikime	1
145	Potsdam	1
146	Redougou	1
147	Seattle	1
148	Shangani	1
149	Sofia	1
150	Solna	1
151	Sterrenbons	1
152	Stratchona	1
153	Szentes	1
154	Telelkebir	1
155	Tiko	1
156	Umbilo	1
157	Urbana	1
158	Vellore	1
159	Virginia	1
160	Welikade	1
161	Weltevreden	1
162	Wentworth	1
163	Westerstede	1
164	Westhapton	1
165	Worthington	1
166	Yoruba	1
167	Yundum	1
168	Zuilen	1
	Total	6624

Supplementary Table 2: *S. Choleraesuis* antibiotic resistance from WGS and datasheets

Antimicrobial resistance of 65 <i>S. Choleraesuis</i> strains		
	WGS	Datasheets
Aminoglycosides	93.8 (61/65)	63.6 (14/22)
Penicillins	87.7 (57/65)	76.7(33/43)
Penicillins + beta-lactamase	1.5 (1/65)	12.5 (2/16)
First-gen cephalosporins	84.6 (55/65)	75.0 (3/4)
Second-gen cephalosporins	0,0	66.7 (4/6)
Fluoroquinolones	0,0	9.4 (3/32)
Tetracyclines	81.5 (53/65)	40.0 (2/5)
Chloramphenicol	1.5 (1/65)	0.0 (0/7)
Sulfonamides	86.2 (56/65)	87.5 (35/40)

Supplementary Figure S1: Antibiotic resistance of *S. Choleraesuis* strains on datasheets



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