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REVIEW

Persistent Infection and Long-Term Carriage of Typhoidal and Nontyphoidal Salmonellae

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SUMMARY The ability of pathogenic bacteria to affect higher organisms and cause disease is one of the most dramatic properties of microorganisms. Some pathogens can establish transient colonization only, but others are capable of infecting their host for many years or even for a lifetime. Long-term infection is called persistence, and this phenotype is fundamental for the biology of important human pathogens, including *Helicobacter pylori*, *Mycobacterium tuberculosis*, and *Salmonella enterica*. Both typhoidal and nontyphoidal serovars of the species *Salmonella enterica* can cause persistent infection in humans; however, as these two *Salmonella* groups cause clinically distinct diseases, the characteristics of their persistent infections in humans differ significantly. Here, following a general summary of *Salmonella* pathogenicity, host specificity, epidemiology, and laboratory diagnosis, I review the current knowledge about *Salmonella* persistence and discuss the relevant epidemiology of persistence (including carrier rate, duration of shedding, and host and pathogen risk factors), the host response to *Salmonella* persistence, *Salmonella* genes involved in this lifestyle, as well as genetic and phenotypic changes acquired during prolonged infection within the host. Additionally, I highlight differences between the persistence of ty-

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phoidal and nontyphoidal *Salmonella* strains in humans and summarize the current gaps and limitations in our understanding, diagnosis, and curing of persistent *Salmonella* infections.

KEYWORDS *Salmonella enterica*, bacterial evolution, enteric pathogens, gene regulation, host-pathogen interaction, immunopathogenesis, pathogenicity islands, persistence, virulence

INTRODUCTION

Members of the genus *Salmonella* are Gram-negative gammaproteobacteria that are facultative intracellular human and animal pathogens and members of the family *Enterobacteriaceae* (1). Initially, the genus *Salmonella* was characterized by its ability to utilize citrate as a sole carbon source and lysine as a source of nitrogen as well as its ability to yield hydrogen sulfide; however, *Salmonella* taxonomy is notoriously confusing and has been changed over the years. Currently, the genus *Salmonella* is classified into two species only, *S. bongori* and *S. enterica*. *S. bongori* is more primordial evolutionarily and was separated from an *Escherichia coli* common ancestor about 100 million to 160 million years ago (2, 3). The horizontal genetic acquisition of *Salmonella* pathogenicity island 1 (SPI-1), which is required for the ability of all salmonellae to invade host intestinal epithelial cells and induce apoptosis in macrophages (4–6), marks an important stage of *Salmonella* speciation (7). A later independent horizontal acquisition of SPI-2 by *S. enterica* subsequent to its speciation from *S. bongori* happened between 40.0 million and 63.4 million years ago (2, 7, 8) and is important for intracellular survival and the systemic phase of infection (9, 10).

The later species, *S. enterica*, is further classified into subspecies, which include *Salmonella enterica* subsp. *enterica* (subsp. I), *Salmonella enterica* subsp. *salamae* (subsp. II), *Salmonella enterica* subsp. *arizonae* (subsp. IIIa), *Salmonella enterica* subsp. *diarizonae* (subsp. IIIb) (11, 12), *Salmonella enterica* subsp. *houtenae* (subsp. IV), and *Salmonella enterica* subsp. *indica* (subsp. VI), while the currently defined separate species *S. bongori* was originally designated subsp. V (13). Determination of *Salmonella* subspecies was first based on phenotypic differences and biochemical traits, such as carbon source utilization, but was later confirmed by DNA-DNA hybridization (14), multilocus enzyme electrophoresis and sequence analysis of housekeeping genes (2, 15, 16), as well as microarray analysis (17). Another *S. enterica* subspecies is known as subsp. VII, which was identified by Boyd and colleagues (18) using multilocus enzyme electrophoresis data. Nonetheless, this subspecies is not distinguished by a unique biochemical profile as for the other *S. enterica* subspecies.

Salmonella isolates are subtyped by the Kauffmann-White scheme (see below for more details) according to three groups of surface structures expressed on the bacterial lipopolysaccharide (LPS), flagella, and capsular polysaccharide (19). As of 2010, 22 antigenically distinct "serovars" or "serotypes" belonging to *S. bongori* have been identified, together with 2,659 different serovars of the species *S. enterica*, of which 1,586 serovars belong to *S. enterica* subsp. I (20). Interestingly, most infections of humans and warm-blooded animals are caused by *S. enterica* subsp. I serovars, while *S. bongori* and other *S. enterica* subspecies are normally associated with infections of cold-blooded animals, including reptiles and amphibians, and only occasionally infect human hosts (Fig. 1). In fact, about 99% of all human *Salmonella* infections are caused by *S. enterica* subsp. I serovars (21).

From a clinical point of view, different serovars belonging to *S. enterica* subsp. I can be classified according to their host specificity and the disease that they cause in their hosts. Most of the *Salmonella enterica* subsp. I serovars elicit localized self-limiting inflammation of the terminal ileum and colon, which is known as gastroenteritis, in healthy humans (22). These nontyphoidal *Salmonella* (NTS) serovars are also recognized as "generalists" due to their ability to colonize and infect a broad range of animal species that serve as their environmental reservoir. Often, animal infections by generalist NTS are asymptomatic; however, some of these NTS serovars could cause symp-

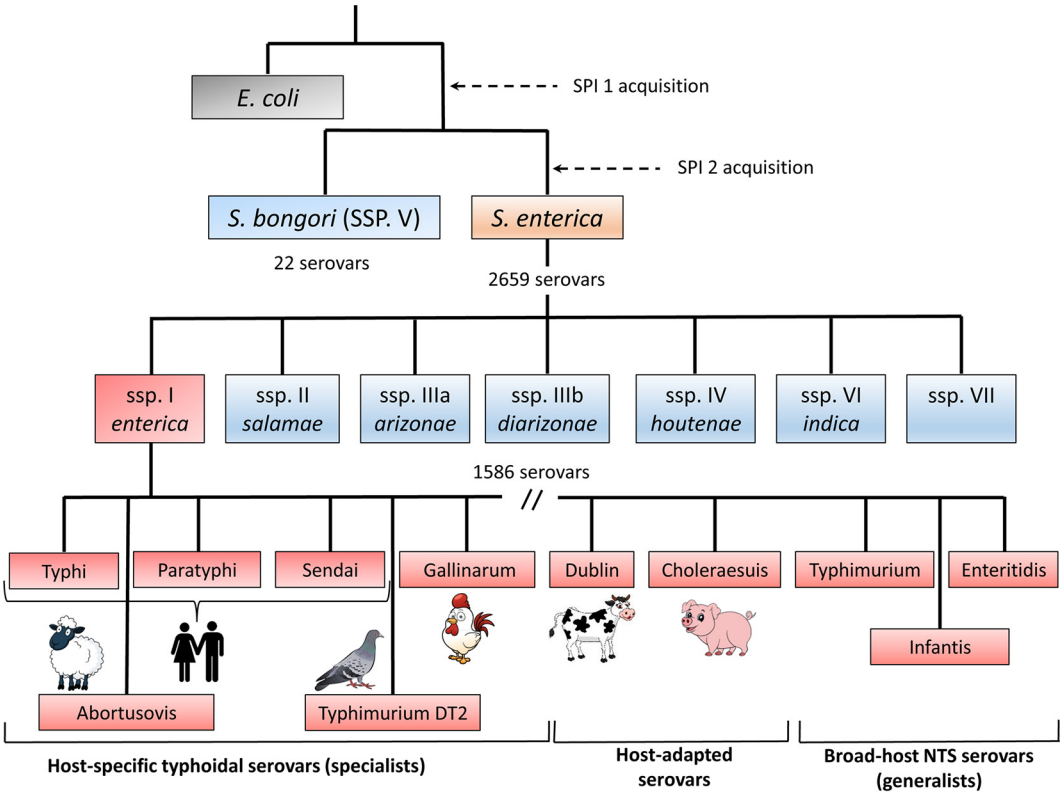

FIG 1 The evolutionary history and phylogenetic and host specificities of *Salmonella*. The currently accepted nomenclature divides the bacterial genus *Salmonella* into two species, *S. bongori*, which was separated from an *E. coli* common ancestor about 100 million to 160 million years ago, and *S. enterica*, which evolved from *S. bongori* between 40.0 million and 63.4 million years ago. Both speciation events were facilitated by the horizontal acquisitions of *Salmonella* pathogenicity island 1 (SPI-1) and SPI-2, respectively. The species *S. enterica* is further classified into 7 subspecies, including *Salmonella enterica* subsp. *enterica* (subsp. I), *Salmonella enterica* subsp. *salamae* (subsp. II), *Salmonella enterica* subsp. *arizonae* (subsp. IIIa), *Salmonella enterica* subsp. *diarizonae* (subsp. IIIb), *Salmonella enterica* subsp. *houtenae* (subsp. IV), *Salmonella enterica* subsp. *indica* (subsp. VI), and *Salmonella enterica* subsp. VII. *S. enterica* subsp. I contains 1,586 distinct serovars, many of which are associated with infections of human and warm-blooded animals (shown in red boxes). *S. bongori* and other *S. enterica* subspecies are frequently associated with infections of cold-blooded animals (shown in blue boxes). Examples of generalist serovars (*S.* Typhimurium, *S.* Enteritidis, and *S.* Infantis), which are capable of infecting a broad range of hosts, and specialist serovars (*S.* Typhimurium DT2, *S.* Gallinarum, *S.* Dublin, and *S.* Choleraesuis), which are host specific, are also indicated. *Salmonella* serovars Typhi, Paratyphi, and Sendai are all human-specific serovars and the causative agents of enteric fever.

tomatic disease, which is dependent on the infecting serovar and the species, genetic background, and immune status of the host. For example, *S. enterica* subsp. *enterica* serovar Typhimurium (*S.* Typhimurium) can cause acute enteritis in pigs and cattle yet causes systemic disease in susceptible mice and colonizes the intestines of adult poultry asymptomatically (23–25).

A small subset of serovars known as "specialists" are capable of infecting and colonizing only a very narrow range of hosts. For example, *S.* Typhi, *S.* Paratyphi A, *S.* Paratyphi B, *S.* Paratyphi C, and *S.* Sendai, collectively known as typhoidal serovars, can infect only humans and higher primates. Human infection with these typhoidal serovars, which have developed in four phylogenetically independent clonal lineages (26), results in a severe and potentially fatal disseminated septicemic infection called typhoid or enteric fever rather than gastroenteritis (27). Other specialist serovars are associated with systemic illnesses in other animal species. For instance, *S.* Gallinarum and *S.* Pullorum, which cause fowl typhoid and septicemic disease in young birds (pullorum disease), respectively, exhibit host specificity for poultry and aquatic birds and are rarely associated with disease in mammalian hosts (28). Similarly, *S.* Typhimurium phage type DT2 is frequently linked to septicemic infections in pigeons (29), and *S.* Abortusovis causes septicemia and abortions in sheep. The third group of serovars includes host-adapted serovars that are normally associated with a specific animal host,

but occasional infections of humans may lead to an invasive septicemic disease similar to bacteremia that develops following infections by typhoidal serovars (30, 31). Examples of such serovars are *S.* Choleraesuis, which causes disease in swine (32), and *S.* Dublin, which accounts for high rates of mortality in young calves and causes fever, reduced milk production, diarrhea, abortion, and sometimes death in adult cattle (33). *Salmonella* phylogenetic and host specificities are summarized and illustrated in Fig. 1.

