Update on molecular epidemiology of *Shigella* infection

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Purpose of review
Shigella spp. are important etiologic agents of diarrhea worldwide. This review summarizes the recent findings on the epidemiology, diagnosis, virulence genes, and pathobiology of *Shigella* infection.

Recent findings
Shigella flexneri and *Shigella sonnei* have been identified as the main serogroups circulating in developing and developed countries, respectively. However, a shift in the dominant species from *S. flexneri* to *S. sonnei* has been observed in countries that have experienced recent improvements in socioeconomic conditions. Despite the increasing usage of molecular methods in the diagnosis and virulence characterization of *Shigella* strains, researchers have been unsuccessful in finding a specific target gene for this bacillus. New research has demonstrated the role of proteins whose expressions are temperature-regulated, as well as genes involved in the processes of adhesion, invasion, dissemination, and inflammation, aiding in the clarification of the complex pathobiology of shigellosis.

Summary
Knowledge about the epidemiologic profile of circulating serogroups of *Shigella* and an understanding of its pathobiology as well as of the virulence genes is important for the development of preventive measures and interventions to reduce the worldwide spread of shigellosis.

Keywords
diarrhea, epidemiology, molecular diagnosis, pathobiology, *Shigella* spp.

INTRODUCTION

Recent epidemiological studies have ratified the permanence of the *Shigella* spp. on the list of the major enteropathogens causing childhood diarrhea [1▪] even in areas with improvements in facilities for health, income, and education [2]. Data collected from developing areas have shown that interventions targeting the major enteropathogens, including the *Shigella* spp., could potentially reduce the burden of diarrhea and its sequelae by about 40% during the first 2 years of life [1▪]. The existence of more than 40 serotypes and subtypes of *Shigella* with distinct epidemiological, immunological, pathological, and virulence characteristics hampers the development of efficient preventive strategies, as demonstrated by the lack of a commercially available multivalent vaccine [3–6]. This review summarizes the most recent developments in the worldwide distribution of *Shigella* spp., the latest advances described for their diagnosis, and the virulence genes as well as the pathobiology involved therein.

CLASSIFICATION OF *SHIGELLA* SPP.

Shigella is a gram-negative, nonlactose-fermenting, nonmotile bacillus of the family Enterobacteriaceae. The genus includes four species, also designated as serogroups A–D; these are *Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*, respectively. The first three contain multiple serotypes [15, 6 (15 subtypes), and 18, respectively], whereas serogroup D contains only a single serotype [7].

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KEY POINTS

- *Shigella* infection remains an important etiologic agent of diarrhea worldwide.
- *S. flexneri* is still the major cause of endemic shigellosis in developing countries, but the prevalence of *S. sonnei* has increased in areas with recent socioeconomic advances.
- Despite the increasing usage of molecular methods, researchers have been unsuccessful in finding a specific target gene capable of differentiating EIEC from *Shigella* as well as distinguish between the four species of *Shigella*.
- *Shigella* has a sophisticated process of adhesion and invasion, which is related to the T3SS; it interferes with its host cell cytoplasm machinery and regulates the inflammatory response.

GEOGRAPHICAL DISTRIBUTION OF *SHIGELLA* SPP.

A classical quantification of the worldwide spread of *Shigella* infections showed that *S. flexneri* was the main serogroup found in developing countries (60%), followed by *S. sonnei* (15%). *S. dysenteriae* and *S. boydii* occur at similar frequencies (about 6%) in these areas. In developed countries, however, *S. sonnei* was the most common serogroup (77%), followed by *S. flexneri* (16%), *S. boydii* (2%), and *S. dysenteriae* (1%) [8].

