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Review article

Emergence of *Salmonella* epidemics: The problems related to *Salmonella enterica* serotype Enteritidis and multiple antibiotic resistance in other major serotypes

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Abstract – Two major changes in the epidemiology of salmonellosis occurred in the second half of the 20th century: the emergence of food-borne human infections caused by *S.* Enteritidis and by multiple-antibiotic resistant strains of *Salmonella*. This review updates information on the *S.* Enteritidis pandemic and focuses on the emergence of *Salmonella,* carrying the SGI1 antibiotic resistance gene cluster, resistant to extended-spectrum cephalosporins, or resistant to fluoroquinolones. The factors responsible for the emergence of these *Salmonella* strains could be either of human origin or related to bacterial genome evolution. However, our increasing understanding of the molecular fluidity of the genome shows that any attempt to counteract bacteria results in further bacterial evolution or adaptation of other bacteria to take place in the new free ecological niche. In these conditions, we can ask who is faster: humans who want to eliminate bacterial pathogens or bacteria that continuously evolve to gain new niches.

***Salmonella* / emergence / antibiotic resistance / virulence / genome evolution**

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1. *SALMONELLA*

The genus *Salmonella* is a typical member of the family Enterobacteriaceae and consists of Gram-negative, nonspore-forming bacilli. Bacteria constituting the genus contain three different types of antigens. The agglutinating properties of the somatic O, flagellar H and capsular Vi antigens are used to differentiate among more than 2 500 serologically distinct types of *Salmonella* [122]. Each year, new serotypes are listed in annual updates of the Kauffmann-White scheme [122]. The genus *Salmonella* consists of only two species, *S. bongori* and *S. enterica,* whith the latter being divided into six subspecies: *entericae*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, *indica*. Within *S. enterica* subsp. I (*S. enterica* subsp. *entericae*), the most common O-antigen serogroups are A, B, C1, C2, D and E. Strains within these serogroups cause approximately 99% of *Salmonella* infections in humans and warm-blooded animals [143]. Serotypes in other subspecies are usually isolated from cold-blooded animals and the environment but rarely from humans [78, 143]. *Salmonella* nomenclature is complex, and scientists use different systems to refer to this genus. The nomenclature used in this review is based on names for serotypes in subspecies I. For example, *Salmonella enterica* subsp. *entericae* serotype Enteritidis, is shortened to *Salmonella* serotype (ser.) Enteritidis or *Salmonella* Enteritidis [23]. *Salmonella* serotypes can be further subdivided by using biotyping and phage typing. A biotype is the biochemical variation between two microbes of the same serotype, whereas the phage type reflects differences between two organisms with the same serotype but different susceptibilities to a lytic bacteriophage [146, 148]. Phage typing has played a central role in epidemiological studies in *S.* Typhimurium and in understanding the evolution of the *S.* Enteritidis pandemic [49].

2. SALMONELLOSIS

The degree of host adaptation varies between *Salmonella* serotypes and affects the pathogenicity for man and animals. Serotypes adapted to man, such as *S.* Typhi and *S.* Paratyphi, usually cause severe diseases in humans as a septicaemic typhoidic syndrome (enteric fever). These serotypes are not usually pathogenic to animals. Serotypes that are highly adapted to animal hosts, such as *S.* Gallinarum (poultry) or *S.* Abortus-ovis (sheep), usually produce very mild symptoms in man. However, *S.* Choleraesuis which has the pig as a primary host, also causes severe systemic illness. In the same way, *S.* Dublin which has a preference for bovines, is primarily responsible for the systemic form of salmonellosis. In young calves this disease causes high mortality, and in adult cattle it results in fever, reduced milk yield, diarrhea, abortion, and occasionally death. Ubiquitous serotypes, such as *S.* Enteritidis or *S.* Typhimurium, which affect both man and animals, generally cause gastrointestinal infections usually less severe

than enteric fever. However, they also have the capacity to produce typhoid-like infections in mice and in humans or asymptomatic intestinal colonisation in chickens [34].

Salmonella has been recognised as causes of intestinal disease for many years, and methods of control are well established. Despite these, *Salmonella* remains the primary cause of reported food poisoning worldwide and recent years have seen massive outbreaks. *S.* Typhi and other human-adapted *Salmonella* are rarely transmitted by food compared to ubiquitous serotypes. For this reason, the human adapted serotypes are often excluded from discussion of *Salmonella* infection. Currently, approximately 30 000 to 40 000 human cases per year of non-typhoidal salmonellosis are reported to Centers for Disease Control and Prevention (CDC) [95]. Taking into account the degree of under-reporting, the CDC estimates the annual number of cases in the United States (USA) to be approximately 1.4 million [95]. In the European Union (EU), the number of human cases reported to Enternet was greater than 100 000 in 1997 [106]. In recent years, the incidence of salmonellosis has shown a sustained decrease across the EU (73 000 cases in 2001) and also in the USA since 1996 [5, 106].

The epidemiology of human disease is dominated, however, by only a few non-typhoidal serotypes. In 2001, approximately 60% of the human cases reported to the CDC were caused by four serotypes, namely *S.* Typhimurium (22.1%), *S.* Enteritidis (17.7%), *S.* Newport (10.0%) and *S.* Heidelberg (5.9%) [5]. Such dominance is even more pronounced in France, where more than 70% of human cases were caused by three serotypes: *S.* Enteritidis (33%), *S.* Typhimurium (32%), and *S.* Hadar (6%) [19].

Two major changes in the epidemiology of non-typhoidal salmonellosis in the EU and the USA occurred in the second half of the 20th century: the emergence of food-borne human infections caused by *S.* Enteritidis and by multiple-antibiotic resistant strains of *S.* Typhimurium. A better understanding of the factors that led to the emergence of these food-borne pathogens may help the design of improved intervention strategies that could reduce the probability that new pathogens could spread in food animal reservoirs [108, 127].

3. EMERGENCE OF S. ENTERITIDIS

3.1. The *S.* Enteritidis pandemic

By the 1980s, *S.* Enteritidis O9,12: g,m had emerged as a major concern for food safety in Europe and the Americas. By 1990 in the USA and by 1993 in Europe, it was the most frequently reported *Salmonella* serotype [5, 19, 33, 127].

