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Virulence Characterization of *Salmonella* Typhimurium I,4,[5],12:i:-, the New Pandemic Strain

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1. Introduction

It is really impossible to estimate the volume of ink that has been spent writing about *Salmonella* since its first description in 1885, by Daniel D. Salmon. Nowadays, performing a search on this bacterial genus using web databases originates more than 17 million references. Before the web era, the dissemination of information on *Salmonella* was extremely complex or even impossible, and this is probably the reason why *Salmonella* taxonomy has been so difficult to establish during the first century after its description. The taxonomy of this bacterial genus still today remains under revision.

Sadly, *Salmonella* is the paradigm of a popular microbe by quite regrettable reason: from the most common citizen to the most qualified microbiologist, everyone has already talked about *Salmonella*. The reason for this is, surely, its incrimination on severe food poisoning outbreaks and in many cases of illness in humans and animals and mortality in humans and animals.

Epidemiological data indicate that the cases of human or animal infections by *Salmonella* are may assume a dramatic dimension. In the European Union, the number of reported human cases is approximately 150 thousand each year, with a consistent tendency to decline during the last four years. In countries where basic health care cannot be delivered, the most

dangerous clinical expression of a *Salmonella* infection, Typhoid fever, affects 16 million people per year, with almost 500,000 fatal cases, as estimated by the World Health Organization (Pang et al., 1998).

In fact, not all *Salmonella* isolates identified worldwide have such devastating consequences to human or animal health. Some of the *Salmonella* serotypes or serovars are strictly adapted to primates (*Salmonella* Typhi, *Salmonella* Paratyphi, *Salmonella* Wien) being referred to as prototrophic for man; others are strictly adapted to some animal species (*Salmonella* Gallinarum-Pullorum, *Salmonella* Abortusovis, *Salmonella* Abortusequi), being referred to as prototrophic to animals; however, the vast majority of the serotypes are zoonotic, being able to infect both animals and humans. *Salmonella* varieties differentiation is based on its antigenic mosaic, in a complex combination of somatic (O), flagellar (H) and capsular (Vi) antigens. Serotyping according to the Kauffmann-White system, established in the middle of the last century, is still recognized as the reference method for discrimination of *Salmonella* varieties. Each combination of different antigens found in a particular *Salmonella* isolate (serotype or serovar) has a specific designation, following the international nomenclature based on one hundred years of scientific contributions, which sometimes originated peculiar designations (Popoff & Le Minor, 1997; Grimont & Weill, 2007).

Salmonella is an infectious and contagious bacterium that may be transmitted to humans, warm blood animals and reptiles by contaminated drinking water, by raw foods consumption, by direct contact with previously infected humans or animals and by iatrogenic accidents.

Food, in particular raw food, is the most common pathway for *Salmonella* infection, especially by zoonotic serotypes. Since the 1950's, zoonotic *Salmonella* became dominant in human salmonellosis cases. Virulence is strictly dependent on the serotype and it also varies with individual competences of each bacterial strain and with host susceptibility. These features explain why some serotypes have a higher prevalence in a particular host. In the last decades, two serotypes of zoonotic *Salmonella* showed a clear dominant incidence: *Salmonella* Typhimurium, firstly found in bovines, pigs, pigeons and secondarily in humans, while *Salmonella* Enteritidis, more common in poultry, but firstly found in humans, with the exception of Europe (EFSA, 2010b). These two serotypes may be discriminated more deeply using epidemiological markers like phage typing, molecular genotyping or other methodologies, including profiling for antimicrobial resistance phenotypes (R-type).

Salmonella Typhimurium is a somatic group B strain with the following antigenic formula: 4,[5],12:i:1,2. It has been early recognized as a serotype with a variable antigenic structure, like the lack of the somatic antigen [5], assuming, in this case, the designation of "variety Copenhagen" (very frequent in pigeons and bovines). The antigenic structure modulation of *Salmonella* Typhimurium may be mediated by plasmids, phages or proto-phages infections or segregations.

By the end of the 1980s, some isolates of a monophasic form of *Salmonella* Typhimurium - serotype I,4,[5],12:i:- gained epidemiological relevance, being more and more frequently referred to in the literature (Machado & Bernardo, 1990). The prevalence of this monophasic serotype has grown, presently being one of the most common *Salmonella* serotypes isolated from humans in several countries (Hopkins, et al., 2010) (Table 1). Other variants have also been found: variants lacking the 1st flagellar phase or lacking both (i and 1,2); and also

variants without the somatic 1 antigen. The possibility that before the 1990s the scarce reports of *Salmonella* I,4,[5],12:i:- isolation, may reflect the difficulties in serotyping it being the isolates probably designated as *Salmonella* Typhimurium. At that time it was frequent to report some *Salmonella* serotypes as “Group B” or “untypable” (Switt et al., 2009).

Years of isolation	Country	Source
1986	Portugal	Chicken carcasses
1993	Thailand	Human
1997	Spain	Human
1991	Brazil	Human
1998	United States	Human
2000	Germany	Human, food, swine, cattle, broiler
2003	Italy	Human, swine
2005	UK	Human
2006	Luxembourg	Pork, pigs

Table 1. First time reports of *Salmonella* 4,[5],12:i: isolation (Adapted from EFSA, 2010b)

2. Occurrence of *Salmonella* I,4,[5],12:i:-

Cases of human infections with serovar I,4,[5],12:i:- have been related to severe illnesses. This serovar was responsible for an outbreak in New York City in 1998, in which 70% of the cases required hospitalization, being also associated with cases of systemic infections in Thailand and in Brazil (Switt et al., 2009).

Some foodborne outbreaks due to *Salmonella* I,4,[5],12:i:- have been reported in Europe. In 2006, Luxembourg signaled two outbreaks caused by a monophasic *S. Typhimurium* DT 193, corresponding to 133 human cases, 24 hospitalizations and one death (Mossong et al., 2007). Pork meat has been incriminated in these *Salmonella* cases (Mossong et al., 2007).

In Germany, the number of *Salmonella* I,4,[5],12:i:- related with human diseases has increased since 2000 (Hauser et al., 2010). Since 2006, the same monophasic variant of the multidrug-resistant *Salmonella* DT 193 strain has been associated with sporadic cases of salmonellosis, with increasing rates of hospitalization (Trupschuch et al., 2010). In 2008, the monophasic I,4,[5],12:i:- variant correspond to up to 42% of all *S. Typhimurium* isolates responsible for human salmonellosis (Hauser et al., 2010).