A recent review [9] focusing on the epidemiology of *Shigella* infection confirms this distribution. However, although *S. flexneri* is still the dominant infecting species in developing areas (44–92% prevalence) [10▪▪,11–15], the frequency of *S. sonnei* has increased in countries that have undergone recent improvements in their socioeconomic status [2,16]. Another interesting aspect of the epidemiology of *Shigella* infection is the variation in the distribution of the species within the same country, with different predominant serogroups expressed according to the area and/or population [17▪▪,18–20]. In Manaus, the capital of the Amazonas state in Brazil, there is still a predominance of *S. flexneri* among the circulating strains, whereas in Belo Horizonte, the capital of Minas Gerais, *S. sonnei* accounts for nearly 90% of *Shigella* strains detected in children suffering from diarrhea [17▪▪,18]. According to the Atlas of Human Development in Brazil 2013, Amazonas has a monthly per capita income of approximately US$ 237, and in Minas Gerais, this value is US$ 329 [21]. The same trend was observed in Israel when comparing the Bedouin population living in conditions similar to those seen in developing countries and the Jewish population living in conditions similar to those in developed countries [19,20].

EPIDEMIOLOGY

Shigellosis is a worldwide endemic disease with millions of infections reported every year. The low infectious dose of the bacterium, the direct person-to-person transmission, contaminated food and water transmission, and its low susceptibility to stomach acids could help explain the wide spread of this disease [22]. The contribution of vectors, such as houseflies, in the transmission of *Shigella* bacteria has also been assessed and added among the causes for spread of this bacterium [23].

Data published in the late 1990s revealed the occurrence of nearly 165 million cases and 1.1 million deaths associated with *Shigella* infection annually. Although the microorganism affects individuals of all ages regardless of socioeconomic status, the vast majority of cases and deaths occur in children under 5 years of age living in less developed areas [8]. Despite the magnitude of these numbers, it is estimated that the burden of the disease is higher, as each reported case of shigellosis accounts for about 25 cases in the community [19].

The role of *Shigella* spp. as enteropathogens causing childhood diarrhea in developing areas is probably the most recognized context in the epidemiology of shigellosis. A recently published 3-year, multicenter, prospective, matched case-control study [1▪] of moderate-to-severe diarrhea evaluated the cause and impact of diarrheal diseases in over 22 000 children from seven different Asian and African countries. The study [1▪] confirmed the permanence of *Shigella* spp. as important etiologic agents of childhood diarrhea. Recent case-control [24–26] and cross-sectional [27–31] studies conducted on children living in areas with poor resources have reported a considerable percentage of *Shigella* strains among bacterial enteropathogens isolated from children with diarrhea.

In developed areas, *Shigella* spp. have been isolated especially during outbreaks of gastroenteritis following ingestion of contaminated food and water [32–35], mainly during events requiring mass gathering of locals [36,37]. Shigellosis is also common among travel-related cases of diarrhea in such areas [38].

Systemic complications due to *Shigella* infection are not frequently observed; however, shigellemia has been detected in immunocompromised patients [39,40]. In addition, the genus has been associated with chronic health sequelae, such as reactive arthritis [41], irritable bowel syndrome, functional constipation, and gastroesophageal reflux disease [42].

Shigellosis is a seasonal affliction [43], and symptoms can range from mild watery diarrhea to severe inflammatory bacillary dysentery [44]. The infecting species is one of the major factors involved in the clinical outcome of the disease [12]. Although all four *Shigella* species may affect both intestinal and extraintestinal manifestations, *S. dysenteriae* serotype 1 (SD1), the producer of Shiga toxin, and *S. flexneri* to a lesser extent are responsible for the most severe forms of the disease. Although *S. flexneri* is the major cause of endemic shigellosis in developing countries, SD1 is responsible for deadly epidemic outbreaks in overcrowded areas with poor sanitation and inadequate hygiene practices. *S. sonnei* and *S. boydii* usually cause milder diarrhea, ranging from watery to bloody [7,12,44].

DIAGNOSIS OF *SHIGELLA* INFECTIONS

Definitive diagnosis of shigellosis is dependent on the identification of *Shigella* from the stool samples. Classical microbiological culture procedures or those associated with modern equipment that improves its performance are still indispensable in routine practice [45–47]. On the other hand, several researchers have developed and assessed molecular tests for the diagnosis of the most prevalent enteropathogens, including *Shigella*, many of which quantify the pathogen load in addition to identifying them [48–56].