GLOBAL EPIDEMIOLOGY OF *S. ENTERICA*

The pathogen *S. enterica* is still a major cause of considerable burdens in both developing and developed countries. The estimated annual prevalence of enteric fever caused by all typhoidal serovars is over 27 million cases, resulting in more than 200,000 deaths worldwide (34). Enteric fever is generally endemic in developing countries in areas that lack adequate sanitation and have a shortage of clean water, enabling the spread of typhoidal salmonellae via the fecal-oral route. In the last years, the occurrence of *S.* Paratyphi A infections is increasing, and in some counties, particularly those in Southeast Asia, this serovar is responsible for up to half of all enteric fever cases (35, 36).

NTS salmonellosis results from infection by a wide range of zoonotic serovars and is mainly (although not exclusively) transmitted by ingestion of contaminated food. While typhoidal *Salmonella* infections affect primarily developing countries, NTS infections are common in the developing as well as in the developed worlds, with an annual estimated global burden of gastroenteritis due to *Salmonella* infection of 78.7 million cases. Each year, 59,000 people die from NTS infections, most of them in developing countries (37). In the United States alone, *Salmonella* accounts for about 1.4 million human infections each year, leading to 116,000 hospitalizations and 600 deaths (38), and the economic burden in the United States due to salmonellosis is estimated to be US$3.66 billion per annum (39). In 2016, the incidences of culture-confirmed salmonellosis were 14.51 and 20.4 cases per 100,000 population in the United States and the European Union, respectively. The top five most prevalent serovars in human illnesses acquired in the United States during 2016 were, in decreasing order, *S.* Enteritidis, *S.* Newport, *S.* Typhimurium, *S.* Javiana, and monophasic *S.* Typhimurium (*S.* 4,[5],12:i:−) (40), and those in the European Union were *S.* Enteritidis, *S.* Typhimurium, monophasic *S.* Typhimurium, *S.* Infantis, and *S.* Derby (41).

Most NTS infections in humans elicit self-limiting gastroenteritis, which is confined to the terminal ileum and colon; however, more-serious complications, such as bacteremia, may result in about 5% of individuals infected with NTS (42). Invasive NTS can further develop to focal infection, such as meningitis, and bone and joint infections. Interestingly, several NTS, including *S.* Choleraesuis, *S.* Dublin, and 9,12:l,v:−, are more likely to cause bacteremia than others (31, 43, 44), and recent reports showed substantial differences in disease manifestations between different serovars (31, 45, 46). In sub-Saharan Africa, invasive salmonellosis and bloodstream infections in both children and adults are predominantly caused by *S.* Typhimurium sequence type 313 (ST313) (47, 48), indicating various potentials of different NTS and strains to cause invasive salmonellosis in humans. Besides serovar-dependent factors, extraintestinal salmonellosis is also more likely to develop in risk groups such as immunocompromised individuals, infants, the elderly, or patients with underlying conditions (31, 49).

The role of food-producing animals as a reservoir of NTS infections in humans has been demonstrated repeatedly. For example, the clonal spread of a multidrug-resistant (MDR) *S.* Infantis strain that has been observed in Israel since 2007 first emerged in the poultry sector before it disseminated to humans (50). Similarly, the pandemic of *S.* Enteritidis infections in the United Kingdom, which lasted over two decades, was linked to poultry meat and eggs (25). In addition to consumption of contaminated food products such as poultry, beef, pork, eggs, milk, seafood, and fresh produce, NTS transmission can occur by person-to-person contact or by contact with pets such as dogs, cats, rodents, reptiles, and amphibians (42, 51–53).

CLINICAL SYMPTOMS AND COURSE OF TYPHOIDAL AND NONTYPHOIDAL *SALMONELLA* INFECTIONS

As mentioned above, human infections with typhoidal or nontyphoidal serovars result in very different diseases. Infection with NTS typically presents as acute gastroenteritis, which appears 4 to 72 h after infection. Symptoms include fever, chills, nausea, vomiting, abdominal cramping, and diarrhea. In healthy individuals, NTS salmonellosis is usually a self-limited disease that lasts 3 to 7 days without the need for medical intervention (24, 54, 55). However, after convalescence, a small subset of patients may develop chronic sequelae such as reactive arthritis or irritable bowel syndrome (56). Moreover, in susceptible patients, NTS infection might spread systemically to other sites in the body and cause febrile illness. Although this occurs more frequently in immunocompromised patients, systemic dissemination of nontyphoidal *Salmonella* strains can happen in otherwise healthy humans as well (57).

Contrary to NTS infections, typhoidal serovars elicit noninflammatory invasive disease resulting in transient primary bacteremia that may lack gastrointestinal symptoms (58). Typhoid and paratyphoid fevers cannot be distinguished clinically, and both become symptomatic usually 8 to 14 days after infection, although the incubation time can vary greatly from 4 to 33 days, depending on the infectious dose (59, 60). Clinical symptoms may last for up to 3 weeks, and fever (>39°C) is the most frequently observed symptom characterizing enteric fever. Additional frequently seen symptoms include headache, malaise, chills, anorexia, weight loss, abdominal pain, cough, and rose spots on the chest. About 50% of patients develop gastroenteritis symptoms, which include diarrhea, nausea, or vomiting, and about 25% of enteric fever patients experience constipation. Occasionally, *S.* Typhi and *S.* Paratyphi infections may also result in other infrequent clinical complications such as meningitis, septic arthritis, and osteomyelitis (58, 61, 62).

Salmonella infection occurs through the fecal-oral route. Following the consumption of food or beverage contaminated with the bacteria, both typhoidal and NTS salmonellae attach to and invade the intestinal epithelium of the distal ileum (63). *Salmonella* transport through the intestinal barrier occurs primarily via the specialized microfold (M) cells found in the lymphoid structures known as Peyer's patches (64) or by active invasion of nonphagocytic cells, mediated by the "trigger" mechanism (65). Active host cell invasion by *Salmonella* requires the function of the evolutionarily conserved type III secretion system (T3SS) encoded on SPI-1 and a large collection of injected effectors, which are directly translocated into the host cell cytoplasm, facilitating cytoskeletal rearrangements and disconnection of epithelial cell junctions (66–68). Active invasion of NTS in the underlying lamina propria and expression of pathogen-associated molecular patterns (PAMPs) elicit in immunocompetent individuals a strong Th1 immune response and the recruitment of a variety of bone marrow-derived phagocytes in an interleukin-8 (IL-8)-dependent manner. This leads to phagocytosis of invading *Salmonella* bacteria by neutrophils and macrophages and further recruitment of T and B cells. Neutrophil recruitment, the generation of reactive nitrogen and oxygen species, antimicrobial peptides, and the bactericidal activity of the phagocytes effectively limit the dissemination of NTS to systemic sites and confine the infection to the lamina propria of the terminal ileum and colon (69–71). Therefore, in immunocompetent patients, most gastroenteritis cases caused by NTS are self-limiting and do not proceed beyond the mucosa of the gastrointestinal tract.

In contrast, invasion of the intestinal mucosa by host-adapted typhoidal serovars does not trigger a mucosal inflammatory response and often does not induce diarrheal disease (72–74). The basically noninflammatory nature of enteric fever is associated with marginal transmigration of neutrophils across the intestinal epithelium, as opposed to massive neutrophil recruitment during gastroenteritis caused by NTS (75). Recent studies were able to demonstrate how differences in the regulation and expression of SPI-1 and flagellum regulons in *S.* Typhi (60, 76–78) and *S.* Paratyphi A (79–81) contribute to the noninflammatory response characterizing typhoidal infection in comparison to the inflammatory-disease-causing NTS.

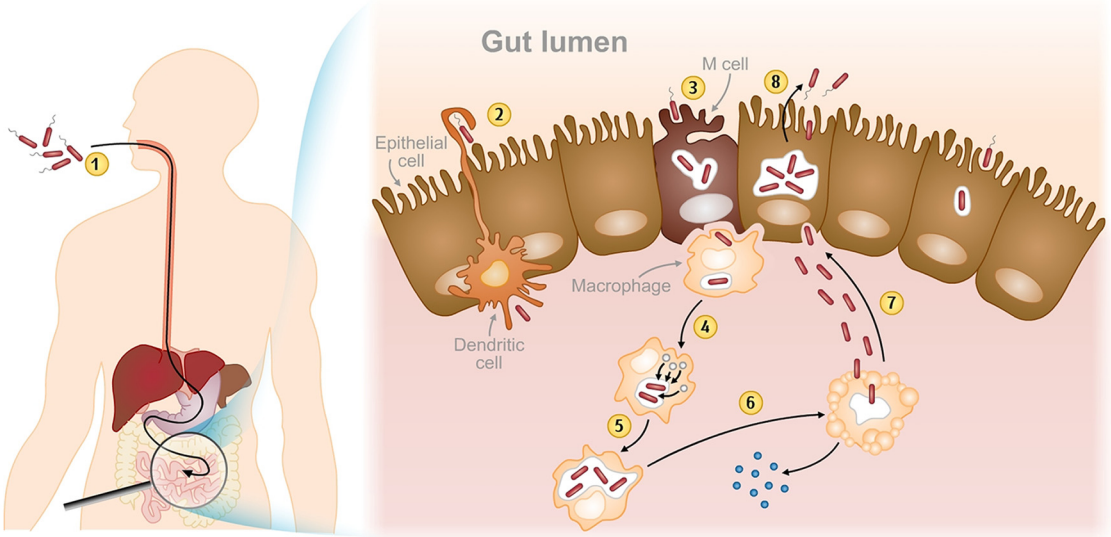

FIG 2 The course of *Salmonella* infection. Disease caused by *Salmonella* occurs after ingestion of food or beverage contaminated with the bacterium (1). After gaining access to the gut lumen, *Salmonella* bacteria can cross the apical pole of the epithelial barrier either by a passive mechanism facilitated by dendritic cells that emit pseudopods between epithelial cells (2) or by invasion through the M cells of Payer's patches in the ileal portion of the small intestine (3). Active crossing of epithelial cells occurs as well and requires the delivery of distinct effector proteins injected directly into host cells using a type III secretion system that is encoded by SPI-1, which also triggers gut inflammation. In immunocompetent individuals, the induced inflammation limits the dissemination of NTS to underlying tissues and systemic sites. However, invasive NTS in immunodeficient patients or typhoidal salmonellae are capable of evading the immune system, enter subepithelial phagocytic cells such as macrophages, and survive within them. Phagocytic cells can then transport *Salmonella* bacteria via the lymphatic system and disseminate the bacteria systemically (mainly to the liver, spleen, and lymph nodes). Within the intracellular environment, *Salmonella* bacteria establish a specialized vacuole known as the *Salmonella*-containing vacuole (SCV), which supports bacterial survival and replication (4 and 5). This stage requires the expression of SPI-2 genes, which encode a second type III secretory system that allows injection of a different set of effectors from the SCV into the host cell cytoplasm. The presence of *Salmonella* bacteria within the cells may lead to cytokine secretion, triggering inflammation and/or programmed cell death (apoptosis) (6). *Salmonella* bacteria may also reseed into the gut by basolateral invasion (7 and 8), excretion into the feces, and bacterial shedding. (Originally posted on http://galmor-lab.com/salmonella/.)