In the USA, *S.* Enteritidis steadily increased in frequency from being the sixth most common serotype in 1963 to becoming the most frequently reported serotype in 1990. Epidemics in the USA are marked by regional differences. *S.* Enteritidis emerged in 1979 in New England and the Mid-Atlantic regions. In the early 1990s, while *S.* Enteritidis rates of infection in the Northeast began to decline, the *S.* Enteritidis epidemic expanded to the Pacific region. Nationwide, the number of *S.* Enteritidis isolates reported to the CDC peaked at 3.8 per 100 000 population in 1995. Although the number of *S.* Enteritidis isolates reported to the CDC had significantly declined to 1.9 by 1999, this rate did not decline further through 2001 and even increased in the south-eastern regions [4].

In England and Wales, there were 200 reported human cases in 1966, which rose to 10 000 in 1981, and peaked at 33 000 in 1997 (more than 70% of human cases of salmonellosis) [33, 149]. Despite a subsequent decline in its incidence, *S.* Enteritidis continues to be the most frequently isolated *Salmonella* serotype in the United Kingdom with 16 465 cases in 2001 [33].

In France, *S.* Enteritidis has become the most common isolated serotype in 1993. The incidence of *S.* Enteritidis human isolates

increased exponentially from 1987 to peak at 6 500 in 1994 and 1997 and subsequently declined to 4 500 cases in 1999 [19]. Similar trends have also been reported from other countries in South America and Europe [33, 127].

3.2. Origin of human contaminations

Investigation of outbreaks and sporadic cases has repeatedly indicated that, when a food vehicle is identified, the most common sources of *S.* Enteritidis infection are poultry and poultry derivatives, particularly, in the case of outbreaks, undercooked and raw eggs [35, 41, 65, 74]. Although contamination of egg products with other *Salmonella* serotypes is a long-standing problem, that has been attributed either to the use of damaged eggs or to contamination at or after breaking, the situation with *S.* Enteritidis is different. Egg shells can be contaminated with *Salmonella* as a result of intestinal carriage but the contents can also become infected by the transovarian route [79, 146].

3.3. Origin of the *S.* Enteritidis pandemic

3.3.1. Symptomless carriers

The factors responsible for the epidemic spread of *S.* Enteritidis are still unclear. One of the factors that may have been important for the epidemiological spread of this pathogen is the difficulty to detect the contamination of the chicken. It is particularly known that *S.* Enteritidis causes symptomless intestinal infections in a wide range of species, especially birds [45, 135]. Acute outbreaks of clinical disease with high mortality may nevertheless occur in chicks younger than two weeks of age [88]. Symptomless carriage may facilitate the spread of infection within a flock by environmental contamination of their intestinal contents [46, 53]. Another difficulty is that the presence of *S.* Enteritidis within contaminated eggs is difficult to detect until the bacteria exceed log 9.0 per egg [79].

3.3.2. Farm practices

The modernisation of chicken farms and globalisation of bird breeding trade have also played a role in the spread of *S.* Enteritidis. For example, the most prevalent molt strategy in the USA is feed removal until hens lose a specific weight. However, hens molted in this way were found to be 100- to 1 000-fold more susceptible to infection by *S.* Enteritidis and excreted significantly higher numbers in their faeces [75]. Several authors reported that the major risk factors were related to disinfection, hygiene barriers and feedmill [37, 69]. Epidemiological investigations in The Netherlands indicated that laying flocks become infected mainly directly from the farm environment and the contribution from the vertical infection route (from infected breeding flocks to progeny) is small [144]. In contrast, Ward et al. have suggested that the spread of the *S.* Enteritidis epidemic in the United Kingdom was related to the introduction of poultry breeding lines infected with phage type 4 in the UK in the early 1980s [150].

3.3.3. Rodent reservoir

However, these data have no bearing on the origins of the epidemic. Several authors have suggested that *S.* Enteritidis was introduced into poultry flocks by rodents where it is endemic since in the distant past it was used as a rodenticide [51, 71]. *S.* Enteritidis was first used to control rodent populations during the *Yersinia pestis* outbreak in San Francisco in 1895 and then occasionally in Europe until 1960 [127]. There is a correlation between the presence of *Salmonella* in mice and the contamination of poultry. Moreover, some recent reports have shown that several wildlife species, especially rodents, are involved in the maintenance of *S.* Enteritidis infection on farms [38, 52, 86]. It is unlikely, however, that there is a causal link between the use of *S.* Enteritidis

as rodenticide and the human cases reported since 1960 [61, 129]. The use of the phage typing system has indeed shown that the majority of human cases in Europe were caused by PT8 before 1980 and by PT4 afterwards. In contrast, *S.* Enteritidis isolates from rodenticides used in the UK in the 1940s belong to PT6 [51] and those found in rodents in 1993 belong to PT23 [61]. Nevertheless, Threlfall et al. point out the fact that the acquisition of the IncX plasmid converts strains of PT4 to PT6a found in rodents [140].

3.3.4. Acquisition of new virulence properties

The dramatic increase of *S.* Enteritidis PT4 infection in Western Europe since 1980 suggested that it might have recently acquired new virulence genes. This hypothesis is strengthened by fingerprinting observations showing a highly clonal structure of the strains investigated, and suggesting that the epidemic in the UK is the result of the efficient spread of just one clone rather than the alteration of conditions which might have favoured the dissemination of several clones or serotypes simultaneously [68]. This clone may have acquired genetic changes, which might have facilitated the spread of PT4. Besides several reports showing that the PT4 spread from experimentally infected chicks to uninoculated chicks occurs at a lower frequency than that of PT8 and PT13, most reports have shown that some PT4 strains tend to be more virulent than other phage types [8, 54, 73]. Additionally, Poppe et al. reported that in orally inoculated one-day old chickens and in laying hens, the clinical UK *S.* Enteritidis PT4 strain was more virulent than the Canadian PT4 strain, which was not epidemic in Canada at this date [123].