The genus *Shigella* has been considered different from *Escherichia coli* since the research conducted by Ewin in the late 1940s when all four species of the *Shigella* genus were described. However, the genetic similarities between *Shigella* spp. and the enteroinvasive *E. coli* (EIEC) have made the specific molecular diagnosis of the *Shigella* group a challenge, especially if the test sample is human stool. *Shigella*-EIEC forms one single pathovar of *E. coli*, in which the same species or serotypes are not necessarily related to each other phylogenetically [57]. According to a whole-genome-based, alignment-free, and parameter-free CVTree approach, all four *Shigella* species are distinct from *E. coli* strains though they both form sister species [58]. Considering the differences between *Shigella* and EIEC with respect to de-novo nicotinic acid synthesis, however, EIEC has been suggested to reflect an earlier stage of the pathotype adaptation process undergone by *Shigella* [59].

These two bacteria can be distinguished by serotyping, but several studies [48–56] have attempted molecular diagnosis without separating the two. Only one tried to differentiate the species with a combination of molecular and serologic methods [60], whereas another study [61] pursued the task of differentiating the bacteria using only molecular DNA techniques. However, the latter research did not comprise the requisite number of test strains, making it difficult to achieve comprehensive differentiation. This goal might be achieved when the whole genomes of both bacteria are sequenced to aid in comparison. In this way, conserved regions of the housekeeping genes could be used to design differentiated probes. To date, the results of the whole genome analysis of the different *Shigella* species have been compared with each other [58,62–65] or with noninvasive *E. coli* [66]. However, so far, this comparison has not been performed against EIEC strains.

The most commonly used target gene for molecular diagnosis of *Shigella* from clinical samples by PCR assays is invasion plasmid antigen H (*ipaH*). This multicopy gene (4–10 copies) is found in chromosomes as well as in invasion plasmids. Its importance in shigellosis has been investigated, and some recent works [67,68] have related its encoded protein as being an immune modulator and host inflammatory. Regardless of the main effect of *ipaH* and its genetic location, this gene is exclusive for *Shigella* and EIEC [57]. As the cell invasive behavior and gut pathobiological effects of these bacteria are indistinguishable, several clinical studies [53] have chosen *ipaH* as the diagnostic target gene.

Several target genes other than *ipaH* have also been used [69–72]; however, none of these studies tried to utilize genes that could diagnose only the *Shigella* species and to differentiate between its four species. However, this topic has been investigated in two recently published articles [73▪▪,74▪▪]. Despite this, there is a lack of more specific and quantitative assays that could finally distinguish EIEC from *Shigella* as well as distinguish between the four species of *Shigella*.

PATHOBIOLOGY

Shigella must be resistant to the stomach acid environment in order to reach its site of infection. Acid resistance is an important virulence trait of the *Shigella* species, and lipopolysaccharide plays a major role in this feature [75] as also the Arg-dependent acid-resistance pathway [76].

Another aspect apart from acidity that must be overcome is temperature. Many bacteria detect host temperature of 37°C and become virulent by turning on metabolic genes in order to survive. A recent study [77], the result of which was emphasized by a review [78], described the role of the RNA thermometer. This describes certain sequences in the mRNA of heat shock proteins and virulence factors, which block protein synthesis at low temperatures

and reverse this blockage when the temperature augments. This process has been evaluated previously with the iron uptake system. The intracellular replication of *Shigella* is iron-dependent, and the outer membrane protein ShuA controls its absorption. According to the authors [77], the gene that encodes ShuA is thermosensitive to RNA thermometers and overexpresses this protein at higher temperatures.

The impact of temperature on the growth of *S. flexneri* [79] and the expression of *Shigella* protein, especially at the elevated temperatures caused during febrile episodes common in shigellosis [80], have also been assessed, displaying the influence of this factor in the upregulation and downregulation of specific proteins.

Apart from adapting to environmental disturbances, *Shigella* spp. must also interfere with the mucin defense barrier in the gut in order to successfully infect the host. This barrier maintains homeostasis and protects epithelial intestinal cells against enteric pathogens. According to Sperandio *et al.* [81], regardless of the mechanism of intestinal mucosa invasion [82▪▪], *Shigella* interferes with the extracellular secretion of gel-forming mucins by promoting their trapping and accumulation at the surface of infected cells, thus increasing the access of bacteria to the cell surface. In addition, Sperandio *et al.* [81] have also found that the wild type of *S. flexneri* impairs the gene transcription of the mucins MUC4, MUC5AC, and MUC15. In contrast, a strain with the mutated form of protein MxiD, a component of the type III secretion system (T3SS), which is required for invasion functionality, induced upregulation of these proteins. Beyond this, *S. flexneri* has also been known to modify the glycosylation pattern of mucin proteins through a T3SS-dependent process.