In France, official data suggest a gradual increase of *Salmonella* I,4,[5],12:i:- isolation rate in humans. After 2005, the frequency of this particular serovar raised from the eleventh to the third place (AFSSA, 2009) and a further significant increase was reported in the first five months of 2010 (Bone et al., 2010). In 2008, several outbreaks of *Salmonella* I,4,[5],12:i:- infections were identified in this country, including 13 family outbreaks, three collective infections and two hospital infections (AFSSA, 2009).

In Spain, the number of *Salmonella* I,4,[5],12:i:- illness cases has consistently increased since 1997, the year of the first report. Nowadays, the monophasic *Salmonella* is at the top five among the most frequently isolated *Salmonella* serovars in Spain (de la Torre et al., 2003). The epidemiological relevance of this serotype makes it a major cause of concern in Spain since the beginning of this Century (Echeita, et al., 1999; Guerra, et al., 2000).

In the UK, human infections by *Salmonella* I,4,[5],12:i:- began to be reported in 2005, when 47 cases occurred. In 2009 151 cases occurred, representing an increase of more than 30%. Almost 30 % of the *Salmonella* I,4,[5],12:i:- isolates had a R-type ASSuT. In Scotland there was also an increase in the number of reports of *Salmonella* monophasic Group B cases.

Since 2008, sporadic and diffuse outbreaks related with ready to eat food have also been described in the UK linked to a DT 191A *Salmonella* I,4,[5],12:i:- strain, which is tetracycline-resistant (Peters et al., 2010). This strain is thought to have originated from infected frozen feeder mice imported into the UK for feeding exotic pets.

In Italy, *Salmonella* I,4,[5],12:i:- is one of the most frequent serotypes related to human cases of salmonellosis (Dionisi et al., 2009). R-type ASSuT represented 75% of the monophasic isolates identified in 2008 and 2009. Almost 50 % of the monophasic isolates were identified as *Salmonella* DT193 and 13% as *Salmonella* U302.

In The Netherlands, *S.* I,4,[5],12:i:- was related to human cases for the first time in 2004. After that, the number of cases has consistently grown, being the third most prevalent serotype responsible for human salmonellosis from 2005 to 2008 (Van Pelt et al., 2009).

There also many cases reported outside Europe. In the USA, *Salmonella* I,4,[5],12:i:- frequency in human infections has consistently increased from 2002, being now ranked in the top six. During 2007, some *Salmonella* I,4,[5],12:i:- outbreaks occurred in the USA, related to frozen chicken pies consumption and also to direct contact with turtles kept as pets (CDC, 2007a, 2007b).

Some cases have also been reported in Canada (Switt et al., 2009).

Salmonella I,4,[5],12:i:- has also been reported in Brazil quite early, in the 1970s. In São Paulo State, the occurrence of the strain in human infections was reported in the 1990s. Since then, the frequency of foodborne outbreaks and of extra-intestinal infections in humans promoted by this serovar showed a consistent tendency to increase (Tavechio et al., 2009).

In Thailand, *Salmonella* I,4,[5],12:i:- has been classified among the top five *Salmonella* serovars responsible for cases of foodborne salmonellosis (Amavisit, et al., 2005; Pornruangwong et al., 2008).

Human cases of infection with this particular serotype seem to be generally linked to raw meat. According to the EFSA zoonoses reports, in 2008 *Salmonella* I,4,[5],12:i:- has been related to 3.1% of *Salmonella* isolations in pig herds; in 2009, the same serotype has been found in 1.2% of the *Salmonella* positive bovine herds, 3.2% of positive pig herds and represented 1.4% of *Salmonella* isolations in poultry meat.

A particularly relevant feature of *Salmonella* I,4,[5],12:i:- is the fact that most virulent isolates exhibit a plasmid-mediated resistance to a wide range of antimicrobial compounds. Similar to its ancestral lineage - *Salmonella* Typhimurium DT104 - the monophasic strain I,4,[5],12:i:- frequently expresses a multiple resistance to ampicillin (A), streptomycin (S), sulphonamides (Su) and tetracyclines (T). This ASSuT antimicrobial resistance pattern is chromosomally-encoded (Hopkins et al., 2010).

The progressive increase of the incidence of this serotype lead some authors to consider *Salmonella* I,4,[5],12:i:- as a possible new pandemic strain (Hopkins, et al., 2010).

Data on the number of salmonellosis cases or outbreaks occurring in livestock due to *Salmonella* I,4,[5],12:i:- is not available. This subject needs to be further studied.

3. Characterization of monophasic *Salmonella enterica* subsp. *enterica* serovar I,4,[5],12:i:-

Serotyping divides *Salmonella* subspecies into subtypes, or serovars, based on the immunologic characterization of surface structures, such as O, H and in some cases Vi-antigens, through the use of polyvalent and monovalent antisera. The full antigenic pool of *Salmonella* [I,4,5,12:i:-] indicates that the somatic O-antigens expressed are I,4,[5],12. The underlined O factor 1 (1) means that this factor is determined by phage conversion, being present only if the culture is lysogenized by the corresponding converting phage. The factor 5 between square brackets ([5]) means that the antigen may be present or absent, not having a relation with phage conversion. So, in this serovar both factors (1 and 5) can be present or absent.

Most *Salmonella* strains are biphasic and express two serologically distinct flagellar antigens. The two antigens were historically designated as phases and the expression of two different phases is mediated at molecular level by an intricate mechanism unique to *Salmonella*. The regulation of phase 1 and phase 2 antigen expressions is under the control of the recombinase Hin. This recombinase facilitates the inversion of a promoter element so that it either (i) transcribes *fljB* (which encodes the phase 2 antigen FljB) and *fljA* (which encodes a repressor of *fliC*, the gene encoding the phase 1 antigen FliC) (Aldridge et al., 2006; Yamamoto & Kutsukake, 2006) or (ii) does not transcribe either of these genes. If the orientation of this promoter does not allow the transcription of *fljB* and *fljA*, the lack of repression of *fliC* transcription leads to the expression of phase 1 flagellar antigens.