Investigations in the laboratory and on farms, have failed to reveal any unique properties of *S.* Enteritidis PT4. It has been possible, however, to observe that *S.* Enteritidis emerged because it was associated with a new food source, namely chicken eggs. We can thus hypothesise that *S.* Enteritidis acquired new genes to increase the efficiency of its infection of the reproductive tract. Interestingly, recent results have demonstrated that repeated passages through the reproductive tract of chickens increased the ability of an *S.* Enteritidis strain PT13a to induce internal contamination of eggs in an oral infection model, as opposed to serial passages through the liver and spleen that did not significantly affect the ability of this strain to cause egg contamination [55]. One gene, which might be related to adaptation to egg contamination, is *yafD*, whose overexpression conferred upon *S.* Typhimurium an enhanced resistance to egg albumen, while disruption of this gene in *S.* Enteritidis rendered the organism more susceptible to egg albumen [91].

3.3.5. Eradication of S. *Gallinarum*

The analysis of *S.* Enteritidis isolates worldwide reveals the existence of two major evolutionary lineages: one found in Western Europe, Japan and South America (PT4) and another found in the USA, Canada, and the Slovak Republic (PT8 and PT13a) [42, 72, 92, 104, 124]. These geographical differences render the pandemic difficult to completely explain as the spread of a single clone of the bacterium. It has recently been proposed that the eradication of *S.* Gallinarum opened an ecological niche, which allowed the introduction of *S.* Enteritidis into poultry flocks [12]. Because the immunodominant epitope of the lipopolysaccharide of *S.* Gallinarum and *S.* Enteritidis is the O9-antigen, mathematical models predict that the coexistence of these two serotypes would prompt competition where the more transmissible bacterium will eliminate the other from the host population [62], either as a result of adaptive immunity, or as a result of microbial competition, which is also partially clonal at the level of the serotype [14]. *S.* Gallinarum may have generated immunity at the flock level against the O9-serotype, thereby excluding *S.* Enteritidis from poultry flocks [126]. According

to this hypothesis, if *S.* Gallinarum had been eradicated from poultry by vaccination rather than by the killing of infected animals, the resulting flock immunity against the O9-serotype might have prevented the emergence of *S.* Enteritidis [82]. However, in this hypothesis, vaccination had to continue even after eradication of *S.* Gallinarum to continue to prevent introduction of *S.* Enteritidis.

3.4. Fluctuation or fall of the *S.* Enteritidis pandemic?

Recent data show a sustained decrease in the number of human cases since 1996 in the USA [5], 1997 in the UK [33] and 1999 in France [19]. In England and Wales, the rate of *S.* Enteritidis infection fell by over 50% between 1997 and 2000 which corresponded to the period of introduction of new live vaccines which were easier to administer to hens in contrast to the initial vaccines based on formalin-killed bacteria [150]. However, vaccination cannot be the sole cause of this decline, because *S.* Enteritidis infections decreased in several countries that do not vaccinate hens. In addition, the proportion of *S.* Typhimurium infections has also declined in a number of countries [5, 19]. This decline might thus also be attributed to the implementation of other preventive measures, including on-farm control programmes, improved hygiene and consumer and food-worker education [145]. One important control is the microbiologic testing of hen houses for the presence of *S.* Enteritidis. If the bacterium is found on a farm during routine environmental testing, eggs may be diverted to pasteurisation. The evidence suggests that proper implementation and overlook of farm-based control programmes can result in a significant reduction of *S.* Enteritidis infections among flocks in poultry houses [152]. However, some reports tend to show that the pandemic has become stabilised rather than showing a real decline. For example, this decline did not occur in Spain, where an increase was recorded [93]. In addition, since 2001 a change in phage type distribution in *S.* Enteritidis infections among European travellers returning from some countries in southern Europe was observed, and a previously rare phage type (PT14b) became one of the most commonly diagnosed in England and Sweden [105, 107]. In the USA, although the number of culture-confirmed *S.* Enteritidis infections reported to the CDC declined in 1999, the number has not decreased since this date and some regions have seen increases again with the appearance of PT4 infections [4, 33]. An explanation for the current variations in *Salmonella* infections could be related to the ubiquity of *S.* Enteritidis and *S.* Typhimurium in poultry flocks leading to flock immunity that could have led to their decline [33]. However, it is possible that, as a serotype becomes less prevalent, the number of immune individuals decreases, which that could itself lead again to an increase in prevalence.

4. SOURCE OF EMERGENCE: MICROBIAL GENOME EVOLUTION

Over the past few years, an increasing number of microbial genomes have been sequenced and compared, allowing for the estimation of the frequencies of mutations influencing their genomic structures [118, 136]. By quantifying these differences, it has been observed that considerable genomic variability is found both in *Escherichia coli* and *Salmonella enterica* [48, 109]. The ratio of rearrangements to substitutions is over 2000-fold higher in their genomes than that which occurs in *Buchnera aphidicola* the obligate bacterial endosymbiont of aphids [98].

An important source of genome evolution is the site-specific recombination mechanism required for the processing of the replicated linear chromosome ends and involved in intra-genomic recombination [83]. Inter-genomic exchange involving homologous recombination probably further increases

the variability seen in these systems. Diversity is also generated by mutagenesis, which may be related to the inactivation of the mismatch pathway normally involved in eliminating errors escaping the replication proofreading process [56]. This mechanism plays a less prominent role in clonal diversity than recombination [132]. However, it is difficult to account for the ability of bacteria to exploit new niches through these genetic mechanisms, and there is growing evidence that lateral gene transfer has played a crucial role in the evolution of bacterial genomes, altering their ecological and pathogenic characteristics [110]. Genome variability could, indeed, result from the acquisition and/or loss of relatively large regions of the genome carrying groups of genes. Such horizontally transmitted DNA fragments include plasmids, genomic islands, temperate bacteriophages, in addition to transposons and insertion sequences. These mobile elements can undoubtedly provide an advantage for the host cell under specific conditions [110].