After crossing the intestinal mucosa via M cells, typhoidal salmonellae cause an initially clinically undetectable infection and transient primary bacteremia in which pathogens gain access to underlying lymphoid tissues and the bloodstream. At this stage, typhoidal serovars reside and multiply within mononuclear phagocytes, spread to the draining mesenteric lymph nodes (MLNs), and from there disseminate to systemic tissues (82, 83). Following dissemination to systemic sites, salmonellae can survive and multiply in phagocytes and epithelial cells of the liver, spleen, bone marrow, and gallbladder (84–87). This intracellular growth requires a second T3SS encoded by SPI-2 and a separate array of effector proteins translocated into host cells (10, 88). Within host cells, salmonellae can then establish and maintain an intracellular replicative niche called the *Salmonella*-containing vacuole (SCV). From systemic sites, *Salmonella* can reseed the intestinal lumen, often through the bile ducts, and be shed in the feces to the environment, ready to infect a new host. Figure 2 illustrates the infection process of *Salmonella*.

LABORATORY DIAGNOSIS OF *SALMONELLA* INFECTIONS

Salmonella Diagnosis by Bacteriological Culture

Clinical symptoms associated with acute gastroenteritis caused by NTS infections are often indistinguishable from those caused by other enteric bacterial pathogens, and the gold standard for *Salmonella* diagnosis still requires isolation of the pathogen from stool samples. In cases of invasive NTS infections that involve systemic dissemination, the pathogen may also be isolated from the blood, lymph nodes, bone marrow, and other systemic sites. Similarly, conclusive diagnosis of enteric fever requires the isolation of typhoidal serovars from either the blood, bone marrow, urine, other sterile sites, or stool. In suspected cases of enteric fever, blood and bone marrow should be sampled

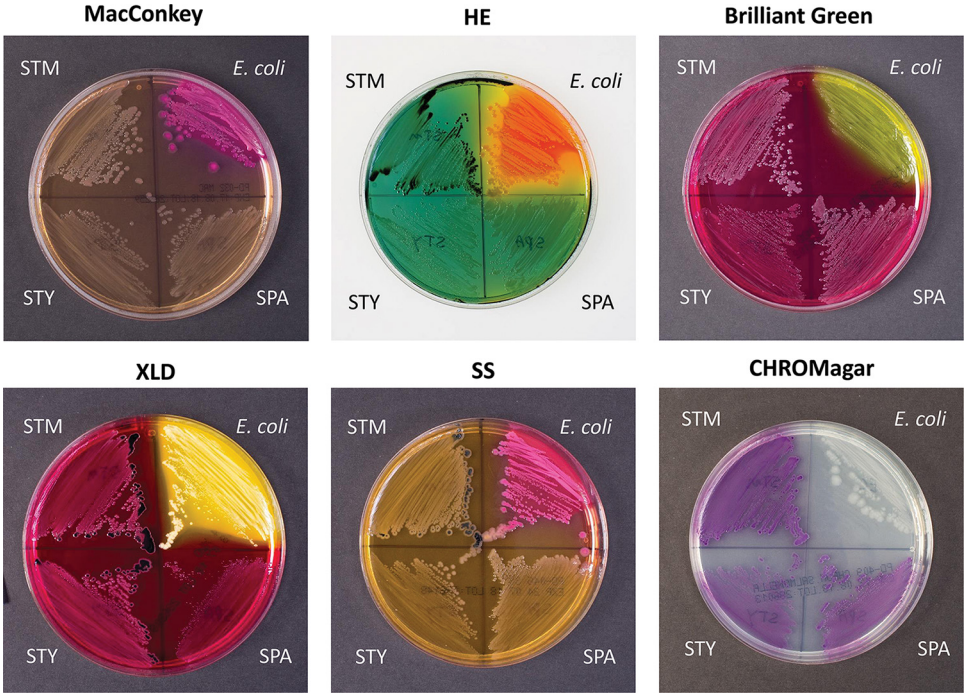

FIG 3 Selective media for *Salmonella* diagnosis. *E. coli* strain R 27, *S.* Typhimurium SL1344 (STM), *S.* Typhi CT18 (STY), and *S.* Paratyphi A 45157 (SPA) were plated on selective media, including MacConkey agar (catalog number PD-032), Hektoen enteric (HE) agar (catalog number AGR-10407), XLD agar (catalog number PD-104), salmonella-shigella (SS) agar (catalog number PD-046), brilliant green agar (catalog number PD-104), and CHROMagar *Salmonella* plus (catalog number PD-409). All plates were obtained from Hy Laboratories Ltd. Plates were incubated at 37°C for 18 to 24 h and imaged using a Pentax K5 camera. Note that *S.* Typhi and *S.* Paratyphi A do not create black colonies on HE and SS agar due to low-level production (*S.* Typhi) or no production (*S.* Paratyphi A) of H_2S.

in the first week of fever, and stool and urine should be sampled in the following weeks. Since typhoidal salmonellae are present in small quantities in patient blood (<15 organisms/ml), the sensitivity of blood cultures is only 40% to 80% (89, 90), and taking more than one blood specimen is recommended.

Different bacteriological media containing lactose and a pH indicator, such as MacConkey agar, have been traditionally used for differentiation of the lactose-nonfermenting salmonellae from other enteric pathogens such as *Escherichia coli*. However, since other enteric pathogens such as *Proteus* spp. and *Shigella* spp. do not ferment lactose as well, inoculation of stool specimens onto additional selective and differential media is needed. Hektoen enteric (HE) and xylose-lysine-deoxycholate (XLD) agars are considered selective, and both agars can detect hydrogen sulfide (H_2S) production (appears as black colonies) (Fig. 3), which is typical of most NTS. Additional highly selective/differential agar media, including salmonella-shigella (SS), bismuth sulfite, or brilliant green agar, may also be used. Nonetheless, since the latter media sometimes inhibit the growth of some *Salmonella* species strains, it is recommended that these media be used in combination with a less selective enteric agar medium, such as MacConkey agar or eosin methylene blue (91).

Over the last 20 years, a range of chromogenic media for detection of *Salmonella* spp. in stool samples has been developed. These media utilize enzyme substrates that create a colored product following hydrolysis, hence resulting in colored colonies that can be easily recognized against the background of the commensal gut flora. For example, CHROMagar *Salmonella* is used for the isolation and differentiation of *Salmonella* spp., which appear lilac on this medium, from other pathogens, which appear blue, colorless, or inhibited on this medium (Fig. 3). The superior performance of chromogenic media has been demonstrated in several studies that reported better sensitivity and specificity over traditional selective/differential media used for *Salmonella* isolation and detection (92–94).

Putative *Salmonella* cultures that are lactose nonfermenters and/or H_2S producers, isolated on selective plates, are often subjected to a secondary screening test involving subculturing of the suspicious *Salmonella* colonies onto triple-sugar iron (TSI) agar, lysine iron agar (LIA), or Hy-Enterotest system tubes containing special culture medium that permits the identification of *Enterobacteriaceae*. This second screen may be unnecessary if a chromogenic medium is used as part of the initial culturing setup. Isolates that yield reactions characteristic of *Salmonella* spp. on TSI agar and/or LIA are further confirmed to the genus level using either a manual identification tool, such as the API 20E system, or an automated bacterial identification system, such as Vitek2, Phoenix, MicroScan, or matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (see below) (91).

Identification of *Salmonella* spp. to the genus level when isolated from stool is usually sufficient, and clinical laboratories are normally asked to submit these isolates to a central public health reference laboratory for serovar typing. However, it is highly recommended that the possibility of typhoidal infection be excluded, given the clinical significance of these pathogens. To this end, *S*. Typhi can be discriminated from other serovars by the typical weak H_2S production seen on a TSI slant and a negative ornithine decarboxylase reaction. Similarly, *S*. Paratyphi A is characterized by negative H_2S (Fig. 3), lysine, and citrate reactions (91).

Salmonella Serotyping

Following the identification of *S. enterica* to the species level, further typing of the isolated serovar is desired. *Salmonella* typing to the serovar level is usually carried out at central reference laboratories. Since *S. enterica* is a highly diverse pathogen, and more than 2,600 serovars have been characterized so far, *Salmonella* typing to the serovar level is very useful for surveillance, epidemiological studies, and outbreak investigations. For many decades, *Salmonella* subtyping has been routinely conducted by serotyping, according to the White-Kauffmann-Le Minor scheme (95). In this method, surface antigens are identified based on agglutination reactions with specific antisera. According to this traditional serotyping scheme, a serovar is defined based on the expression of somatic (O) and flagellar (H) antigens. A third group is a capsular antigen (Vi) that is expressed by only a few *S. enterica* serovars, including *S*. Typhi and *S*. Dublin (96). The somatic antigens are presented on the lipopolysaccharide (LPS) envelope and are used to divide all *S. enterica* serovars into 46 serogroups (97) that, together with 114 distinct flagellar antigens, define more than 2,600 different serovars (20).

The expression of flagellar antigens, encoded by the *fliC* and *fljB* genes, occurs at two stages, known as phase I (H1 antigens) and phase II (H2 antigens). Expression of H1 and H2 flagellar antigens is controlled by a phase variation mechanism (98–100), and serovars that express two flagellin types are defined as diphasic, while those with only one phase of flagellar antigens are called monophasic. Reporting of a *Salmonella* serovar is conducted according to a conventional formula that comprises a sequential list of the antigens O, H1, and H2 separated by colons. In cases where the Vi antigen is present, it is indicated in brackets following the listing of the O antigens. *S. enterica* serovar Typhi, for example, is reported as "9,12,[Vi]:d:−," where its O antigens are "9" and "12," the capsular antigen is "Vi," "d" is the phase 1 H1 antigen, and the minus sign indicates that since *S*. Typhi is monophasic, it does not express H2 antigens. *S. enterica* serovar Heidelberg, which expresses the O antigens "1," "4", "12," and sometimes "5"; the H1 antigen "r"; and the H2 antigens "1" and "2," is reported as "1,4,[5],12:r:1,2."

Despite this method being considered the gold standard of *Salmonella* serotyping, it is labor-intensive and time-consuming (typing of a single isolate requires a minimum of 3 days) and fails to provide any information regarding the phyletic relationships and population structures between same-serovar isolates. Thus, several molecular methods to classify *Salmonella* serovars were developed to complement traditional serotyping. Such molecular typing approaches include pulsed-field gel electrophoresis (PFGE) (101, 102); multilocus sequence typing (MLST), which is based on variation in the sequences

of seven housekeeping gene fragments (103, 104); ribotyping (105); clustered regularly interspaced short palindromic repeat (CRISPR) typing (106, 107); as well as other molecular methods. Since several comprehensive reviews about molecular *Salmonella* typing approaches have been published in the last few years (108–111), and because more-modern next-generation sequencing methods are now emerging (see below), which are expected to replace these techniques in the near future, I do not further discuss these techniques here.