Strains expressing both flagellar types are called biphasic. In contrast, strains defined as monophasic fail to express either phase 1 or phase 2 flagellar antigens. *S.* [I,4,5,12:i:-] possess only the phase 1 of the H-antigen "i" and lacks the second phase H antigen, encoded by *fljB*, which either is not present or contains mutation(s) affecting its expression. In 2007, Zamperini et al. screened *S.* 4,[5],12:i:- isolates for phase 1 and phase 2 antigen genes, *fliC* and *fljB*, and found that 100% of the isolates were positive for *fliC*, while 11% were positive for *fljB*. Approximately 89% of these isolates contained complete or partial deletions of the phase 2 flagellin gene, *fljB*, whereas 96% possessed the upstream gene, *hin*, which encodes the DNA invertase involved in "flipping" the *fljB* promoter.

Phage typing is a method also used for *Salmonella* typing based on the lysis of isolates with a panel of bacteriophages. Since this technique does not depend on the presence of the second phase H antigen, monophasic *Salmonella* reactions are performed with the same panel of phages used for *Salmonella* serovar Typhimurium (Echeita et al., 2001; Amavisit et al., 2005; Mossong et al., 2006). Thus, all phage types that have been recognized so far within monophasic *Salmonella* have also been found in *S.* serovar Typhimurium.

For example, the multidrug-resistant *Salmonella* 4,[5],12:i:- strain detected in Spain in 1997 was lysed by the *S.* Typhimurium phage 10 (Echeita et al., 2001). Phage type U302 was also detected among *S.* 4,[5],12:i:- isolates in other countries, such as Denmark (Ethelberg et al., 2004) and Italy (Dinosi et al., 2009). This phage type has been considered closely related to DT104 (Briggs & Fratamico, 1999).

However, *S.* 4,[5],12:i:- isolates have also been classified in other phage types linked to *S.* Typhimurium. In Germany, Hauser et al. (2010) analyzed *S.* [4,[5],12:i:- isolates obtained from different sources (human, swine and pork) and classified 70% of strains as DT193 and 19% as DT120. In another study, Hopkins et al., (2011) screened a large number of *S.* 4,[5],12:i:- strains from different countries (France, The Netherlands, England and Wales, Germany, Italy, Spain and Poland), obtained from similar sources as described by Hauser et al. (2010), and were able to identify 16 different phage types. However, the most commonly identified phage types were DT193, DT120 and RDNC ("Reaction Does Not Conform"). DT193 was the most common phage type identified in England and Wales, France, Germany, Spain and the Netherlands, while DT120 predominated in Italy and Poland. In other studies, *S.* 4,[5],12:i:- DT193 strains were also isolated from human cases of infection and/or pigs in United Kingdom, Luxembourg, United States and Spain (Hampton et al., 1995; Gebreyes & Altier, 2002; de la Torre et al., 2003; Mossong et al., 2006), while monophasic DT120 strains were identified in Italy (Dionisi et al., 2009).

According to serological characterization, it is difficult to identify the origin of the monophasic strains. This strain may be a new variant of the rare serovar Lagos (4,[5],12:i:-), or a new variant of the very common serovar Typhimurium [4,5,12:i:1,2], or even a new variant of other serovars with similar antigenic pools, such as *S.* Agama [4,12:i:1,6], *S.* Farsta [4,12:i:e,n,x], *S.* Tsevie [4,12:i:e,n,z15], *S.* Cloucester [1,5,12,27:i:l,w], *S.* Tumodi [1,4,12:i:z6] or as *S.* 4,5,27:i:z35, an unnamed serotype (Switt et al., 2009). However, large scale studies suggest that *S.* 4,[5],12:i:- is genetically related to Typhimurium [4,5,12:i:1,2], and is likely to have originated from a *S.* Typhimurium ancestor (Echeita et al., 2001; Zamperini et al., 2007). Monophasic *Salmonella* could have evolved by two distinct pathways. It could represent ancestral forms which did not acquire, through evolution, a second flagellar antigen or the required switching mechanism. Alternatively, it could originate as mutants of biphasic *Salmonella*, which have lost either the switching mechanism or the ability to express the second flagellar antigen (Burnens et al., 1996).

The atypical *fljB*-negative and multidrug-resistant *S.* 4,[5],12:i:- which emerged and spread in Spain in 1997 had a unique sequence specific for *S.* Typhimurium phage types DT104 and U302 and also an IS200 fragment located in a Typhimurium serovar-specific location. Both facts strongly suggest that these strains are monophasic variants of *S.* Typhimurium (Echeita et al., 2001).

On other hand, *S.* 4,[5],12:i:- DT193 and DT120 strains were classified as monophasic variants of *S.* Typhimurium due to the presence of a Typhimurium-specific fragment of the malic acid dehydrogenase gene (Hopkins et al., 2011). However, these strains were negative for the DT104- and U302-specific region. Houser et al. (2010) indicated that phage type DT193 and DT120 isolates of both serovars presented genetic differences and represent different Pulsed-field Gel Electrophoresis (PFGE) clusters. Such differences seem to indicate that the *S.* Typhimurium phage type DT193 lineage was not a direct ancestor of the monophasic phage type DT193. In contrast, *S.* 4,[5],12:i:- phage type DT120 strains showed a higher genetic similarity with the *S.* enterica Typhimurium phage type DT120 strains, suggesting that this biphasic subtype was the recent common ancestor of the monophasic variant.

Different mutations and deletions have been associated with the lack of phase 2 flagella expression in *S.* 4,[5],12:i:- isolates. Specifically, some Spanish *S.* 4,[5],12:i:- isolates appear

to be characterized by the deletion of a large fragment, that included *fljB*, *hin*, and a DNA invertase essential for *fljB* expression (Garaizar et al., 2002). Most USA isolates characterized so far also present deletions that eliminate *fljB* but maintain *hin* (Zamperini et al., 2007; Soyer et al., 2009). These genetic differences among American and Spanish *S.* 4,[5],12:i:- isolates have also been made evident by PFGE typing (Soyer et al., 2009). Genetic data indicate that American and Spanish isolates represent different clonal groups with distinct genome deletion patterns. This is consistent with the observation that most Spanish *S.* 4,[5],12:i:- isolates are phage type U302 (Echeita et al., 2001). Moreover, PFGE and Multiple-Locus Variable Number Tandem Repeat Analysis (MLVA) techniques showed that Spanish phage type U302 strains seem to be more homogeneous than groups constituted by isolates from other countries (Soyer et al., 2009), supporting a clonal origin. Soyer et al. (2009) suggested that Spanish *Salmonella* 4,[5],12:i:- strains might have emerged from a multidrug-resistant *S.* Typhimurium strain, while American *S.* 4,[5],12:i:- strains might have emerged from a non-drug-resistant *S.* Typhimurium strain, through independent events.