Virulence associated genes showing evidence for an origin outside the bacteria in which they are identified and which may be present on such a mobile element, are referred to as a pathogenicity island (PAI). Recent studies have shown that acquired PAI are major contributors to the virulence nature of many pathogenic bacteria [60, 63], including *Salmonella* where they are referred to as *Salmonella* Pathogenicity Islands (SPI). These chromosomally encoded regions typically contain large clusters of virulence genes and have the potential to increase the virulence of a micro-organism or even to transform a benign organism into a pathogen [100]. Until now, 12 SPI have been described. Some of them are conserved throughout the genus *Salmonella*, while others are specific for certain serotypes like SPI-8 for *S.* Typhi or for certain subspecies like SPI-6, 9, 10 and SGI-1 for subspecies I serotypes [70]. PAI are often associated with tRNA loci, which may represent target sites for the chromosomal integration of these elements [63]. The sequences flanking PAI also frequently contain short direct repeats reminiscent of those generated upon the integration of mobile elements. Open reading frames within PAI sometimes display sequence similarity to bacteriophage integrases, suggesting that lysogenisation by bacteriophages encoding virulence determinants can result in the conversion of a strain into a pathogenic variant. For example, horizontal transfer of the *sopE1* gene by lysogenic conversion with the SopEphi phage increased the enteropathogenicity of *S.* Typhimurium in the bovine ligated ileal loop model, suggesting that the horizontal transfer of type III dependent effector proteins may have contributed to the emergence of epidemic cattle-associated *S.* Typhimurium clones [18, 156]. Another important source of diversity is provided by the acquisition of integrons. They may be part of mobile elements such as transposons, plasmids, and chromosomal genomic islands. Integrons usually carry one or more antibiotic resistance gene cassettes and can sometimes be complex such as the class 1 integron found in SGI1 [21]. In such a case, it looks more like an antibiotic resistance gene cluster and to date SGI1 variants have been identified carrying up to six antibiotic resistance genes, conferring a multiple-antibiotic resistance profile to antibiotic families of clinical importance such as β-lactams, aminoglycosides, phenicols, sulfonamides, tetracyclines, and trimethoprim [21, 22]. In addition, the structures carrying these various antibiotic resistance genes may undergo recombinational, gene replacement and gene capture events, which can lead to a wide variety of antibiotic resistance gene clusters [22, 43, 44].

5. EMERGENCE OF MULTIPLE-ANTIBIOTIC RESISTANCE IN *S. ENTERICA* SEROTYPES

An inevitable side effect of antibiotic use, which is associated to the adaptability of bacteria and microbial genome evolution, is the emergence and dissemination of resistant bacteria, not only in pathogenic bacteria

but also in the endogenous flora of man and animals. Resistant commensal bacteria of food animals might contaminate, like zoonotic bacteria, meat products, thus reaching the intestinal tract of humans [108]. Resistance genes against antibiotics that are or have only been used in animals, were soon after their introduction not only found in animal bacteria, but also in the commensal flora of humans, in zoonotic pathogens like *Salmonella*, and in strictly human pathogens, like *Shigella* [9]. There is evidence, indeed that resistance determinants can transfer between unrelated bacteria such as *Bacteroides* on the one hand and *Salmonella* and *Escherichia* on the other [108]. Therefore, not only does the clonal spread of resistant strains occur, but there is also a transfer of resistance genes between human and animal bacteria [108].

Resistance can be caused by a large number of mechanisms, involving decreased antibiotic accumulation, physical modification or destruction of the antibiotics, and alteration of the enzyme target of antibiotic action. Recently, a mechanism of resistance involving the active efflux of antibiotics by multidrug efflux pumps was also elucidated [84, 121, 131].

5.1. Emergence of multiple-antibiotic resistant *S.* Typhimurium DT104

Multiple-antibiotic resistant *S.* Typhimurium definitive phage type (DT) 104 emerged during the last decade as a global health problem because of its involvement in diseases in animals and humans [31, 125, 141]. Multiple-antibiotic resistant strains of this phage type were first detected in the United Kingdom in cattle and humans in the late 1980s, but have since become common in other animal species such as poultry, pigs and sheep. Human infections with multiple-antibiotic resistant DT104 isolates have been associated with the consumption of chicken, beef, pork, sausages and meat paste [147]. The *S.* Typhimurium DT104 epidemic is now worldwide with a considerable number of outbreaks since 1996 in the USA and Canada [16, 59, 125].

These multiple-antibiotic resistant strains are generally resistant to ampicillin (Ap), chloramphenicol (Cm)/florfenicol (Ff), streptomycin (Sm)/spectinomycin (Sp), sulfonamides (Su), and tetracyclines (Tc). Genes associated with these resistance properties have been found to be chromosomally encoded [138]. Additional resistance to trimethoprim (Tm), occasionally seen among *S.* Typhimurium DT104 strains, may be encoded by a non-conjugative but mobilisable plasmid of approximately 4.6 MDa which also encodes resistance to Su [139]. Transferable apramycin resistance has also been described in some DT104 strains [90].

5.1.1. The Salmonella *genomic island 1 (SGI1)*

The ApCmFfStSpSuTc multiple-antibiotic resistance profile of *S.* Typhimurium DT104 is conferred by an antibiotic resistance gene cluster carried by a chromosomal genomic island called *Salmonella* genomic island 1 (SGI1) [20, 21]. The 43 kb-size SGI1 is located between the *thdF* and *int2* genes of the chromosome of *S.* Typhimurium DT104 and is flanked by an imperfect 18-bp direct repeat (Fig. 1). The *thdF* gene codes for a thiophene-and furan-oxidation protein. The *int2* gene, a prophage CP-4-like integrase gene, is part of a retron sequence which has to date been reported only in strains of serotype Typhimurium [20]. The antibiotic resistance gene cluster is located near the 3' end of SGI1 and constitutes a complex class 1 integron belonging to the In4 group. Class 1 integrons contain a 5'-conserved segment (5'-CS) which consists of the *intI1* gene encoding the site-specific integrase and the associated *attI1* site, the primary site of recombination, and a 3'-conserved segment (3'-CS) of variable length but generally consisting of *qacE1* encoding low level resistance to some antiseptics, the *sul1* gene encoding sulfonamide resistance, and orf5, a gene of unknown function [50].