Serological Diagnosis of Enteric Fever

Serodiagnosis of typhoid fever has been performed for more than a century using the Widal tube agglutination test. In this procedure, killed *Salmonella* serovar Typhi and Paratyphi A bacteria are detected with serum that measures agglutinating antibodies to the LPS (O) and flagellar (H) antigens. The efficacy of the Widal test is controversial, as it is considerably limited by poor sensitivity and by cross-reactivity with other *Salmonella* serovars, resulting in a low predictive value for typhoid fever (112, 113). Furthermore, infections with non-*Salmonella* pathogens, including malaria, dengue, and brucellosis, were also shown to lead to cross-reactivity in regions where enteric fever is endemic (113). In spite of these flaws, due to its low cost and simplicity, the Widal test is still commonly used as a diagnostic test in regions that lack advanced laboratory infrastructure (114).

Other currently commercially available enteric fever point-of-care diagnostic tests include Typhidot and Typhidot-M, which test IgM and/or IgG antibodies against a 50-kDa outer membrane protein antigen in a dot blot enzyme-linked immunosorbent assay (ELISA) format, and Tubex, which measures antibodies against *S*. Typhi LPS antigen by quantifying interference of binding between O9 monoclonal antibodies and LPS-coupled magnetic particles (115, 116). Several other enteric fever rapid diagnostic tests were recently reviewed (117) and are not discussed further. Overall, the diagnostic accuracy for enteric fever using the currently available enteric fever rapid diagnostic tests was evaluated by a meta-analysis to have only moderate sensitivity (69 to 85%) and specificity (79 to 90%), and the authors of that study concluded that the moderate sensitivity and specificity of these tests do not justify their replacement of blood culture for enteric fever diagnosis and that there is an urgent need to develop rapid diagnostic tests for typhoid fever with better performance (116, 117). Thus, laboratory diagnosis of typhoid fever is still dependent mostly on the detection of bacteria in the blood by PCR (see below) or culture, despite its limited sensitivity.

PCR-Based Molecular Methods for *Salmonella* Detection

Microbiological culture has excellent specificity; however, this approach has lower sensitivity and often requires 24 to 72 h of incubation for result interpretation. Hence, molecular approaches for *Salmonella* identification characterized by high sensitivity and a short turnaround time have been developed. PCR-based assays have been clinically validated for diagnosis of gastrointestinal NTS infection (118), invasive NTS infection (119), and typhoidal *Salmonella* infection in the blood of patients with enteric fever (reviewed in reference 120) and are increasingly being used for diagnosis of *Salmonella* infections. Lin and associates successfully demonstrated the feasibility of a bacterial enrichment culture-based real-time PCR for detection and screening of NTS infection in children presenting with diarrhea. Enrichment culture-based real-time PCR reached 85.4% sensitivity and 98.1% specificity, in comparison to 53.7% sensitivity and 100% specificity for detection with routine bacterial culture methods (118). Tennant et al. have shown that PCR based on the O and H antigen-encoding genes was 100% sensitive and specific in identifying *S*. Typhimurium and other serovars using purified isolate DNA as a template, demonstrating that a PCR-based method could support surveillance of invasive disease caused by NTS (119). Although the sensitivity of PCR for the detection *S*. Typhi and *S*. Paratyphi in blood samples is variable (120), several studies have reported a sensitivity of over 90% in PCR assays used to detect typhoidal salmonellae in blood culture, even without an enrichment step (121–124). Similarly,

100% sensitivity was reported for bone marrow samples that tested positive for *Salmonella* (125), and successful results (95.4% sensitivity) have also been reported for the efficacy of nested PCR targeting the flagellin gene (*fliC*) to detect *S.* Typhi in urine (126) and blood (127) samples. Other useful *Salmonella*-specific genes that were used for *Salmonella* detection in clinical samples include the *S.* Typhi Vi capsular gene *viaB* (128), *hilA* (a regulatory gene controlling the expression of SPI-1 genes) (129), the tetrathionate genes *ttrC-ttrA* (130, 131), the 23S rRNA gene (132), and the CS54 island-borne gene *ratA* (133).

Recently, a few PCR-based multiplex detection panels have been marketed. These platforms allow direct identification of gastrointestinal pathogens, including *Salmonella*, in clinical stool specimens and include the bioMérieux Biofire Filmarray system (134), the BD Max system (135), the Savyon Diagnostics gastrointestinal infection panel (GIP) (136), the Luminex xTAG system (136), and Allplex gastrointestinal panel assays (137). These panels allow markedly improved turnaround times compared with culture-based methods; however, *Salmonella* culturing is still required for serovar classification and susceptibility testing. Since the experience with these newly developed panels is still rather limited, confirmatory testing prior to reporting of *Salmonella* infection with these kits is still recommended. In addition, a significant level of experience and a sufficient volume of tests should be accumulated before considering the replacement of traditional *Salmonella* identification methods with a single molecular/commercial test.

Salmonella Diagnosis by MALDI-TOF MS

Recently, MALDI-TOF MS arose as a rapid and robust method for microbial identification and diagnosis. The usage of mass spectrometry for identification and typing of bacteria was recently reviewed (138, 139), and therefore, it is only briefly summarized here. MALDI-TOF MS-based instruments allow identification of pathogens by ionizing extracted molecules of whole bacterial cultures without a specific protease digestion step (in contrast to liquid chromatography-tandem mass spectrometry [LC-MS/MS]). The bacterial culture is normally treated with a strong solvent, such as formic acid or acetonitrile, and extracted molecules (mainly ribosomal proteins) are analyzed on a MALDI platform for mass spectrometry detection. Laser energy that is being shot on the extracted proteins generates ions that travel through a flight tube within a specific time, which is dependent on their mass-to-charge ratio (*m/z*) (140). Signals (resulting in mass spectral peaks) from mass spectrometry analysis are next compared against a manufacturer-provided database (spectrum library) containing mass spectrometry patterns of designated bacteria that create a unique proteomic fingerprint of well-characterized reference strains. The Clinical and Laboratory Standards Institute (CLSI) recently reported microbiology guideline methods for the identification of cultured microorganisms using MALDI-TOF MS (guideline M58) (141), and commercial MALDI-TOF MS performs well for the identification of salmonellae to the genus level. Both the Bruker Biotyper and bioMérieux Vitek MS systems are MALDI-TOF MS systems that were FDA approved for *in vitro Salmonella* identification. Nonetheless, the currently available systems and databases cannot identify *Salmonella* to the serovar level (142), and the bioMérieux Vitex MS system presents a manufacturer's recommendation for confirmatory testing when *Salmonella* identification is made. Because of the short time to results, comfort of operation, and cost-effectiveness associated with MALDI-TOF identification, many laboratories have begun to apply MALDI-TOF MS analysis, rather than performing additional screening tests (e.g., TSI agar and/or LIA), and MALDI-TOF MS can be performed on colonies picked directly from the selective media (e.g., XLD, SS, and HE media) according to the FDA-approved package insert (143, 144). With the increasing popularity of MALDI-TOF MS-based approaches, improved detectability, and more user-friendly instrumentation and software, MS will surely become more dominant in *Salmonella* identification and typing in the not-far future.

Salmonella Typing Using Next-Generation Sequencing

In recent years, significant improvements in DNA and genome sequencing technologies, sharp decreases in sequencing costs, and the development of tools and bioinformatics pipelines for sequence assembly and comparative genomics analyses have made whole-genome sequencing (WGS) of bacterial genomes an affordable and advantageous approach for *Salmonella* typing. Noticeably, this approach offers the highest genomic resolution possible and discriminatory power for even highly genetically close strains, which is lacking in conventional subtyping methods. In the last 5 years or so, the number of sequenced *Salmonella* genomes has been growing exponentially, largely due to the implementation of high-throughput genomic sequencing by the FDA (145), PulseNet International (146), the Wellcome Trust Sanger Centre (147), and Public Health England (148). As a result, WGS-based analysis is becoming the method of choice by public health laboratories for bacterial pathogen characterization and subtyping for epidemiological studies. This approach is also very useful in studying cases of persistent and recurrent infections (149–154).

The currently used sequencing platforms typically generate millions of short sequences of 100 to 600 bp, known as "reads," for a single bacterial genome. The obtained reads can then be further connected (assembled) into longer sequences known as "contigs" and are annotated using various assembler algorithms and tools, such as Velvet (155), SPAdes (156), or SOAPdenovo2 (157). More and more assembled *Salmonella* genomes are being deposited in the public domains, and the number of *Salmonella* genomes that have been deposited as short-read sets currently exceeds 110,000 (158).

One frequently used approach for exploiting WGS data is the identification of single nucleotide polymorphisms (SNPs) that diverge between even closely related isolates. SNP analysis utilizes nucleotide changes at specific positions in the bacterial genome to discriminate between strains. Often, SNPs (particularly those that are synonymous) are stable in the bacterial genome and therefore can be used to reveal the evolutionary history of a specific bacterium, distinguish between isolates, and trace outbreaks. These are analyzed by comparing the sequence data from isolates of interest against a reference genome, while nucleotides that vary within the data set are identified in a process known as "SNP calling." This approach was successfully applied for studying phylogenetic relationships and disease outbreak investigations (159–161). SNP analyses were also used to determine the phylogenetic relationship between longitudinal isolates from patients who were persistently infected with *S.* Typhimurium (154). Nevertheless, differences in assembly pipelines and references genomes used can pose difficulties in the standardization of SNP-based analyses across laboratories.

Alternative WGS-based approaches are whole-genome multilocus sequence typing (wgMLST) and core genome multilocus sequence typing (cgMLST), which are basically an extended concept of the traditional MLST method (162) allowing genome-wide gene-by-gene comparisons between isolates of interest. While wgMLST uses the entire genome and requires the assembly of a pangenome allele database that contains genes present in all isolates, cgMLST schemes balance between the number of analyzed loci and the highest possible resolution by including common loci harbored by the majority of isolates (usually 95% to 99% of all genes present across the studied population). An important strength of this approach is that both wgMLST and cgMLST schemes are shared among laboratories using online databases and pipelines, such as EnteroBase (http://enterobase.warwick.ac.uk) (158) and PGAdb-builder (http://wgmlstdb.imst.nsysu.edu.tw/) (163). These platforms allow users to upload WGS data to generate wgMLST or cgMLST profiles and to perform cluster analysis of these profiles. This methodology provides high-resolution and reproducible isolate typing and has been successfully implemented for analyzing disease clusters, evolutionary relationships between isolates, and outbreak investigations (164–167).

Importantly, WGS data can be used not only for outbreak investigations and evolutionary relationship studies but also for *Salmonella* serotyping. New Web-based

tools such as SeqSero (www.denglab.info/SeqSero) were developed for determining *in silico Salmonella* serotypes using high-throughput genome sequencing data. SeqSero can use both raw sequencing reads and genome assemblies and is based on databases of *Salmonella* serotype determinants (*rfb* gene cluster and *fliC* and *fljB* alleles) (168). Another Web-based serotyping tool is the *Salmonella in silico* typing resource (SISTR) (https://lfz.corefacility.ca/sistr-app/) for typing and subtyping draft *Salmonella* genome assemblies, which is also able to integrate sequence-based typing analyses for MLST, ribosomal MLST (rMLST), and cgMLST. The SISTR tool was shown to provide serovar prediction using a genoserotyping approach with an accuracy of over 94.6% on a data set comprised of 4,188 finished genomes and WGS draft assemblies (169). Serovar prediction is also offered by EnteroBase (158).