Strains belonging to *S.* 4,[5],12:i:- that have been recently implicated in infections both in humans and farm animals have been further typed using genetic techniques. Guerra et al. (2000) studies showed a high genetic homogeneity among 16 Spanish *S.* 4,[5],12:i:- isolates using techniques such as ribotyping, RAPD (Random Amplified Polymorphic DNA analysis) and plasmid profiling. However, the use of PFGE techniques could originate degrees of heterogeneity that may range from moderate to high, even when applied to strains from a single country (Agasan et al., 2002; de la Torre et al., 2003; Zamperini et al., 2007; Soyer et al., 2009). For example, at least 13 different *Xba*I PFGE types were found among 32 *S.* 4,[5],12:i:- isolates from Georgia (Zamperini et al., 2007), 44 different *Xba*I PFGE types were detected among 148 isolates from Germany (Hauser et al., 2010) and at least 11 *Xba*I PFGE types were found among 23 Spanish *S.* 4,[5],12:i:- isolates (de la Torre et al., 2003). Despite their heterogeneity, *S.* 4,[5],12:i:- strains have been reported to be less heterogenic than *S.* Typhimurium strains (Guerra et al., 2000; Agasan et al., 2002; Soyer et al., 2009; Hauser et al., 2010).

Studies also have showed the occurrence of common genetic profiles among *S.* 4,[5],12:i:- isolates obtained from different sources and countries. Zamperini et al. (2007) studies revealed the same PFGE profile among poultry and bovine *S.* 4,[5],12:i:- isolates. In 2011, Hopkins et al. compared isolates from humans, pigs and pork using PFGE and detected several prevalent genetic profiles common to these three sources (STYMXB.0131, STYMXB.0083, STYMXB.0079, STYMXB.0010, STYMXB.0022). Moreover, these profiles were detected in isolates from different countries. One PFGE profile, STYMXB.0010, was identified in isolates obtained in all the countries surveilled in the referred study. However, the authors also found some country-specific differences in the distribution of PFGE patterns. For example, nine of the 12 STYMXB.0079 strains originated from Italy, three of the five Polish strains were STYMXB.0010 and six of 10 strains from the Netherlands were STYMXB.0131. The STYMXB.0079 profile was also the predominant one among 146 human *S.* 4,[5],12:i:- isolates obtained in Italy by Dionisi et al. (2009).

Typing of monophasic strains using molecular techniques, such as PFGE, have showed that these strains differ from *S.* Lagos strains (Soyer et al., 2009). Data also showed the occurrence of some profiles common to *S.* Typhimurium. Zamperini et al. (2007) examined isolates of *S.* 4,[5],12:i:- and *S.* Typhimurium collected from animal sources that presented

the same PFGE profile. In another study, Agasan et al. (2002) compared the PFGE profile of *S.* 4,[5],12:i:- strains found in humans in New York City to the profile of *S.* Typhimurium isolates, including *S.* serovar Typhimurium DT104, and found that *S.* 4,[5],12:i:- isolates were related to some of the *S.* Typhimurium isolates examined. Amavisit et al. (2005) compared the PFGE profiles of human isolates identified as *S.* Typhimurium DT104, Typhimurium U302 and 4,[5],12:i:-, showing that four of the *S.* 4,[5],12:i:- isolates presented the same or similar profiles as *S.* Typhimurium phage type U302.

In another study, Alcaine et al. (2006) used Multilocus Sequence Typing (MLST) to show that ST6 type comprises not only bovine and human *S.* 4,[5],12:i:- isolates but also Typhimurium isolates obtained in the United States. ST6 was unique to *S.* Typhimurium and 4,[5],12:i:-, which supports the initial findings based on characterization of Spanish isolates (de la Torre et al., 2003), that *S.* [4,5,12:i:-] may have emerged from a *S.* Typhimurium ancestor. A MLST technique based on four genes applied to American and Spanish isolates belonging to *S.* 4,[5],12:i:- and to *S.* Typhimurium classified the vast majority of isolates as ST1 (Soyer et al., 2009). Similar results were obtained with other molecular fingerprinting techniques e.g. RAPD analysis, plasmid profiling (Sala, 2002; de la Torre et al., 2003), MLST (Alcaine et al., 2006) and MLVA/Variable Number of Tandem Repeats (VNTR) typing (Laorden et al., 2009; Torpdahl et al., 2009; Hauser et al., 2010; Hopkins et al., 2011). All these studies lead to the conclusion that *S.* 4,[5],12:i:- isolates belong to a single genetic lineage or clone and seem closely related to *S.* Typhimurium (Zamperini et al., 2007; Dionisi et al., 2009; Hopkins et al., 2011).

Overall, the genomic characterization of *S.* 4,[5],12:i:- isolates suggests that this serovar is likely to gather several clones or strains that have independently emerged from *S.* Typhimurium during the last two decades, and have changed through multiple independent events involving different clonal groups (Garaizar et al., 2002; Laorden et al., 2009; Laorden et al., 2010). Although the driver for this evolution remains to be enlightened for many epidemic strains antimicrobial resistance may be implicated (Zaidi et al., 2007; Bailey et al., 2010).

4. Antimicrobial resistance traits of *Salmonella* I,4,[5],12:i:-

The characterization of zoonotic bacteria virulence factors, including the presence of antimicrobial resistance traits, is of major importance for assuring the safeguard of health in the wider concept of “one health”. The dissemination of antimicrobial resistant bacteria is a well-recognized hazard for public and animal health.

Several *Salmonella* serovars are frequently related to human and animal diseases, and this genus is recognized worldwide as a major foodborne pathogen. Gastroenteritis due to *Salmonella* is usually characterized by mild to moderate self-limiting symptoms, such as diarrhea, abdominal cramps, vomiting and fever. However, some strains are responsible for severe infections, such as septicemia, osteomyelitis, pneumonia, and meningitis that occur, especially in children and in elderly and immunocompromised individuals (Folley and Lynne, 2008a).