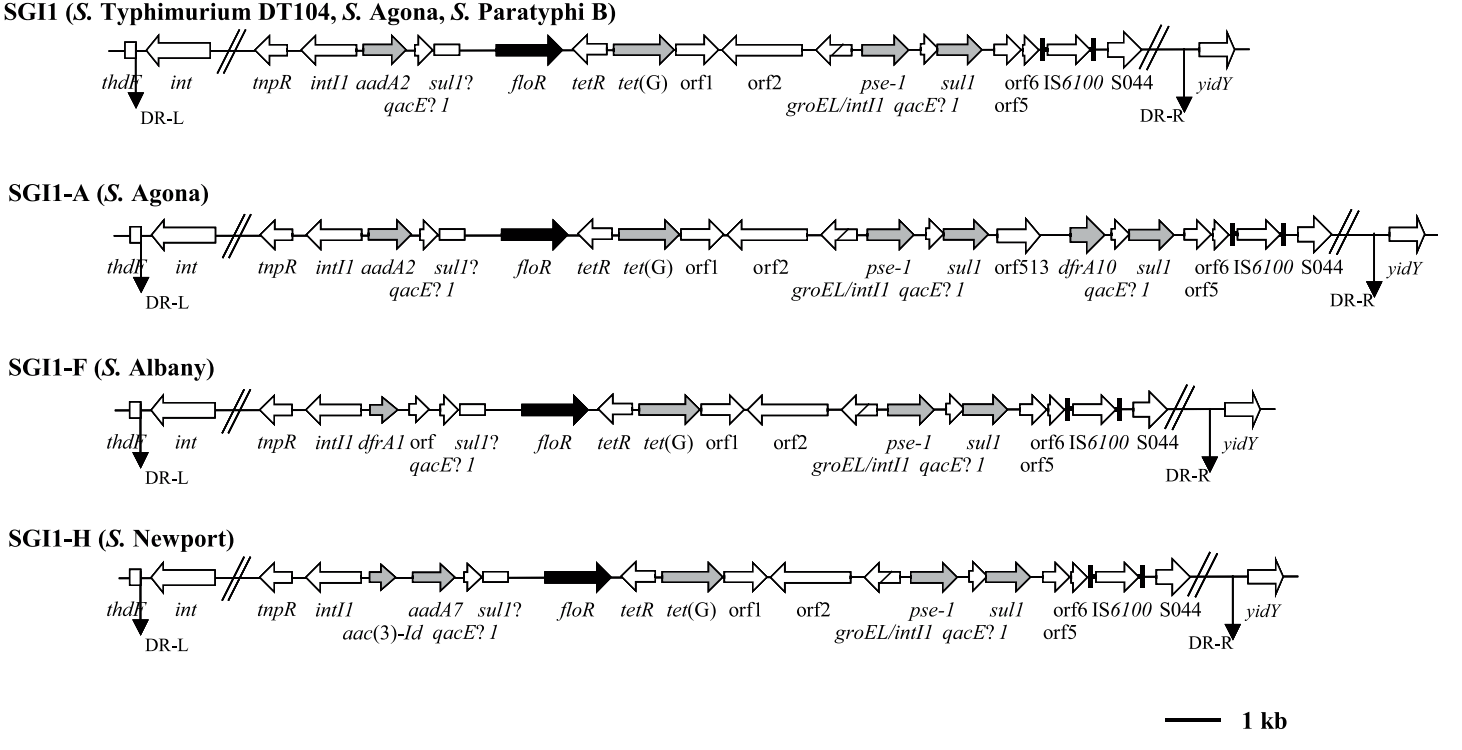

Figure 1. Genetic organization of various antibiotic resistance gene clusters of *Salmonella* Genomic Island 1 (SGI1) in different *Salmonella enterica* serotypes. SGI1 and variants (SGI1-A, SGI1-F, SGI1-H) are always located between the *thdF* and *yidY* chromosomal genes. DR-L and DR-R are the left and right repeats, respectively, bracketing SGI1. The first SGI1 gene *int* codes for a putative integrase probably involved in site-directed integration of SGI1 at the 3' end of *thdF*. The various antibiotic resistance gene clusters mediate resistance to ampicillin (*pse-1*), cloramphenicol and florfenicol (*floR*), gentamicin (*aac(3)-Id*), streptomycin and spectinomycin (*aadA2*, *aadA7*), sulfonamides (*sul1*), tetracyclines (*tet*(G)), and trimetoprim (*dfrA1*, *dfrA10*).

One or more gene cassettes consisting of the coding region(s) and the downstream 59-base element (59-be), which is responsible for recognition and mobilisation of cassettes, are found between the 5'-CS and 3'-CS [50]. Transposon Tn*402* is a mobile class 1 integron that contains the 5'-CS and a transposition module (*tni* region) consisting of four genes required for transposition [25]. In addition, Tn*402* is bound by inverted repeats of 25 bp, IRi at the integrase end, and IRt at the *tni* end. Several class 1 integrons appear to have originated from a Tn*402*-like ancestor by incorporation of the common part of the 3'-CS including *qacE1*, *sul1*, and orf5. Most of these integrons, though still bound by IRi and IRt, have lost part or all of the *tni* module and are deemed defective transposon derivatives [25]. The In4 group has a 3'-CS that includes a copy of IS*6100* but no transposition genes and most members are bound by IRi and IRt [114, 115]. The antibiotic resistance gene cluster of SGI1 is bound by IRi and IRt and thus, can be considered a complex In4-type integron [22]. Furthermore, the multiple-drug resistance region is surrounded by 5-bp direct repeats, strongly suggesting that it was integrated through transposition [22, 114]. Interestingly, in SGI1 there is a duplication of a part of the 5'-CS, leading to a second *attI1* site followed by a gene cassette. At the first *attI1* site, the cassette carries the *aadA2* gene, which confers resistance to Sm and Sp, and a 3'-CS with a truncated *sul1* (*sul1*) gene. At the second *attI1* site, the cassette contains the β-lactamase gene bla_{PSE-1} conferring resistance to Ap and a 3'-CS with a complete *sul1* gene conferring resistance to Su. Flanked by the two cassettes are, first the *floR* gene, which confers cross-resistance to Cm and Ff, and second the tetracycline-resistance genes *tetR* and *tet*(G) [6, 17]. According to the deduced amino acid sequence homologies and the topology of the deduced protein, the *floR* and *tet*(G) genes encode efflux pumps belonging to the 12-transmembrane segment (TMS) family export proteins of the major facilitator superfamily (MFS) reviewed by Paulsen et al. [117]. Variant antibiotic resistance gene clusters of SGI1 have recently been described in *S.* Typhimurium DT104 [22]. In some cases, there is only one 5'-CS and hence, a single gene cassette, either *aadA2* (SGI1-C) or bla_{pse-1} (SGI1-B), and *floR* and *tet*(G) are not present.