There is no doubt that WGS-based platforms and pipelines will continue to develop, while more and more Web-based pipelines for genome assembly and analysis will become the new gold standards for clinical and public health practices. These techniques will allow a rapid, cost-effective, and consistent approach to the study of phylogenetic relationships, evolutionary origins, population structures, and epidemiological tracing that will eventually replace traditional serotyping and subtyping approaches.

PERSISTENCE, CARRIAGE, REINFECTION, AND RECURRENT INFECTION

The ability of pathogenic bacteria to infect higher organisms and cause infectious disease is one of the most dramatic characteristics of microorganisms. However, the interaction of a host and a disease-causing bacterium can result in different outcomes. Although most *Salmonella* infections are short-term episodes, a certain fraction of *Salmonella* infections in humans can lead to persistent infection or prolonged carriage of this pathogen. A comprehensive understanding of these phenomena is highly important not only from a public health point of view (as these carriers serve as biological reservoirs) but also for a more complete understanding of *Salmonella* biology and its complex interactions with the host. Therefore, from here on, this review focuses on long-term infection by *Salmonella* and comparison of this phenomenon between typhoidal and NTS serovars.

One possible outcome of a host-pathogen interactions is clearance, which happens if the pathogen is unable to colonize (establish an infection) and grow in the host or if the host is able to actively eradicate the invading pathogen by the function of the innate and adaptive immune systems. Nevertheless, if the pathogen is not cleared, it will colonize one or more niches of the host. Pathogen colonization may be transient or maintained for a prolonged period. Long-term sustainability or stable colonization by the pathogen in its host is referred to as "persistence." In this state, the pathogen is not fully cleared, but its growth is kept in check by the host immune system, which restricts its infection to a privileged niche. Pathogen colonization may subsequently inflict a damage or pathological response in the host, and in this case, disease symptoms develop. If pathogen persistence in the host is asymptomatic, this host-pathogen relationship is known as "carriage." In the carriage state, the pathogen persists in the host without causing any apparent signs of active disease; nonetheless, carrier individuals are often contagious, and their carried pathogen can be transmitted to naive hosts, establishing a new infection cycle. Because carriers are asymptomatic, identification of carriers is difficult and poses a potential public health concern.

Carriage of *Salmonella* and other pathogens can be temporary (convalescent) or chronic (permanent). Most confusingly, different studies have determined different periods to distinguish between temporary and chronic carriage of *Salmonella*. Some reports referred to temporary carriage as asymptomatic shedding that lasts up to 3 months (170, 171), while others set the time limit as 12 months (172, 173). Similarly, while chronic or permanent carriage was defined as *Salmonella* excretion for more than 3 months (170, 171), others defined chronic carriage only for cases in which shedding lasts for more than 12 months (172, 173). Hence, the entire field can benefit from more-consistent terminology that should be used not only for *Salmonella* infections but

also for infections by other enteric pathogens. Therefore, I suggest adopting a borderline of 12 months to distinguish between temporary and chronic carriage of enteric pathogens in humans.

Symptomatic persistence of a pathogen may cause continuous expression of pathology or discontinuous disease. Relapsing episodes of a disease originating from a single infection event is called "recurrence," and this state is distinguished from a different scenario of "reinfection," in which the same host is repeatedly infected by the same agent on independent occasions. The above-mentioned scenarios represent different and distinct encounters with the host and are expected to involve different host and pathogen mechanisms. In this review, I focus on long-term infections caused by typhoidal and nontyphoidal serovars that can lead to recurrence (symptomatic disease) and carriage (asymptomatic disease) in the human host.

CARRIAGE RATE AND DURATION OF SHEDDING IN TYPHOIDAL AND NONTYPHOIDAL *SALMONELLA* INFECTIONS

Today, it is widely accepted that asymptomatic *S.* Typhi carriage may develop following acute typhoid fever convalescence or even subsequent to a subclinical infection. Robert Koch, the famous German microbiologist and the founder of modern bacteriology, was the first to articulate the nontrivial idea that a disease might originate from nonsick infected people and applied this notion to typhoid fever epidemiology. Koch endorsed this idea on 28 November 1902 during a scientific meeting in Berlin, Germany, and accurately predicted that the main reservoir of *S.* Typhi is humans who are symptom-free yet secrete live pathogens (174). At about the same time, the concept of *S.* Typhi carriage by asymptomatic individuals was practically demonstrated in reality by two persons, known as "Typhoid Mary" in the United States and "Mr. N" in England. In the early 20th century, Mary Mallon (Typhoid Mary) worked as a cook at different households in the New York City metropolitan area. Mary Mallon was the first known asymptomatic carrier of *S.* Typhi in the United States, and during her work, she infected 51 to 57 people in nine different epidemics (175, 176). One more example of an asymptomatic *S.* Typhi carrier was "Mr. N," who was employed as a milker and cowman in southeast England and was found accountable for a 207-case outbreak of typhoid fever, which peaked in the year 1899 and continued until 1909 (177). Following Koch's concept, J. C. G. Ledingham reported in 1910 that 55 of 482 patients (11%) with acute typhoid fever excreted *S.* Typhi up to 6 weeks after convalescence, while 8 patients (1.6%) continued to excrete the bacteria for more than 3 months (170), emphasizing that *S.* Typhi shedding could extend beyond clinical recovery from symptomatic typhoid fever. Another large study that was published in 1948 and included 417 cases of typhoid fever over a period of 28 years found that 11.9% of the patients became temporary carriers, while 3.5% were chronic carriers of *S.* Typhi (171). A later comprehensive work showed very similar results and reported that up to 10% of convalescing untreated *S.* Typhi-infected patients continued to shed *S.* Typhi bacilli in the feces or urine up to 3 months after the resolution of acute illness. A clinically significant percentage (1 to 4%) of individuals infected with *S.* Typhi became asymptomatic chronic carriers, who kept shedding 10^4 to 10^{10} *S.* Typhi bacteria per g of stool for more than 12 months (172, 178). Some chronic carriers will continue to periodically excrete high levels of these bacteria in their stools and urine for decades, in the absence of clinical symptoms (171, 178). Interestingly, up to 25% of *S.* Typhi carriers do not report any stage of acute disease and possibly have developed only subclinical infection by *S.* Typhi (27, 170, 178).

Since *S.* Typhi is a human-restricted pathogen, these chronic carriers are thought to serve as its natural reservoir, necessary for the existence of this pathogen in the human population (179), and therefore, the persistence phenotype is of special concern from a public health point of view.

Asymptomatic carriage of *S.* Paratyphi A and B is less well described than that of *S.* Typhi, but a recent study that was conducted in Nepal suggested a similar incidence of persistence for *Salmonella* serovars Typhi and Paratyphi A in regions of endemicity

(180). Vogelsang and Boe, who monitored 1,055 paratyphoid B patients for over 28 years, found similar rates of carriage as for *S.* Typhi-infected patients, with about 11% and 2% of patients being temporary and chronic carriers, respectively (171). As in the case of *S.* Typhi infections, the role in outbreaks of food handlers who are chronic *S.* Paratyphi A carriers is dominant. This was recently demonstrated in an unusual outbreak of 37 cases of paratyphoid A in young Israeli travelers who visited Nepal. The source of the outbreak was most likely a Jewish New Year dinner that the travelers all consumed at the same establishment in the city of Pokhara, which was probably contaminated by one of the food handlers who worked in the venue (181, 182).

In contrast to the well-documented incidents of persistent *S.* Typhi infections, long-term NTS infections are far less well studied, and the occurrence of NTS carriers in the general population is rather vague. Infections by NTS in healthy humans usually produce only a brief symptomatic illness. Thus, to define a persistent NTS infection, one must first determine the duration of a "normal" short-term NTS infection. This issue was addressed by Sirinavin and colleagues, who reported in 2003 that 98.8% (254 of 257) of adults infected with NTS had eradicated their initial NTS infection within 12 days from the first positive stool sample (183, 184). Eradication of *S.* Typhimurium within 12 days postexposure was also reported in 18 asymptomatic carriers in a *S.* Typhimurium food-associated outbreak in Kanagawa, Japan, while clearance of the pathogen within 25 days was reported in symptomatic but antibiotic-untreated patients (185). Similar results were obtained in a survey that included 3,000 asymptomatic adults from 16 hotels in Bangkok, Thailand. This study identified 142 persons (4.7%) who were infected and shed NTS bacilli in their stool, which were cleared within 21 to 28 days (183). However, these periods were shorter than those in a previous study that reported that the median duration of NTS shedding was about 5 weeks (172). These discrepancies most likely reflect variations in the infecting serotype, age, and sex of the studied population and antibiotic treatment, which were all shown to affect the shedding period (see below).

In a cross-sectional study that involved 1,002 healthy schoolchildren in India, the NTS carriage rate from a single stool sample was found to be 1% (186). A meta-analysis of 32 studies that included 2,814 patients from 10 countries who were infected with NTS also found that persistent excretion beyond 12 months occurred in $<$1% of the subjects (172). In a recent retrospective study that included 48,345 culture-confirmed NTS cases that occurred in Israel between 1995 and 2012, we found that at least 2.2% of all reported cases of salmonellosis were long-term infections that persisted 30 days or more, with a median persistence period of 55 days (154). Since the persistence period was determined using the documented time intervals between at least two positive cultures, the actual time of persistence is expected to be even longer. Importantly, in contrast to *S.* Typhi, lifelong persistence of NTS was not found, and the maximal identified time of NTS carriage was 8.3 years. The majority (93%) of the persistently infected patients were immunocompetent, and 65% were symptomatic with relapsing diarrhea, suggesting a persistent manifestation, distinct from the known asymptomatic carriage of typhoidal *Salmonella* (154).

HOST AND PATHOGEN FACTORS AFFECTING *SALMONELLA* PERSISTENCE

Multiple studies have shown that both the age and the sex of patients play a critical role in *S.* Typhi persistence (171, 178). In a 27-year-long study that included 32 chronic carriers, it was demonstrated that 75% and 90% of all *S.* Typhi and *S.* Paratyphi B chronic carriers, respectively, were women (171). Ames and Robins reported that the overall prevalence of *S.* Typhi carriage in women (3.8%) is almost 2-fold higher than that in men (2.1%) and that while only 0.3% of patients younger than 20 years old developed *S.* Typhi carriage, this percentage increased to 10% in the 50- to 59-year-old group (187).

As with *S.* Typhi, the rate of carriage of NTS was reported to be higher (up to 69%) in women than in men in multiple studies (172, 173, 188). Age was also found to play a crucial role in the duration of NTS shedding. In patients younger than 5 years old, the

median duration of shedding was 7 weeks, whereas in older children and adults, the median period of excretion was much shorter and lasted for only 3 to 4 weeks (172). Many later studies confirmed that among children and infants, the excretion duration is significantly prolonged (189–191). Moreover, children are likely to shed higher numbers of NTS cells, which can reach 10^6 to 10^7 organisms per g of feces, while adults tend to excrete much lower numbers of 10^2 to 10^3 salmonellae per g of stool (192).