Generally, salmonellosis cases caused by *Salmonella* I,4,[5],12:i:- strains are severe, requiring hospitalization (EFSA, 2010b). The control of severe infections requires antimicrobial therapy, generally with fluoroquinolones or ceftriaxone, administered to children in order

to avoid the cartilage damage frequently associated with fluoroquinolone therapy (Folley and Lynne, 2008a). Therefore, *Salmonella* represents a bacterial genus of special concern regarding antimicrobial resistance dissemination.

This serotypes' resistance profiles may vary, worldwide, from 100% susceptible to multidrug resistance. Although *S. Typhimurium* resistance levels have been decreasing in several European countries, the incidence of resistant *S. Typhimurium* I,4,[5],12:i:- strains seems to be escalating (Switt et al., 2009). There are only a few studies available on antimicrobial resistance traits and genes present in antimicrobial drug-resistant *Salmonella* serotype 4,[5],12:i:- isolates, which have identified some specific resistance genes and genetic mechanisms. The limited data available still hasn't allowed researchers to identify the common ancestor responsible for the emergence of 4,[5],12:i:- isolates with a multidrug resistance pattern (MDR), information essential to understand resistance evolution and dissemination (Switt et al., 2009).

Antimicrobial resistance in *Salmonella* spp. may be due to several resistance determinants that can be located either in the bacterial chromosome or in plasmids (Folley and Lynne, 2008a; Switt et al., 2009). These genetic determinants can be responsible for the expression of intrinsic resistant mechanisms, related to the production of β -lactamases, to the modification of the antimicrobial compound by bacterial enzymes, to the variation of bacterial permeability, to the presence of efflux pumps or to the modification of target receptors (Folley and Lynne, 2008a).

Antimicrobial resistance may also result from the expression of acquired resistance mechanisms, emerging through the occurrence of point mutations in chromosomal genes or the acquisition of mobile elements such as plasmids, transposons, and genomic islands (Switt et al., 2009). The transfer of resistance determinants may occur directly from the same or different bacterial species/genera, or indirectly through the environment (Folley and Lynne 2008a; EFSA, 2010b). Intestinal microbiota from humans and animals is often exposed to antimicrobial compounds of different classes, concentrations and exposure frequencies, used for therapy, prophylaxis or metaphylaxis. This exposure may derive from food/feed products or from the environment (Martins da Costa et al., 2007). Emergence, selection and dissemination of antimicrobial resistant bacteria are still mainly attributed to the selective pressure of antibiotic misuse and abuse (Monroe & Polk, 2000; Sayah et al., 2005), so intestinal bacteria can become resistant to some antimicrobial compounds, and therefore transmit these resistant traits to *Salmonella*, which occupies the same ecological niche.

The presence of one or the combination of several of the above mentioned mechanisms may also confer a MDR profile to bacteria. These MDR profiles may comprise major antimicrobial compounds, hampering the treatment of severe *Salmonella* infections (EFSA, 2010b).

In 1997, MDR *Salmonella* 4,[5],12:i:- isolates were identified for the first time in Spain (Guerra et al., 2001). The most frequent MDR pattern is the ASSuT tetraresistance pattern, isolated from 30% of the human infection cases in the last 5 years and also from farm animals (Lucarelli et al., 2010; EFSA, 2010b). This pattern emerged in Italy during the 2000s, and has already been identified in Denmark, the United Kingdom, the United States, Spain, France, and the Czech Republic (Lucarelli et al., 2010). Genes responsible for this MDR phenotype are present in a chromosomal resistance island that usually includes the *bla*TEM,

strA-strB, *sul2* and *tet(B)* genes (Hauser et al., 2010; Lucarelli et al., 2010), having some strains additional resistances (Lucarelli et al., 2010; EFSA, 2010b).

Other multiresistant patterns identified in 4,[5],12:i:- isolates worldwide are the ACKGSuTm (showing resistance to ampicillin, chloramphenicol, kanamycin, gentamicin, sulfamethoxazole and trimethoprim) and ACKGSuTm with additional resistance to nalidixic acid patterns, found in Thailand (Switt et al., 2009); the ACSuGSTTm (showing resistance to ampicillin, chloramphenicol, sulfamethoxazole, gentamicin, streptomycin and tetracycline) and ACGSSuTSTm patterns, found in Spain (Echeita et al., 1999); the ACSSuT pattern, found in the United States (Agasan et al., 2002; Switt et al., 2009); and the ACSSpSuT pattern, found in the United Kingdom and other countries (Lucarelli et al., 2010). The isolation of multiresistant isolates was also described in Brazil (Switt et al., 2009) and Germany (Hauser et al., 2010).

The MDR phenotypes include 4,[5],12:i:- strains harboring class 1 integrons or large resistant plasmids, resistant to ampicillin, chloramphenicol, gentamicin, streptomycin, sulfamethoxazole, tetracyclines and trimethoprim. These resistance traits are mainly due to the expression of *bla*TEM-1, which codes for broad spectrum *b*-lactamases responsible for resistance to penicillin and amino-penicillins; of *bla*CTX-M-1, which codes for extended-spectrum β -lactamases; of *cmlA1*, which codes for an efflux pump responsible for chloramphenicol resistance; of *aac*(3)-IV and *aadA2*, which code for enzymes that modify gentamicin and streptomycin active sites, impairing the action of these drugs; of *aadA1*, *sul1* and *sul2*, which code for enzymes responsible for resistance to sulfonamides; of *sul3* and *tet(A)*, which code for an efflux pump mechanism responsible for tetracycline resistance; and of *dfrA12*, which codes for an enzyme responsible for resistance to trimethoprim (Folley and Lynne, 2008a; Guerra et al., 2001; Switt et al., 2009).

It is important to refer that, despite the road book aiming at controlling antimicrobial use and abuse, antimicrobial resistance remains a worldwide problem for both human and veterinary medicine. In this context, the boundaries between human and animal health, as well as between living organisms and the environment are insubstantial. Besides data from clinical studies, resistant bacteria have been described from a variety of environmental sources, including domestic sewage, drinking water, rivers, and lakes (Sayah et al., 2005).