5.1.2. Origin of the SGI1 antibiotic resistance gene cluster

Several authors have speculated on the origin of the *S.* Typhimurium DT104 antibiotic resistance gene cluster and consecutive spread of multiple-antibiotic resistant *S.* Typhimurium DT104 strains [3, 39, 40]. The use of antimicrobial agents in agriculture, particularly in intensive calf rearing in the 1970–1980s, might have contributed to the emergence of multiple-antibiotic resistant *S.* Typhimurium DT104 strains. The genes included in the antibiotic resistance gene cluster of *S.* Typhimurium DT104 strains confer resistances to drugs belonging to four out of the five classes of antimicrobials most frequently used in veterinary medicine (tetracyclines, β-lactams, aminoglycosides and sulfonamides) and co-selection of the entire cluster may thus result from the use of any of these drugs. While some genes in the cluster, such as *aadA2*, bla_{PSE-1}, or *sulI*, are widely distributed among the *Enterobacteriaceae*, the remaining two genes, *floR* and *tet*(G), are most probably not of enterobacterial origin.

Ff is a veterinary antimicrobial agent that has been used in aquaculture in Asia since the early 1980s. A *floR* homolog was first identified on a plasmid in *Pasteurella piscicida* recently renamed *Photobacterium damselae*, a fish pathogen [80]. In addition, the class G tetracycline resistance gene associated with the *floR* gene in the *S.* Typhimurium DT104 antibiotic resistance gene cluster was first identified in *Vibrio anguillarum*, also a fish pathogen [157]. The *tet*(G) gene has also been detected on plasmids of *Photobacterium damselae* [81]. Based on these data, and on the fact that the *floR* and *tet*(G)

genes in *S.* Typhimurium DT104 have a similar G+C content (58%), Angulo et al. [3] suggested that the resistance determinants of multiple-antibiotic resistant *S.* Typhimurium DT104 strains have the same origin and may have emerged amongst bacteria in aquaculture and subsequently been horizontally transferred to *S.* Typhimurium DT104. However, Davis et al. [40] suggested an origin in *Pseudomonas* sp. Indeed, *tet*(G) also occurs in bacteria of this genus [130], and similarly *floR* is closely related to the *P. aeruginosa* chloramphenicol-resistance gene *cmlA* [6, 24]. Moreover, the bla_{PSE-1}-encoded β-lactamase is a common feature of hospital *P. aeruginosa* isolates [120]. Thus, the hypothesis that *S.* Typhimurium DT104 acquired resistance genes horizontally from nosocomial pseudomonads might also be worth considering. The association of *floR* with the *tetR* and *tet*(G) genes has nevertheless not yet been described in bacteria other than of the *S. enterica* serotypes. One might expect to find it in *E. coli* isolates also, if this was a general event.

Using pulsed-field gel electrophoresis (PFGE), several studies have concluded that multiple-antibiotic resistant *S.* Typhimurium DT104 has probably been spread clonally in European countries and the United States [27, 85, 141]. Evidence to the contrary has been found by others, including, Markogiannakis et al. [94] who also using PFGE, have shown that six distinct clones are present among Greek multiple-antibiotic resistant *S.* Typhimurium DT104 isolates. In a recent review, Tauxe also stated that multiple-antibiotic resistant *S.* Typhimurium DT104 represents a cluster of strains with related lysotyping patterns, including DT104, DT104a, DT104b, and U302 and thus, the epidemic would be more accurately described as being due to a cluster of related strains [137]. It is also interesting to note that strains showing the same macrorestriction pattern still exhibit genetic diversity if other analysis methods are used, such as infrequent restriction site PCR (IRS-PCR) and amplified fragment length polymorphism (AFLP) [21]. The occurrence of SGI1 in several clones would suggest a potential for horizontal transfer of this genomic island.

5.2. Emergence of variant SGI1 antibiotic resistance gene clusters in other serotypes

Horizontal transfer of SGI1 has been supported by its discovery in other *S. enterica* serotypes, namely *S.* Agona, *S.* Paratyphi B, *S.* Albany, and *S.* Newport [22, 43, 44, 97]. In these serotypes, SGI1 has the same chromosomal location as in *S.* Typhimurium DT104, i.e. it is inserted at the 3' end of the *thdF* gene. However, all serotypes were shown to lack the retron sequence found so far only in *S.* Typhimurium. Interestingly new SGI1 variants were identified in *S.* Agona, *S.* Albany, and *S.* Newport (Fig. 1). These clusters of antibiotic resistance genes were likely generated, according to sequence analysis, as a result of chromosomal recombination events or by antibiotic resistance gene cassette replacement at one of the *attI1* sites.

In *S.* Agona strains, three SGI1 variant antibiotic resistance gene clusters, SGI1-A (complex class 1 integron containing the entire antibiotic resistance gene cluster), SGI1-D (class 1 integron containing only the *aadA2* gene cassette), and SGI1-G (class 1 integron containing only the bla_{PSE-1} gene cassette), the 3'-CS (designated 3'-CS1) is followed by a 2 154-bp common region (CR) originally described in In6 and In7 [112, 133]. There is a unique region adjacent to the CR that includes *dfrA10* (Tm resistance), as in In7 [112]. Following this unique region is a second partial copy of the 3'-CS, designated 3'-CS2. The CR itself contains a gene, designated orf513, whose deduced product is thought to be a putative transposase [22], which is postulated to interact with the 27-bp boundary sequence and mobilise antibiotic resistance genes [113].

The variant SGI1-F from *S.* Albany represented the first example of replacement of one of the *attI1* sites of the gene cassette [43]. In this isolate, the Sm/Sp resistance

aadA2 gene cassette at the first *attI1* site was replaced by a *dfrA1* gene cassette and an open reading frame of unknown function. The *dfrA1* and orf gene cassettes may have been introduced by homologous recombination with a class 1 integron containing the same array of gene cassettes from another bacterium [116]. Another possibility is the exchange between *aadA2* and the two gene cassettes which would imply excision, mediated by the integron-encoded integrase, of *aadA2* and its replacement by the other gene cassettes [64]. The array of gene cassettes found at the first *attI1* site of the *S.* Albany strain was interestingly the same as those recently reported in integrons from *Vibrio cholerae* isolated in Thailand and India [36, 142].