In contrast to the extended shedding time in children (temporary carriers), chronic carriage of both typhoidal and nontyphoidal serovars is normally associated with adulthood, and individuals aged 50 years and older are more likely to become long-term carriers (171, 173, 178, 187, 193). A conceivable explanation for this discrepancy is that while salmonellae are cleared more slowly from the digestive system of young children, long-term persistence takes place in the gallbladder, possibly on gallstones, which are rare in children (194, 195). Indeed, gallbladder abnormalities and especially cholelithiasis (gallstones) were found to be significant risk factors for carriage of typhoidal serovars (171, 194, 196) and possibly of NTS (173). The prevalence of gallstones has been found to be up to 90% in *S.* Typhi carriers (197), and in an area of endemicity, 3.5% of patients who were subjected to cholecystectomy were infected with *S.* Typhi in their gallbladder (180). Another possible explanation for the longer temporary persistence in children younger than 5 years old might be the less-well-developed microbiota characterizing children in comparison to the more complex microflora in the adult intestines.

Interestingly, when it boils down to persistence, not all NTS are alike. Several independent studies have shown that various NTS may result in different durations of persistence (154, 185, 191). *S.* Typhimurium, for example, was found to cause a significantly shorter excretion period than other NTS (198), and in a meta-analysis, it was shown that the median duration of *S.* Typhimurium shedding (for 1,124 patients in 14 studies) was 7 days shorter than the duration of shedding in patients who were infected with other serovars (1,294 subjects from 16 studies) (172). Moreover, we recently showed that certain NTS tend to cause persistent infections more frequently than others and that the prevalence of *Salmonella* serovars Mbandaka, Bredeney, Infantis, and Virchow among cases of persistent salmonellosis in Israel was significantly higher than their prevalence in sporadic short-term infections. In contrast, the frequency of persistence of *Salmonella* serovars Typhimurium and Enteritis was found to be lower than expected, based on their occurrence among sporadic infections (154).

An additional factor that is expected to play a major role in *Salmonella* persistence is antibiotic therapy. Longer persistence of NTS infection was found in patients treated with antibiotics, including ampicillin, chloramphenicol, neomycin, and streptomycin (185, 188, 199–201). Noteworthy, a similar effect of antibiotic treatment was also demonstrated in the mouse model. Antibiotic treatment prior to mouse infection enhanced the occurrence of gut inflammation and increased gastrointestinal bacterial loads in both the chronic model (202) and the acute infection mouse model (203). Moreover, the administration of a single dose of antibiotic to chronically infected mice that were not shedding detectable amounts of *Salmonella* bacteria reactivated *Salmonella* excretion and induced a "supershedder" phenotype, in which a subset of infected mice shed high numbers of bacteria ($>10^8$ CFU/g) in their feces (202). These observations in both humans and mice demonstrate that dysbiosis of the intestinal microbiota due to antibiotic therapy is a significant risk factor for persistence and that the intestinal microbiota is an important player in modulating persistent *Salmonella* infections.

The protective activity of the gut microbiota against pathogen colonization is expected to occur by multiple mechanisms, including direct inhibition of gut colonization of pathogens, nutrient depletion, and stimulation of the immune response (204). The contribution of the microbiota to NTS gut colonization was further demonstrated using a mouse model of gastroenteritis (streptomycin-pretreated mice). Mice that harbored a low-complexity gut flora failed to clear *S.* Typhimurium infection from the gut lumen, and clearance of the pathogen was reached only by transferring a normal complex microflora to the microbiota-deficient mice (203). These results suggested

that, besides inhibition of pathogen colonization, the microbiota may facilitate pathogen clearance.

SITE OF PERSISTENCE BY TYPHOIDAL AND NONTYPHOIDAL *SALMONELLA* SEROVARS

Long-term persistence and shedding of salmonellae in the stool and urine require the establishment of extraintestinal infection in a permissive niche. Dissemination of invasive *Salmonella* bacteria enables the pathogen to access the gallbladder, and the biliary tract and the gallbladder are considered to be the primary persistence sites of *S.* Typhi during chronic carriage (194, 205). As mentioned above, gallstones and chronic inflammation of the gallbladder (cholecystitis) are thought to facilitate persistence in this site, and about 90% of chronically infected carriers were diagnosed with gallstones (197). Several reports have shown that *Salmonella* can adhere to and form a biofilm on cholesterol, which is the main component of gallstones (194, 206). Moreover, individuals with preexisting biliary diseases, including common bile duct stones, are at a higher risk of becoming carriers (194, 196). These observations support the notion that chronic carriage of *S.* Typhi is facilitated by biofilm formation on cholesterol gallstones and that the gallbladder comprises its main persistence site (205). Persistence of *S.* Paratyphi A in the gallbladder has also been reported (180, 207), indicating that persistence in this site is not specific to *S.* Typhi only.

Infection of the biliary tract and the gallbladder is likely to occur through seeding from the liver during the acute phase of the disease, when typhoidal serovars disseminate within macrophages to systemic sites. In a murine model of chronic infection, it was shown that *S.* Typhimurium persistence occurs in the gallbladder lumen and epithelium (84, 86). Using this model, colonization of the gallbladder by *S.* Typhimurium induced a localized inflammatory response mediated by neutrophils that were recruited to the gallbladder lumen (86).

Two possible models could be considered to explain *S.* Typhi shedding from the gallbladder. The first model suggests that the main persistence niches are *Salmonella* biofilms attached to gallstones. Detachment of bacteria from the cholesterol gallstone biofilm promotes entry to the ileum via the common bile duct, followed by excretion in the stool and urine (194). An alternative model proposes that salmonellae can either grow to high numbers extracellularly in the gallbladder lumen or invade the gallbladder epithelium and replicate there without further crossing the lamina propria and mucosa (86). A host caspase-1-dependent response to these epithelium-replicating bacteria results in cell death and apical release of the proinflammatory cytokine IL-18, compromising epithelial integrity. Infected gallbladder epithelial cells disintegrate from the monolayer (epithelial sloughing) and burst, releasing intracellular bacteria into the gallbladder lumen, which from there are secreted to the intestine together with the bile (86, 208).

Nevertheless, the gallbladder is most likely not the sole niche of *S.* Typhi persistence, as suggested by the clinical observation that even though cholecystectomy significantly improves cure rates, it does not always lead to the clearance of the pathogen in human carriers (209). Additional clinical evidence demonstrated that other systemic tissues, including the liver (210), biliary tree, and bone marrow (211), can also serve as persistence sites for typhoidal *Salmonella*. Urinary carriage mainly in patients with abnormalities of the urinary tract and kidneys was also reported as a possible persistence niche (194, 206, 212). Furthermore, chimpanzees infected orally with *S.* Typhi exhibited 50 days of persistence of the bacterium in the mesenteric lymph nodes (MLNs) (213), suggesting another possible site for typhoidal persistence.

In contrast to *S.* Typhi, much less is known about the persistence sites of NTS, and their putative persistence in the gallbladder is not clear. Musher and Rubenstein reported in 1973 that gallstones were documented in about 40% of NTS carriers (173); however, in our recent study, abnormalities in the biliary tract were not found to be associated with prolonged NTS infections (154), suggesting additional NTS persistence sites other than the gallbladder. Indeed, in pigs, the highest level of persistent salmo-

nellae was found in tonsils and jejunal and ileocecal lymph nodes, while no bacteria were isolated from the muscle, spleen, or liver, indicating that lymph nodes are the main persistence site in the porcine host (214, 215).

A murine model used to study *S.* Typhimurium infection suggested that infected hemophagocytic macrophages provide a survival niche during NTS persistence at 8 weeks postinfection (216–218). Hemophagocytic macrophages are phagocytes that ingest nonapoptotic cells of the hematopoietic lineage, and these results propose that splenic hemophagocytes might serve as a permissive niche for NTS persistence. Overall, it seems that while the gallbladder is the main persistence site for typhoidal *Salmonella*, NTS may persist in additional sites, including the lymph nodes, possibly while inhabiting hemophagocytic macrophages.

SALMONELLA PERSISTENCE IN NONHUMAN HOSTS

Salmonella persistence and asymptomatic carriage are common in many animals, including food-producing livestock and domestic pets. These animals are often the sources of NTS food-borne outbreaks. Poultry are some of the most significant environmental reservoirs for *Salmonella enterica* serovars (219). Chickens can be infected either with host-specific *Salmonella* serovars, such as *S.* Pullorum and *S.* Gallinarum, which cause a typhoid-like systemic disease in chickens, or with a wide range of NTS, most of which are carried in the animal's intestinal tract asymptomatically. A recent survey carried out in China showed that the prevalences of *Salmonella* species carriage in poultry were 12.4% in geese, 6.8% in ducks, 10.4% in turkeys, and 9.8% in chickens. Poultry-associated salmonellae belonged to 20 different serovars, and the most prevalent serovars were *S.* Pullorum, *S.* Typhimurium, and *S.* Enteritidis (220). It was shown that *S.* Pullorum persisted within macrophages in the spleen and reproductive tract for over 40 weeks in chickens (221) and that SPI-2 is required for long-term persistence of *S.* Pullorum in chickens (222).

Multiple NTS were found in asymptomatic sheep, cattle, and camels (223). More than 50% of cattle that were recently screened for *Salmonella* in Burkina Faso (West Africa) (224) and more than 20% of cattle screened in northeast Spain (225) were found to be contaminated with diverse NTS, indicating that persistent *Salmonella* infection in livestock is not uncommon and that bovine peripheral lymph nodes are most likely their main site of persistence (226).

Swine carriage of NTS is also significant, as 21.5% of the fecal samples obtained from pigs in Italy (227) and 30.5% of the fecal samples obtained from pigs in the United Kingdom (228) were contaminated with *Salmonella* spp. Tracking of fecal shedding in pigs that were infected with *S.* Typhimurium and *S.* Choleraesuis showed that both serovars were shed in high numbers in the first 2 weeks postinfection, but the numbers then declined, and *Salmonella* excretion became sporadic but continued for up to 5 months following infection.

Salmonella species are also very common in reptiles, and as many as 90% of reptiles contain *Salmonella* as part of their natural intestinal flora. Reptile-borne salmonellae are often excreted in the feces and are responsible for 76,000 to 140,000 salmonellosis cases in the United States annually. *Salmonella* serovars commonly encountered in reptile-related salmonellosis are *Salmonella* serovars Java, Stanley, Marina, Poona, and Pomona, while *Salmonella enterica* subsp. *arizonae* (subsp. IIIa) is often isolated from snakes (229).

Taken together, *Salmonella* persistence is not unique to humans. *Salmonella* spp. can colonize the intestinal and reproductive tracts or be part of the normal flora of many food-producing, wild, and pet animal species, leading to persistence and key environmental reservoirs of these bacteria.