5. *Salmonella* virulence factors

Salmonella enterica includes many serovars that cause disease in avian and mammalian hosts (Eswarappa et al., 2008). Also, *Salmonella* sp. is one of the most frequent bacterial food-borne pathogens affecting humans. In both animal and human hosts, infections may be present in a variety of presentations, from asymptomatic colonization to inflammatory diarrhoea or typhoid fever, depending on serovar- and host-specific factors. Colonization of reservoir hosts often occurs in the absence of clinical signs; however, some *S. enterica* serovars threaten animal health due to their ability to cause acute enteritis or to translocate from the intestine to other organs, causing fever and septicaemia (Stevens, 2009). Also, while certain serovars of *S. enterica* are ubiquitous and cause disease in humans and in a variety of animals, other serovars are highly restricted to a specific host (Hensel, 2004). For example, ubiquitous serovars such as Typhimurium and Enteritidis tend to produce an acute but self-limiting enteritis in a wide range of hosts, whereas host-specific serovars are associated with severe systemic disease that may not involve diarrhoea, usually affecting healthy adults of a single species (e.g. *S. Typhi* in humans, *S. Gallinarum* in poultry) (Stevens, 2009).

Differences in virulence among *Salmonella* serovars and variations in the evolution of *Salmonella* spp. infections in several host species have been attributed to the acquisition and expression of virulence genes (Zhao, 2001). *Salmonella* spp. virulence requires the coordinated expression of complex arrays of virulence factors that allow the bacterium to evade the host's immune system. All *Salmonella* serotypes share the ability to invade the host by inducing their own uptake into the intestinal epithelial cells. In addition, *Salmonella* serotypes associated with gastroenteritis trigger an intestinal inflammatory and secretory response, whereas serotypes that cause enteric fever give rise to systemic infections through their ability to survive and replicate in mononuclear phagocytes (Ohl & Miller, 2001).

Many virulence phenotypes of *Salmonella enterica* are encoded by genes located in distinct chromosome regions, organized in 12 pathogenicity islands (Bhunia, 2008; Eswarappa et al., 2008; Saroj et al., 2008). These gene clusters, known as *Salmonella* pathogenicity islands (SPIs), are thought to be acquired by horizontal gene transfer. They present a G-C content that differs from the remaining chromosome, suggesting acquisition by horizontal transfer. While some SPIs are conserved throughout the genus, others are specific for certain serovars (Amavisit et al., 2003; Bhunia, 2008; Eswarappa et al., 2008). According to Saroj et al., (2008), pathogenicity islands can be transferred between bacteria of different genera, leading to an accumulation of different virulence mechanisms in some strains. Therefore, the occurrence of SPIs varies between serovars and strains (Hensel, 2004). Pathogenicity islands often contain multiple genes functionally related, and required for the expression of a specific virulence phenotype, which suggests that the acquisition of a pathogenicity island during evolution may in one "quantum leap" open up new host niches for the pathogen (Ohl & Miller, 2001; Eswarappa et al., 2008). According to Bhunia (2008), the virulence genes responsible for invasion, survival, and extraintestinal spread are distributed in the *Salmonella* pathogenicity islands. For instance, the virulence genes that are involved in the intestinal phase of infection are located in SPI-1 and SPI-2. Many pathogenicity islands, including SPI-1 and SPI-2, encode specialized devices for the delivery of virulence proteins into host cells, termed type III secretion systems (TTSSs) (Eswarappa et al., 2008). The remaining SPIs are required for causing systemic infections, intracellular survival, fimbrial expression, antibiotic resistance, and Mg²⁺ and iron uptake (Bhunia, 2008).

Besides the SPIs, some virulence factors can be encoded in virulence plasmids. Six serovars (Typhimurium, Gallinarum, Gallinarum biovar Pullorum, Enteritidis, Dublin, Choleraesuis and Abortusovis) typically harbor virulence plasmids of 60-95 kb that contain the *spv* locus, which holds some of the genes that are involved in intracellular survival and multiplication of this facultative intracellular pathogen (Tierrez & Garcia-del Portillo, 2005). The typical virulence plasmid of *S. Typhimurium* (pSLT90), is about 90-95 kb, and belongs to the FII incompatibility group.

Regarding the monophasic *S. Typhimurium*, this serotype has only recently emerged, but it comprises a wide variety of different strains (Soyer et al., 2008). For that, consistent data on virulence mechanisms are limited. Nevertheless, several studies have already shown that not only *Salmonella* serotype 4,[5],12:i:- isolates are genetically and phenotypically closely related to *Salmonella* serotype Typhimurium (Agasan et al., 2002; Amavisit et al., 2005; de la Torre et al. 2003; Delgado et al., 2006; Echeita et al., 2001; Zamperini et al., 2007) but also, virulence genes of monophasic *S. Typhimurium* and their variability are identical to those found in *S. Typhimurium* (Garaizar et al., 2002; Hauser et al., 2009; Soyer et al., 2009; Hauser

et al., 2010). For example, studies developed by del Cerro et al. (2003) and Guerra et al. (2000), demonstrated that strains of monophasic *S. Typhimurium* presented an homology regarding virulence plasmid genes *spvC*, *invE* and *invA* invasion genes, *stn* enterotoxin genes, *shyA* cytolysin genes and genes associated with survival within macrophages (*pho*), when compared to those typically found in *S. Typhimurium*

For all these reasons, it should be noted that, presently, most of the knowledge on SPIs and other *Salmonella* virulence genes of monophasic *S. Typhimurium* is based on observations made in serovar Typhimurium. This serovar is considered a model organism for genetic studies, and a wide variety of classical and molecular tools are available for the identification and characterization of potential *Salmonella* virulence genes.

5.1. *Salmonella* Pathogenicity Islands

As above referred, there are at least twelve chromosomally-encoded *Salmonella* pathogenicity islands (SPIs) (Table 2), as follows:

- SPI-1 is a 43-kb chromosomal locus that was acquired by horizontal gene transfer from other pathogenic bacteria during evolution. It contains 31 genes with a major role in the invasion of host cells and induction of macrophage apoptosis. It also encodes components of the Type III secretion system (TTSS) designated as the Inv/Spa-Type III secretion apparatus that includes the secretion apparatus components, effectors, chaperones, and regulator (Amavisit et al., 2003; Bhunia, 2008; Eswarappa et al., 2008). The major genes present in SPI-1 are *invA*, *invB*, *invC*, *invF*, *invG*, *hilA*, *sipA*, *sipC*, *sipD*, *spar*, *orgA*, *sopB*, and *sopE*. *invABCD* genes, responsible for the expression of several invasion factors that promote bacterial attachment and invasion of M-cells, allowing them to cross the epithelial barrier which is the preferential route of *Salmonella* translocation. For example, InvA is an inner membrane protein involved in the formation of a channel through which polypeptides are exported. InvH and HilD are accessory proteins involved in *Salmonella* adhesion. InvG is an outer membrane protein of the TTSS that plays a critical role in bacterial uptake and protein secretion.