The variant SGI1-H from *S.* Newport strains isolated from French patients with gastroenteritis represented the second example where gene cassette replacement took place in one of its *attI1* sites [44]. In these strains, the Sm/Sp resistance gene cassette *aadA2* inserted at the first *attI1* site was replaced by two other aminoglycoside resistance gene cassettes. The first cassette contained a new resistance gene encoding an AAC(3)-I aminoglycoside 3-*N*-acetyltransferase which confers resistance to gentamicin (Gm) and sisomicin (Sc). This gene has been named *aac*(3)-*Id*. The second cassette harboured the Sm/Sp resistance gene *aadA7*. The array of resistance gene cassettes found in the integron of the *S.* Newport strains were the same as those recently reported in an integron of a *Vibrio fluvialis* strain (GenBank accession no. AB114632). The natural aquatic environment of pathogenic *V. fluvialis* strains is surface water and it seems likely that antibiotic resistance gene exchange between different bacterial species such as *Vibrio* and *Salmonella* probably took place in such aquatic environments. Thus, the SGI1 complex class 1 integron could contribute to the capture in the *Salmonella* chromosome of a wide diversity of resistance gene cassettes and thus generate diverse antibiotic resistance gene clusters.

5.3. Re-emergence of high-level fluoroquinolone resistance

The increasing rates of resistance to traditional anti-*Salmonella* agents (i.e. Ap, Cm, and trimethoprim-sulfamethoxazole) have turned the treatment of invasive salmonellosis into a clinical dilemma. The emergence of resistance to fluoroquinolones (FQ) among nontyphoid *Salmonella* is of particular concern, since this class of antimicrobial agents constitutes the drug of choice for treating potentially life-threatening *Salmonella* infections caused by multiple-antibiotic resistant strains in adults [3].

In *Salmonella*, quinolone resistance was initially attributed to point mutations in the *gyrA* gene encoding the A subunit of gyrase, whose complex with DNA is the primary target of quinolones. Resistance mutations of *gyrA* occur in a region of the gene product between amino acids 67 and 106, termed the quinolone resistance-determining region (QRDR). Amino acid changes at Ser-83 (to Phe, Tyr, or Ala) or at Asp-87 (to Gly, Asn, or Tyr) are the most frequently observed in nalidixic acid (Nal)-resistant strains [30]. Double mutations at both residues 83 and 87 have been identified in clinical isolates of an *S.* Typhimurium DT204 clone showing high-level resistance to FQ [67]. These strains were mainly isolated between 1991 and 1995 from animals and humans in limited areas in Europe and are highly resistant to ciprofloxacin (Cip) (MIC of 32 μg/mL). These isolates have in addition an altered *gyrB* gene encoding the B subunit of gyrase [66]. This consists of a single mutation in the QRDR of *gyrB* leading to amino acid change Ser464Phe [10]. These isolates also carry a fourth mutation in the QRDR of *parC* encoding the ParC subunit of topoisomerase IV, which is the secondary target for quinolones. The mutation identified led to amino acid change Ser80Ile [10].

FQ resistance in *S.* Typhimurium has also been attributed to active efflux mechanisms [57, 119], and especially to the participation of the AcrAB-TolC efflux system. Indeed, inactivation of the genes coding for either

the AcrB inner membrane multidrug transporter or the TolC outer membrane channel in *S.* Typhimurium DT204 strains resulted in a 16- to 32-fold reduction of resistance level to several FQ (Cip MIC of 2 μg/mL) [10, 11]. Similar results were obtained using the efflux pump inhibitor Phe-Arg-β-naphthylamide [89]. Thus, using efflux pump inhibitors together with FQ may be promising in combination therapy against high-level FQ-resistant *S.* Typhimurium. Further reversion of the *parC* (Ser80Ile) mutation resulted in a further 16- to 32-fold decrease of resistance levels to FQ [11]. High level resistance to FQ in *Salmonella* is thus essentially explained by the combination of two major resistance mechanisms, i.e. multiple target gene mutations and active efflux mediated by AcrAB-TolC.

Recent reports suggest that high level FQ resistance is re-emerging in *S.* Typhimurium, *S.* Choleraesuis, and *S.* Schwarzengrund in different parts of the world [28, 76, 102, 111]. Polyclonal re-emergence of high level FQ-resistance is not only reflected by the several serotypes in which this resistance is now encountered, but also by the diversity of the target gene mutations identified: two different double mutations in *gyrA* [111], one single mutation in *gyrB* [28], three different single mutations in *parC* [102], and for the first time one single mutation in *parE* [87].

5.4. Emergence of resistance to extended spectrum cephalosporins

Extended spectrum (E-S) cephalosporins are the drugs of choice for children because they cannot be treated with FQ. *Salmonella* and *E. coli* exhibiting resistance to E-S cephalosporins are an emergent problem worldwide. Before 1996, resistance to E-S cephalosporins was rarely reported among *Salmonella* sp. In 2000, the emergence of domestically acquired infections by E-S cephalosporin-resistant *Salmonella* carrying a plasmid-mediated CMY-2 AmpC β-lactamase was reported by the National Antimicrobial Resistance Monitoring System (NARMS) in the USA [47]. Molecular and phenotypic analysis of E-S cephalosporin-resistant strains revealed several distinct serotypes and chromosomal DNA patterns, suggesting that this resistance phenotype is present among genetically diverse strains [47, 158]. It has been recently demonstrated that the bla_{CMY} genes in *Salmonella* reside on different large plasmids [26].

The occurrence of CMY-2-mediated cephalosporin resistance in *Salmonella* has now been reported in Canada [2], Spain [103], Romania [99], and Taiwan [154]. In 2002, the CDC investigated an outbreak of multiple-antibiotic resistant *S.* Newport infections associated with eating raw or undercooked ground beef [155]. This multidrug resistance phenotype included resistance to Sm, sulfamethoxazole, Tc, Ap, Cm, and decreased susceptibility to E-S cephalosporins. These resistant strains have also been detected in cattle [128]. An increase in prevalence of E-S cephalosporin-resistant strains could be in part related to the use in food animals of ceftiofur, which is an E-S cephalosporin approved for used in veterinary medicine [151]. Since the bla_{CMY} genes confer decreased susceptibility to both ceftiofur and ceftriaxone, the use of ceftiofur has the potential to select for *Salmonella* cross-resistant to ceftriaxone, another E-S cephalosporin used in human medicine. In fact, many plasmids containing bla_{CMY-2} genes have been reported to confer additional resistance to aminoglycosides, phenicols, Tc, and Su. Ceftriaxone-resistant *Salmonella* strains recently isolated from animals in Canada [2] and retail foods in the USA [151] have also been reported to be resistant to florfenicol. The bla_{CMY-2}-carrying plasmids studied were recently shown to also carry the Ff resistance gene, *floR,* on a genetic structure previously identified in *E. coli* plasmids in Europe [32]. These data indicated that the use of different antimicrobial agents, including phenicols, might serve to maintain multiple-antibiotic resistance plasmids on which E-S cephalosporin resistance determinants co-exist with other resistance genes in *Salmonella*.