ANIMAL MODELS TO STUDY PERSISTENT SALMONELLOSIS

Traditionally, *Salmonella* virulence has been studied in commonly used mouse backgrounds such as BALB/c or C57BL/6. These strains carry two point mutations in the gene *Nramp1* (natural resistance-associated macrophage protein [also known as

Slc11a1]) which make the mice susceptible to infection by intracellular pathogens, including *Leishmania*, mycobacteria, and *Salmonella* (230, 231). The *Nramp1* gene encodes a metal ion transporter that is expressed mainly in macrophages and dendritic cells, localized in the phagosome, and thought to combat infection by limiting the availability of iron, manganese, and possibly other metals to the intracellular pathogens (232). Infection of *Nramp1*$^{-/-}$ mice with virulent strains of *S.* Typhimurium causes pathological symptoms similar to those of the acute phase of human infection with typhoidal *Salmonella*, and the mice die within 7 to 10 days postinfection. Therefore, these genetic backgrounds are inappropriate for investigation of long-term *Salmonella* infection. Nonetheless, *Salmonella* persistence can be effectively studied in the 129X1/SvJ mouse strain, which harbors a wild-type *Nramp1* allele. Oral infection of this mouse strain with up to 10^8 CFU of *S.* Typhimurium leads to a sublethal systemic infection and bacterial persistence in reticuloendothelial macrophages found in the spleen, liver, gallbladder, and MLNs, accompanied by sporadic shedding in the stool. Chronic infection in this mouse model was elegantly demonstrated by the isolation of *S.* Typhimurium for 1 year following oral infection (217) and was also used to exhibit the role of cholesterol gallstones in *Salmonella* persistence (194). For this purpose, the mice were fed a highly lithogenic diet that led to the development of cholesterol gallstones, which supported biofilm formation following *S.* Typhimurium infection. Moreover, these mice were found to have enhanced gallbladder colonization and much higher fecal shedding than gallstone-free mice (194).

Another interesting animal model for *S.* Typhi carriage was developed in guinea pigs whose gallbladders were infected with *S.* Typhi. This pathogen was recovered from the bile and feces of infected yet asymptomatic animals for up to 5 months postinfection, while antibiotic therapy was unable to eliminate this carrier state (233).

THE HOST IMMUNE RESPONSE TO *SALMONELLA* PERSISTENCE

Different studies have suggested that chronic infection with various bacterial, viral, and parasitic agents involves a cytokine switch from a Th1 to a Th2 immune response (234–237). Although this model might represent an oversimplification of the immune response (238), the establishment of persistence can be schematically described as two distinct phases, characterized by a different balance of Th1/Th2 responses (Fig. 4). In the early phase, during acute infection, a robust Th1 and a low Th2 immune response are mounted to diminish the growth of the invading bacteria. This phase is characterized by the secretion of tumor necrosis factor alpha (TNF-α), IL-12, gamma interferon (IFN-γ), and nitric oxide (NO), required for *Salmonella* growth control by the host (239–241). In the second stage of infection, during convalescence, the balance between Th1 and Th2 responses is skewed toward a Th2-type response with a lower Th1 response, and an equilibrium between the host and the persistent pathogen is developed. This new balance is needed for the maintenance of an immune *status quo* and persistence-permissive conditions. The Th2 cytokine IL-10 inhibits reactive oxygen and nitrogen species production and the secretion of TNF-α and IL-12 by activated macrophages. An increase in IL-10 levels leads to a decrease in IFN-γ levels, compromising bacterial clearance by macrophages (241) and facilitating the survival of intracellular pathogens and maintenance of the carrier state. Evidence for a Th2 inflammatory response bias was also found in chicks persistently infected with *S.* Enteritidis. In these natural-carrier-state birds, the expression of genes transcriptionally linked to the Th1 axis (type I interferon and transforming growth factor β [TGF-β]) was shown to be downregulated, whereas genes linked to the Th2 response (IL-4, IL-5, and IL-13) were induced (242).

Although the persistence phase likely involves a lower Th1 response, IFN-γ is still required to maintain this sensitive equilibrium, as administration of anti-IFN-γ antibodies to persistently *S.* Typhimurium-infected mice led to a reactivation of acute infection in these animals (217), suggesting that some basal level of IFN-γ is still required to keep persistent salmonellae in check.

The late host immune response to *Salmonella* persistence following acute infection may be further divided into two distinct stages. Using a mouse model of persistence

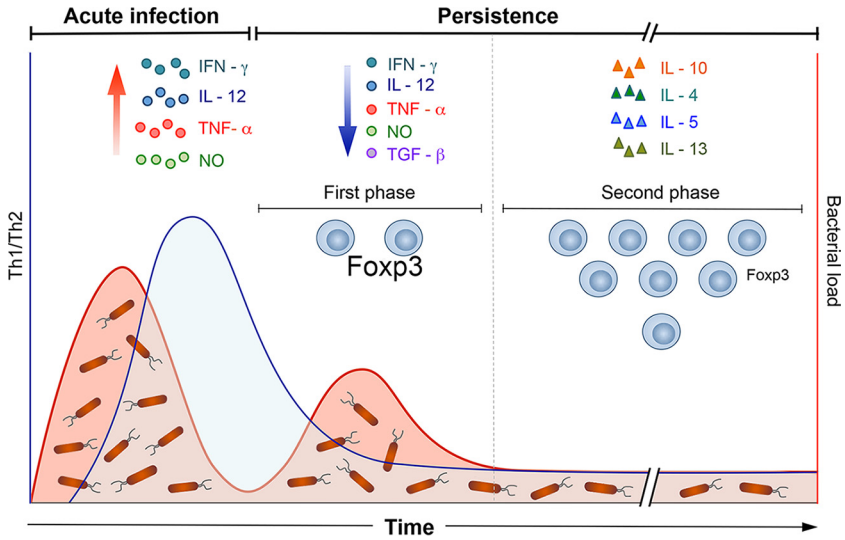

FIG 4 Host response to persistent *Salmonella* infection. The host immune response to acute and persistent infection is illustrated by the Th1-to-Th2 ratio (left *y* axis) and by the levels of the prototypic cytokines secreted in response to the different stages of infection. The bacterial load (shown by the right *y* axis) increases during acute infection. As a result, a strong Th1 response (secretion of IFN-γ, IL-12, TNF-α, and nitric oxide) is elicited, reducing the bacterial burden toward convalescence. During *Salmonella* persistence, a reduced Th1 response but an induced Th2 response occurs with the secretion of IL-10, IL-4, IL-5, and IL-13. At the early stage of persistence, due to low activity of effector T cells (shown as blue double circles), a moderate increase in the *Salmonella* burden occurs. At the second phase of persistence, a robust activation of effector T cells and a reduction in the suppressive potency of FOXP3$^+$ Treg cells lead to decreases in the bacterial loads in privileged niches and an equilibrium between the pathogen and the host throughout persistence.

(129SvJ × C57BL/6 mice), Johanns and colleagues demonstrated that in the first persistent phase, during the first 3 to 4 weeks after infection, the bacterial burden at systemic sites is progressively increasing. At this stage, the activation of protective immune components, such as effector T cells, is delayed, and this phenomenon is associated with increased regulatory T (Treg) cell suppressive potency. In contrast, at later time points of infection, a reduction in the bacterial burden in systemic sites is observed. The reduction in bacterial loads is correlated with a robust activation of effector T cells and a reduction in the suppressive potency of FOXP3$^+$ Treg cells. These results suggest that dynamic regulation of Treg cell suppressive potency affects the course of persistent *Salmonella* infection in this mouse model (243).

Another bacterial phenotype that may contribute to a restrained immune response toward persistence is biofilm. Since adherent bacteria are not accessible for antibody opsonization and can block polymorphonuclear leukocyte signaling, several studies have shown the role of biofilms in skewing the T cell response, facilitating persistence (244).

Although the host immune response to *Salmonella* persistence is primarily based on innate and T cell-mediated activity, mice persistently infected with *Salmonella* were shown to carry high antibody titers (217). Similarly, high antibody titers against the *S.* Typhi Vi antigen were found in human carriers of this pathogen (245, 246), indicating adaptive immunity against *S.* Typhi infection, which may help to keep the persisting bacteria intracellular, inaccessible to the humoral arm of the immune system. A model summarizing the host immune response to persistent infection is illustrated in Fig. 4.

SALMONELLA GENES INVOLVED IN PERSISTENCE

A large array of *Salmonella* genes have been found to contribute to prolonged infection and maintenance of persistence in various animal models. The involvement of multiple genes highlights the complexity and the multifactorial nature of *Salmonella* persistence, which needs to adjust to various environmental conditions in the host,

complex immune responses, and the microbiota population. In this section, I emphasize the main *Salmonella* factors that were shown to be involved in persistence.

One of the largest groups of genes shown to contribute to persistence is fimbrial and nonfimbrial adhesins, used for attachment and colonization. Different fimbrial operons (*lpf*, *bcf*, *stb*, *stc*, *std*, and *sth*) were found to facilitate long-term intestinal carriage of *S*. Typhimurium in genetically resistant mice (247), while a different set of operons, including *stc*, *bcf*, and *sth*, was found to contribute to long-term systemic infection (248). Two genes carried by the *S*. Typhimurium pathogenicity island CS54 have been attributed a role in *Salmonella* persistence and fecal shedding. A *shdA* (encoding a fibronectin binding factor) mutant strain was demonstrated to be impaired in persistence in the gastrointestinal tract and shedding in the mouse model (249). In addition, a *ratB* (adjacent to *shdA*) mutant strain was also found to be defective in colonization in the cecum and in fecal shedding in mice (250). Similarly, the autotransporter adhesin MisL encoded on SPI-3, which functions as a fibronectin and collagen IV binding factor, was also found to contribute to *S*. Typhimurium intestinal persistence in the mouse model. A *misL* mutant strain was shown to be shed in significantly lower numbers than the wild type and was impaired in mouse cecum colonization (251). Besides adhesins, LPS, such as the O12 antigen, was also found to be critical for persistence and fecal shedding of *S*. Typhimurium in the murine model (252).

A recent gene expression study in pigs identified 37 *Salmonella* Typhimurium genes that were expressed in the tonsils, ileum, and ileocecal lymph nodes 3 weeks after oral inoculation. This study found that the *efp* and *rpoZ* genes, which are involved in protein biosynthesis, were specifically expressed in the ileocecal lymph nodes during *Salmonella* persistence. Additionally, STM4067, *dnaK*, and *aroK* were identified as factors potentially contributing to persistence (20 days postinfection) in pigs (253).

Bile is a lipid-rich fluid with detergent-like properties produced in the liver and concentrated inside the gallbladder. Since the gallbladder is one of the main persistence sites of *S*. Typhi, *Salmonella* resistance to bile is expected to contribute to systemic persistence. Although bile acids are bactericidal, *Salmonella* is resistant to bile (254), and it was shown to trigger several downstream persistence pathways required for biofilm formation on gallstones and cholesterol, including the *Salmonella* O-antigen capsule genes *yihU-yshA* and *yihV-yihW* (206). An immunoscreening technique known as *in vivo*-induced antigen technology (IVIAT) was also applied to identify bacterial biomarkers expressed during *S*. Typhi carriage. This approach was able to successfully identify 13 *S*. Typhi immunogenic antigens, including membrane proteins, lipoproteins, and hemolysin-related proteins, that were found to be immunoreactive using *S*. Typhi carrier sera but were not identified in healthy individuals in Bangladesh, an area where typhoid is endemic. A putative ATP binding protein, YncE, demonstrated the highest immunoreactivity in this screen, suggesting specific induction in chronic *S*. Typhi carriers (255).