There are two kinds of effector proteins secreted by the TTSS. One subclass consists of InvJ and SpaO, which are involved in the protein secretion through the TTSS. The other subclass modulates host cytoskeleton and induces its uptake. SipB and SipC are the major proteins, which interact with host cytoskeletal proteins to promote *Salmonella* uptake. Inv/Spa are also responsible for macrophage apoptosis. SipA is an actin-binding protein. SopB is an inositol phosphate phosphatase and SopE activates GTP-binding proteins. HilA is the central transcriptional regulator of genes located on SPI-1 (Bhunia, 2008).

- SPI-2 is a 40-kb segment that encodes for 32 genes, only present in members of *S. enterica*, and other type III secretion systems involved in systemic pathogenesis (Amavisit et al., 2003; Eswarappa et al., 2008; Bhunia, 2008). The gene products are essential for systemic infection and mediate bacterial replication, rather than survival within host macrophages (Bhunia, 2008). The majority of these genes are expressed during bacterial growth inside the host-cells. SPI-2 carries genes for Spi/Ssa and TTSS apparatus, i.e., SpiC, which inhibits the fusion between the *Salmonella*-containing phagosome and the lysosome (Bhunia, 2008).
- Type III Secretion Systems are expressed by many bacterial pathogens to deliver virulence factors to the host cell and to interfere with or subvert normal host cell

signaling pathways (Marcus et al., 2000). The TTSS structural genes (including *invG*, *prgH* and *prgK*) encode proteins that may form a needle-like structure and are responsible for contact dependent secretion or for the delivery of virulence proteins to host cells (Zhao, 2001; Bhunia, 2008). This needle-like organelle located in the bacterial periphery has four parts: a needle, outer rings, neck, and inner rings. The needle is constituted by PrgI and a putative inner rod protein, PrgJ; the outer rings structure by InvG; the neck by PrgK; and the base by PrgH that forms the inner rings. The inner membrane components include InvC, InvA, SpaP, SpaQ, SpaR, and SpaS proteins (Bhunia, 2008). When *Salmonella* adheres to a target cell, this needle-like structure is assumed to form a channel with its base anchored in the cell wall and its tip puncturing the membrane of the host cell. Through this channel, *Salmonella* effectors proteins such as SipC, SipA, SopE/E2, and SopB, are injected into the host cell cytoplasm, promoting actin polymerization and membrane remodelling which allows the active uptake of bacteria by the host cell (Zhao, Y., 2001).

- SPI-3 is a 17-kb locus conserved between *S. enterica* serovar Typhi and Typhimurium that is also found in *S. bongori*, being variable in other serovars. SPI-3 harbors 10 genes, including the *mgtCB* operon, which is regulated by PhoPQ and is required for intra-macrophage survival and virulence and for magnesium uptake under low magnesium concentrations (Amavisit et al., 2003; Bhunia, 2008; Eswarappa et al., 2008). PhoQ is a sensor and PhoP is a transcriptional activator that expresses different genes that are required for bacterial survival inside the macrophage, as well as in various stressing environments including carbon and nitrogen starvation, low pH, low O₂ levels, and the presence of defensins. In addition, PhoP regulates genes such as *spiC* and *tassC* that prevent lysosome fusion with the *Salmonella*-containing vacuole. PhoQ regulon activates *pags* genes that are essential for adaptation during the intracellular life cycle (Bhunia, 2008).

Salmonella present in the subcellular lamina propria are either engulfed by the macrophages or by the dendritic cells, which allows its extraintestinal dissemination. The survival of *Salmonella* within macrophages is generally considered to be essential for the translocation of bacteria from the gut-associated lymphoid tissue to the mesenteric lymph nodes and from there to the liver and spleen.

- SPI-4 is a 27-kb locus located next to a putative tRNA gene, containing 18 genes. It is thought to encode genes for the Type I secretion system and is suspected to be required for intramacrophage survival (Amavisit et al., 2003; Bhunia, 2008).
- SPI-5 is a 7.6-kb region and encodes six genes. It appears that SPI-5 encodes effector proteins for TTSS. SopB, which is translocated by TTSS, is an inositol phosphatase involved in triggering fluid secretion responsible for diarrhea. Thus, it is believed that SPI-5 is possibly responsible for enteric infections (Bhunia, 2008; Eswarappa et al., 2008).
- SPI-6 is a 59-kb locus present in both serovars Typhi and Typhimurium. It contains the *saf* gene cluster responsible for fimbriae development, *pagN* responsible for invasion traits, and several genes with unknown function (Bhunia, 2008).

In many studies, bacterial motility was found to be essential for adherence or invasion. In many systems, flagella provide the driving force that enable the bacteria to penetrate the host mucus layer and reach the host cell surface more rapidly (Zhao, 2001). *Salmonella* expresses different types of fimbriae that promote adhesion to M-cells and colonization of intestinal epithelial cells. Type I fimbriae (Fim) binds to α -D-mannose

receptor in the host cell; long polar fimbriae (Lpf) bind to cells located in the Peyer's patch; and plasmid-encoded fimbriae (Pef) and curli, thin aggregative fimbriae, aid in bacterial adhesion to intestinal epithelial cells. Curli helps bacteria to autoaggregate, which enhances survival in the presence of stomach acid or biocides (Bhunia, 2008).

- SPI-7 or Major Pathogenicity Island (MPI) is a 133-kb locus specific for serovar Typhi, Dublin, and Paratyphi. Its genes encode for Vi antigen, a capsular polysaccharide that illicit high fever in typhoid fever infections. SPI-7 also carries the *pil* gene cluster responsible for type IV pili synthesis and the gene that encodes for the SopE effector protein of TTSS (Bhunia, 2008).
- SPI-8 is a 6.8-kb locus that appears to be specific for serovar Typhi. It carries genes for putative bacteriocin biosynthesis but its functional traits have not been fully investigated (Bhunia, 2008).
- SPI-9 is a locus of approximately 16-kb that carries genes for type I secretion system and a large putative RTX (repeat in toxin)-like toxin (Bhunia, 2008). SPI-9 is present in *S. Typhi*, and also as a pseudogene in *S. Typhimurium* (EFSA, 2010)
- SPI-10 is a 32.8-kb locus found in serovars Typhi and Enteritidis. It contains genes that encode for Sef fimbriae (Bhunia, 2008).
- *Salmonella* Genomic Island 1 is a 43-kDa locus that contains genes responsible for antimicrobial resistance. It was identified in *S. Typhimurium* DT104, Paratyphi and Agona, which are resistant to multiple antibiotics. The DT104 strain has been implicated in outbreaks worldwide. It includes genes responsible for five antimicrobial resistance phenotypes (ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracycline) that are clustered in a multidrug resistance region and are composed of two integrons (Bhunia, 2008).
- High Pathogenicity Island (HPI) contains genes responsible for siderophore biosynthesis, required for iron uptake. The HPI is found in *S. enterica* (Bhunia, 2008).