Besides the emergence of multidrug resistance plasmids carrying bla_{CMY-2} and *floR*, other extended-spectrum β-lactamases have been reported in recent years in *Salmonella* [1, 7, 101].

5.5. Conclusions

Taken together, antibiotic resistance genes can be propagated by integrons, transposons, mobile genomic islands that can reside in the chromosome, and on plasmids [134]. These mobile elements can collect and recombine numerous resistance gene cassettes in almost any combination as shown for SGI1 [22]. Consequently, treatment with one antimicrobial agent can enrich the population for bacteria resistant not only to that specific agent, but also to all antimicrobial agents whose resistance genes are genetically linked to the agent used (i.e. present as a cluster of genes on the same mobile element). The consequences were not realised in the past by most clinicians, who, in treating with an aminoglycoside, assumed they were selecting for strains resistant to only that antimicrobial agent. This may also explain why, despite periodic cycling of antimicrobials in hospitals, the prevalence of multiple-antibiotic resistant bacteria does not diminish but indeed continues to increase [134].

Antimicrobial resistance can involve not just obvious pathogens but also commensal bacteria, which may act as an enormous reservoir of antimicrobial resistance genes [134]. Thus, antimicrobial use in medicine and agriculture affects the general ecology of bacterial communities, including interactions between bacteria and their environment but also mechanisms by which antimicrobial resistance genes spread and persist. Their use, especially in food animals, can also have adverse effects on human health [9]. One fact that can decrease the impact of antibiotic resistant strains is that for some mechanisms in particular those conferring resistance to FQ in *S.* Typhimurium could have an important fitness cost and could therefore be counter-selected under in vivo conditions [58]. Thus, whereas it is clear that genotypes resistant to an antibiotic are selectively favoured in the presence of this antibiotic, they often have lower growth rates than susceptible genotypes in an antibiotic-free environment. However, one mechanism that can increase the impact of antibiotic resistant strains, involves virulence traits linked to antibiotic resistance gene clusters. For example, besides the antibiotic resistance cluster, SGI1 also carries genes coding for proteins of unknown function that could potentially be involved in the virulence colonisation or infectivity of the multiple-antibiotic resistant *S.* Typhimurium DT104 [21]. This hypothesis might explain the current worldwide epidemic of this pathogen. DT104 was indeed an uncommon phage type before acquiring the multiple-antibiotic resistance phenotype. In this hypothesis, the antibiotic resistance cluster might be regarded as only the small visible tip of an iceberg and spread of multiple-antibiotic resistant *S.* Typhimurium DT104 might thus now occur even without the added selective pressure imposed by the use of antibiotics.

6. FUTURE PERSPECTIVES

Effective prevention and control programmes must involve coordinated and simultaneous attacks on the problem from several directions. Vaccination and competitive exclusion are important methods to aid reducing *S.* Enteritidis poultry infection [37, 96, 153]. The prevalence of *Salmonella* in animals may also be reduced by the genetic selection of animals resistant to disease but also to the asymptomatic carrier state [13, 15, 77]. However, our increasing understanding of the molecular fluidity of the genome suggests that any attempt at extensive biological intervention will result in a further evolution, as the bacterium attempts to overcome the obstacle placed in its ecological path. It has been shown that horizontal gene transfer of foreign DNA coding for novel phenotypes is an important factor in the rapid evolution of bacterial pathogens. In addition, if bacteria in a particular ecologic niche are destroyed, other bacteria

resulting from adaptation to this new free niche, will replace the original flora. This may have occurred in the replacement of the avian adapted serotype *S*. Gallinarum by zoonotic bacteria, although this has by no means been completely proven and remains in dispute. Similar changes occur in response to the use of antimicrobial agents. The continuous exchange of bacteria between humans and their environment suggests that imposition of any selection on bacteria will result in proliferation of bacterial stains that tend to resist the initial stress. Once acquired, these additional genes will be lost very slowly and in contrast may be transmitted to many other bacteria. In the past, the use and often the misuse of antimicrobials in both humans and animals have given rise to a selection unprecedented in the history of microbial evolution. As a result, humans are facing the emergence of infectious bacteria displaying resistance to many, and in some cases all, effective antimicrobials. The use of antibiotics in livestock, fish and poultry has accelerated the development of antibiotic-resistant bacteria, complicating treatment for both animals and humans. Chemotherapeutic selection may have additional consequences for virulence evolution through the acquisition of linked virulence genes.

Given this information, what might be done to assist combating *Salmonella*? It is clear that the continuous development of existing surveillance measures (control programmes, traceability of the food chains) and epidemiological expertises is required, both for food-borne pathogens, but also for sentinel organisms, present in the normal flora, and which may represent a huge reservoir of resistance. It is also necessary to understand the mechanisms of evolution to form the foundation for a predictive science of infectious disease enabling us to anticipate the emergence of problems for public health and to evaluate the influence of future changes in animal husbandry with regards to their potential of altering the pathogen population and genome. Genome evolution is indeed one great source of emergence. A second source is the ability of a pathogen to infect multiple hosts, particularly hosts in different taxonomic orders or wildlife [29]. In the case of antimicrobial resistant bacteria, it is of prime importance that all sectors using antibiotics (medicine, veterinary, horticulture) cooperate to minimise the proliferation of resistant bacteria, which may more generally have important consequences for virulence evolution and disease control.

The knowledge of the potential risks, even the perception of the risks, should, however, not mask the real health hazard. We should remember that current food presents much less microbial health hazards than food five decades ago and that the increase in lifespan is partly related to this improvement.

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