Another important bacterial factor for successful persistence is the ability of *Salmonella* to import iron, since this metal is required for growth and colonization of host tissues. The main iron transporters of *Salmonella* are the FepBDGC ferric iron transporter and the siderophores enterobactin and salmochelin. All three systems were shown to be required for *Salmonella* to evade nutritional immunity in macrophages and to establish persistent infection in the mouse model (256).

To identify *Salmonella* genes involved in long-term infection by *S*. Typhimurium, Lawley and colleagues used a high-complexity transposon-mutagenized library of *S*. Typhimurium to infect 129X1/SvJ *Salmonella*-resistant mice. This microarray-based negative-selection screen found 118 candidate genes that contribute to *S*. Typhimurium systemic infection at 28 days postinfection. Among these genes, several SPI-1 invasion and translocation effector genes (*sipB*, *sipC*, and *sipD*) were also confirmed in a mouse competition assay to be required for 30-day systemic infection. Similarly, SPI-2 effector genes, including *sseK2*, *sseJ*, *sseI*, and *sopD2*, were also found to be required for infection lasting longer than 2 weeks, while *sseJ* and *sseK2* were also confirmed in a competitive-index experiment at 30 days postinfection (248). Interestingly, SseI was

independently shown to be required for the maintenance of long-term systemic infection in mice by inhibiting the normal migration of primary macrophages and dendritic cells and in this way counteracts the capacity of the host to clear systemic *Salmonella* infection (257). Collectively, these studies indicate the possible involvement of both T3SS-1 and T3SS-2 translocated effectors in persistent infection by *Salmonella* in the mouse model.

Several *Salmonella* factors providing resistance against host antimicrobial peptides, including Mig-14 (258), RcsC and VirK (259), and YdeI (260), were shown to contribute to *Salmonella* persistence in mice at late stages of infection, suggesting that the ability of *Salmonella* to resist the activity of antimicrobial peptides such as polymyxin B and cathelin-related antimicrobial peptide (CRAMP) is an important phenotype for host persistence.

In summary, during persistence, the pathogen experiences a set of environmental conditions and various stresses posed by the host. It is expected that a specific gene expression profile and possibly genetic and phenotypic changes will be the result of such selective pressures, as explained below.

GENETIC AND PHENOTYPIC CHANGES ACQUIRED DURING *SALMONELLA* PERSISTENCE IN HUMANS

One of the most interesting questions about bacterial persistence is whether the pathogen changes during prolonged infection and becomes more adaptive to the persistence lifestyle. Although it may be assumed that the *Salmonella* genome is rather stable, comparative genomics have demonstrated that during persistence, the *Salmonella* genome is dynamic and undergoes chromosomal rearrangements, losses and gains of horizontally acquired genes, and single nucleotide polymorphism (SNP) changes, which may affect the "regular" genome organization and gene expression.

A previous study showed the occurrence of large-scale chromosomal rearrangements mediated by recombination between *rrn* operons in *S.* Typhi during gallbladder persistence, in strains isolated from the same carrier at different time points. This study demonstrated that chromosome rearrangements arise within the human host over time and change the replichore (the chromosomal halves on either side of the *ori-dif* axis) balance of the persisting pathogen (261). Such large-scale rearrangements may cause chromosome asymmetry (making one replichore longer than the other), extend the generation time, and limit pathogen survival outside the host (262).

Recently, we reported a comparison of genome sequences of longitudinal isolates obtained from patients who were persistently infected with *S.* Typhimurium, where the time intervals between related isolates were 33 to 95 days. This analysis identified that the number of SNPs in the core genome ranged from 0 to 10, showing an average substitution rate of 1 SNP per 24 days (154). A similar SNP substitution rate was found in patients who were persistently infected with invasive *S.* Typhimurium pv. ST313 in sub-Saharan Africa (263) or in *S.* Typhimurium associated with short- and long-term carriage, which showed an estimated mutation rate of 1.49×10^{-6} substitutions per site per year (264). Although this is a relatively low SNP rate, we were able to demonstrate that in independent cases, SNPs were found to be nonsynonymous substitutions or nonsense mutations in global virulence regulatory genes, including *dksA*, *rpoS*, *hilD*, *melR*, *rfc*, and *barA*, which may infer a pleotropic change in the transcriptional landscape of persistent *Salmonella* infection (154). Similarly, a nonsense mutation in another global regulator, *flhC*, encoding a master regulator of flagellum biogenesis, was reported by Octavia and colleagues for a 55-day *S.* Typhimurium persistence case (264). Moreover, we demonstrated that recrudescent isolates may harbor different compositions of plasmids and bacteriophages, suggesting loss and/or horizontal acquisition of mobile genetic elements during prolonged infection within the human host (154).

Not surprisingly, SNPs in regulatory genes and changes in the mobile genetic element composition were associated with clinically relevant phenotypic differences.

For example, when we compared the motilities of longitudinal isolates and their abilities to form biofilm, replicate within macrophages, and grow under nutrient-limiting conditions, we observed in multiple cases differences between recurrent isolates obtained from the same patient. Furthermore, gaining a multidrug resistance phenotype during persistence was also documented and linked to the horizontal acquisition of a large plasmid conferring extended-spectrum-beta-lactamase activity (154), suggesting that the acquisition of antibiotic resistance may be an important driving force in the development of persistent NTS infection. These results show that although a relatively low mutation rate occurs in the core genome, SNPs in global regulatory genes and changes in plasmid and prophage compositions may change virulence-associated and resistant phenotypes in a way that could have an adaptive advantage for persistent salmonellae.

TREATMENT OF PERSISTENT *SALMONELLA* INFECTIONS

Antibiotic administration was proven over and over again to be ineffective against the persistence of typhoidal serovars (196, 265), and the most effective treatment option currently known for chronic typhoidal infection is the removal of the gallbladder (cholecystectomy) (209). Administration of antibiotics is also not effective in the eradication of NTS persistence and may actually prolong the duration of NTS shedding (172, 185). A therapeutic approach that included cholecystectomy combined with antibiotic treatment of NTS carriers seemed to be less effective in NTS carrier patients than in typhoidal carriers, as such therapy cured only 5 out of 12 (41%) NTS carriers (173). This therapeutic failure may result from a NTS persistence site, outside the gallbladder, demonstrating that, currently, there is no proven protocol to eradicate persistent NTS infections.

CONCLUSIONS

Salmonella enterica is one of several bacterial pathogens notoriously known to cause persistent infection in the human host. *Helicobacter pylori*, for example, infects more than half of the human population worldwide and can colonize the human gastric mucosa for decades or for the entire life span of the host (266). *Mycobacterium tuberculosis*, which is estimated to infect one-third of the world's population, can establish persistent colonization that may manifest as acute disease, chronic disease, or clinically asymptomatic infection (267). Despite the profound impact on public health globally, there is only limited information regarding pathogen and host factors contributing to persistent infection. Therefore, understanding the biology of persisting pathogens and their complex interactions with the host and microbiota is of special interest. Thus far, only a few studies have addressed the biology of *Salmonella* persistence in humans and the virulence mechanisms required for long-term infection. Similarly, the host immune responses to prolonged infection are still poorly understood. Although typhoidal and nontyphoidal *Salmonella* serovars belong to the same biological species, these groups elicit clinically distinct acute infections but also vary in the nature of the persistent infections that they cause in humans. Table 1 highlights the currently known differences in persistent infections caused by typhoidal and nontyphoidal serovars discussed in this review. Nonetheless, many key aspects about persistent *Salmonella* infections are unknown, and several important open questions are waiting to be addressed. What is the site(s) of persistence of NTS in the human host? What is the metabolic state of persistent salmonellae, and are they metabolically active or found in a dormancy-like state during persistence? In this context, Helaine et al. reported a few years ago that many intracellular *Salmonella* bacteria inhabiting bone marrow-derived macrophages and splenocytes do not multiply but appear to be in a dormancy-like state, which could serve as a possible reservoir of persistent bacteria (268). Another set of questions regards the genetic variation among *Salmonella* isolates causing persistent infection. What are the bacterial factors that account for the predisposition of certain NTS for causing long-term infection in humans? Are persisting bacteria subjected to selection forces within the host that lead to a selection of certain

TABLE 1 Comparison of typhoidal and nontyphoidal *Salmonella* persistence in humans

Characteristic	Description	
	Typhoidal persistence	Nontyphoidal persistence
Causative serovars	*S. enterica* serovars Typhi and Paratyphi A, B, and C	Multiple nontyphoidal *S. enterica* serovars, some of which are probably predisposed to causing persistent infection, including *Salmonella* serovars Mbandaka, Bredeney, Infantis, and Virchow
Prevalence and duration of persistence	1–4% of individuals infected with *S.* Typhi may become temporary carriers who shed *S.* Typhi for more than 3 mo; lifelong carriage occurs infrequently	More than 2% of patients infected with NTS become temporary carriers who shed the bacteria for 1 to 12 mo; in rare cases, individuals carry the bacteria for several years; lifelong carriage is currently not known
Clinical manifestation	Asymptomatic infection	In most cases, NTS persistence presents as symptomatic disease with relapsing gastroenteritis
Host risk factors	Older age (>50 yr old), female sex, gallbladder abnormalities, and gallstones	Older age (>50 yr old), female sex, and antibiotic therapy
Site(s) of persistence	The gallstone surface, lumen, or epithelial cells of the gallbladder; the liver, biliary tree, mesenteric lymph nodes, and bone marrow are also possible sites of persistence	Possibly hemophagocytic macrophages in the lymph nodes; carriage in the gallbladder is uncertain for noninvasive NTS
Genetic changes	Chromosomal rearrangements between the *rrn* operons were reported for *S.* Typhi	1.49×10^{-6} substitutions/site/yr and change in the composition of mobile genetic elements were frequently found among recurrent isolates
Possible treatment	Removal of the gallbladder (cholecystectomy)	Currently, there is no effective treatment against persistent NTS; antibiotic therapy is neither recommended nor efficient to treat NTS persistence

subpopulations (including antibiotic resistance acquisition)? Why can't NTS serovars persist for a lifetime in humans, while typhoidal serovars can? Correspondingly, the role of the human immune system in NTS persistence, the changes in the host response to persistent *Salmonella* infection, and a better mechanistic understanding of the equilibrium kept between the host and the pathogen during persistence are all currently unknown and require further investigation.

From a public health standpoint, laboratory diagnosis is particularly challenging for persistent infections in the absence of symptoms. Normally, convalescing patients are not screened for *Salmonella* shedding after the disease has passed, and currently, there are no approved guidelines for routine surveillance of patients who have recovered from *Salmonella* infection. This makes identification of patients with persistent salmonellosis or carriers very difficult. Thus, we still do not know the prevalence of persistent invasive NTS (e.g., ST313), especially in Sub-Saharan Africa. What is the role of NTS carriage in outbreaks, and what is the contribution of human carriers to the environmental reservoir of NTS?

The development of additional animal models, systems biology approaches, and single-cell sequencing technologies, including dual RNA sequencing, which can determine the simultaneous transcriptional landscapes of both the pathogen and the host (269), is expected to advance our future understanding of *Salmonella* persistence and possibly provide novel surveillance and therapeutic approaches against *Salmonella* persistence, which are very limited at present.

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