Islands	Salmonella serovars	Length Kb)	Functions
SPI - 1	<i>S. enterica</i> and <i>S. bongori</i>	43	TTSS, invasion of host cells
SPI - 2	<i>S. enterica</i>	40	TTSS, systemic infection
SPI - 3	<i>S. enterica</i> and <i>S. bongori</i>	17	Mg ²⁺ uptake, macrophage survival
SPI - 4	<i>S. enterica</i> and <i>S. bongori</i>	27	Macrophage survival
SPI - 5	<i>S. enterica</i> and <i>S. bongori</i>	7.6	Enteropathogenicity
SPI - 6	<i>S. enterica</i> subsp. <i>enterica</i>	59	Fimbriae
SPI - 7	<i>S. Typhi</i> , <i>S. Dublin</i> , <i>S. Paratphy</i>	133	Vi antigen
SPI - 8	<i>S. Typhi</i> ,	6.8	Unknown; putative bacteriocin biosynthesis
SPI - 9	<i>S. Typhy</i>	16.3	Type I secretion system and RTX – like toxin
SPI - 10	<i>S. Typhi</i> , <i>S. Enteritidis</i>	32.8	Sef fimbriae
SGI - 1	<i>S. Typhimurium</i> (DT104), <i>S. Paratyphi</i> , <i>S. Agona</i>	43	Antibiotic resistance genes
HPI	<i>S. enterica</i> subsp. IIIa, IIIb, IV	?	High affinity iron uptake

Table 2. Main properties and functions of *Salmonella* pathogenicity islands (SPI) (Adapted from Hensel, 2004 and Bhunia, 2008)

Presently, there are over 30 *Salmonella* specific genes that have been used as targets for PCR (Polymerase Chain Reaction) to detect and characterize *Salmonella*. These include *invA* gene sequences that are highly conserved among all *Salmonella* serotypes, other gene sequences also present throughout the genus, and fimbriae protein-encoding genes and antibiotic resistance genes (Table 3).

Gene	Description
<i>invA</i>	Triggers internalization required for invasion of deep tissue cells
<i>InvE/A</i>	Invase proteins
<i>phoP/Q</i>	Intramacrophage survival and enhanced bile resistance
<i>stnB</i>	<i>Salmonella</i> enterotoxin gene
<i>irob</i>	Iron regulation
<i>slyA</i>	Salmolysin
<i>hin/H2</i>	Flagellar phase variation
<i>afgA</i>	Thin aggregative fimbriae
<i>fimC</i>	Pathogen related fimbrae gene of <i>S. enterica</i>
<i>sefA</i>	Major subunit fimbrial protein of serotype Enterica strains
<i>pefA</i>	Fimbrial virulence gene of <i>S. Typhimurium</i>
<i>spvA</i>	Virulence plasmid region
<i>spvB</i>	Virulence plasmid region
<i>spvC</i>	Virulence plasmid region that interacts with the host immune system and is responsible for an increased growth rate in host cells
<i>rep-FIIA</i>	Plasmid incompatibility group
<i>sprC</i>	Virulence gene
<i>sipB-sipC</i>	Junction of virulence genes <i>sipB-sipC</i>
<i>himA</i>	Encodes a binding protein
<i>his</i>	<i>Salmonella</i> genus specific histidine transport operon
<i>prot6e</i>	Virulence plasmid region specific for <i>S. Enteritidis</i>
ST M3357	Regulatory protein whose start codon sequence determines the DT phenotype exhibiting enhanced virulence

Table 3. Genes Used for the PCR Identification of *Salmonella* spp. (Adapted from Levin, 2010)

6. Conclusions

Salmonella spp. is one of the major foodborne pathogen responsible for outbreaks worldwide (EECDC, EFSA, 2009; Switt et al., 2009), being estimated to be the main pathogen responsible for foodborne mortality in the United States (Mead et al., 1999). This bacterial genus includes 2,500 identified serotypes, distributed between 2 species: *Salmonella enterica* and *Salmonella bongori* (Foley and Lynne, 2008a). The emergence of new pathogenic strains and serotypes has been described (EFSA, 2010b; Hauser et al., 2010). Due to their increased virulence, these strains can rapidly spread among production animals and humans, representing a major public health issue (EFSA, 2010b; Hauser et al., 2010). In the mid-1990s the emergence of *Salmonella enterica* subsp. *enterica* serotype I,4,[5],12:i:-, a monophasic variant of *Salmonella* Typhimurium, has been reported in Europe (Foley et al., 2008b; Hauser et al., 2010; Switt et al., 2009). Nowadays it seems to be one of the major serotypes

responsible for human salmonellosis cases worldwide (EECDC, EFSA, 2009; Switt et al., 2009). It has also been isolated from several animal species, such as poultry, cattle, swine, and turtles, and also from food products, such as poultry and pork products.

In 2010, the European Food Safety Authority (EFSA) Panel on Biological Hazards (BIOHAZ) published a Scientific Opinion alerting for the increasing number of outbreaks in the European Union member states promoted by “*Salmonella* Typhimurium-like” strains. The Panel has recommended that these strains should be further typed and characterized, particularly in terms of antimicrobial resistance (EFSA, 2010b).

Studies aiming at fully characterizing the monophasic variants of *Salmonella* Typhimurium-like strains (4,[5],12:i:-) isolated from different sources, such as food products, animals and the environment, in terms of molecular typing, antimicrobial resistance, virulence traits and immune response modulation, are extremely relevant. Data provided by such studies will have repercussions in preventive and therapeutic strategies, both in human and veterinary medicine.

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