Following infection, the ability to adhere to the host cell is one of the crucial activities for intracellular pathogens. However, few studies have tried to investigate the molecular mechanism of adhesion of *Shigella*; and until recently, no adhesins had been described. Faherty *et al.* [83] reported the role of the factors OspE1 and OspE2, proteins that were overexpressed on the outer membrane, following the contact of bacterium with bile salts in the gut lumen. Another protein that was newly described as an adhesin was IcsA [84▪]. The polarly distributed IcsA was customarily related only for cell-to-cell spreading [85,86], and icsP (outer membrane protease) site specificity [87]. The mechanism of action of IcsA involves the neural Wiskott–Aldrich syndrome protein activation. The activation of neural Wiskott–Aldrich syndrome protein stimulates the Arp2/3 complex, which initiates de-novo actin nucleation and polymerization to form F-actin comet tails and allows bacterial cell-to-cell spreading [88]. However, some authors have shown that IcsA is activated by bile salts and that it functions as an adhesin. This study [84▪] also found an IcsA insertion mutant that caused an attenuated form of shigellosis, proving that its adhesion feature is necessary for *S. flexneri* infection. We believe that in the near future, proteins other than the ones already mentioned would be described as adhesins, as its field has not been explored as thoroughly as the invasion process.

Several recent reviews [67,82▪▪,89▪,90–93] have focused on the *Shigella* pathogenesis mechanism. *Shigella* spp. initially infect colonic cells through entry into the microfold cells. However, some studies [94] have suggested that these bacteria can also use epithelial cells as a portal of entry to the lamina propria. Inside the microfold cells, the bacterium is transcytosed toward a vacuole until it gets to invade resident macrophages and dendritic cells. The vacuole membrane is then disrupted into the cytoplasm, signaling the start of bacterial replication, associated with a massive inflammatory response, which culminates in macrophage death. Released by dead macrophages, *Shigella* invades the surrounding epithelium through its basolateral surface. Once inside, its vacuole is disrupted again, and dissemination and replication processes are repeated. In order to spread to neighboring cells, it induces actin polymerization at one pole of the bacterium and forms a comet tail, a motile structure that aids in propagation. In addition, *Shigella* alters host inflammatory response.

Shigella is the prototype of a perfect pathogen. After its sophisticated process of invasion related to the T3SS, it interferes with its host cell cytoplasm machinery in order to survive during infection [75].

Most of the regulatory processes in *Shigella* pathogenesis originate from a plasmid. Among many proteins, this virulence plasmid encodes T3SS, in which its protein structure encompasses the export apparatus, a membrane-embedded basal structure, a needle that protrudes out of the bacterium surface, a tip complex that caps the needle, and the translocon that is mounted between the tip complex and the gut cell [89▪].

The T3SS is responsible for secreting approximately 30 different effectors, but it is not always on acting mode. This apparatus is activated when the bacterium enters the host and when it needs to spread from cell-to-cell. However, during cytoplasmic replication, the T3SS is turned off. The on and off switch is regulated by the protein complex encompassed by membrane expression of Ipa antigen (MxiE) and its cytoplasmatic chaperone IpgC [95].

MxiE-IpgC is also related to the bacterial effector secretion mechanism. Upon contact of the host cell by the tip complex formed by invasion plasmid antigens B (IpaB) and D (IpaD), a signal is generated toward the basal structure through the needle, in order to activate bacteria effector secretion. The protein MxiC, also called the gatekeeper, is considered the signal receiver, but the component that could link it to the needle was unknown. Cherradi *et al.* [96▪] showed that the missing component was the inner rod protein, MxiI. The proposed idea was that, before any host contact, the complex MxiC-MxiI would form a plug-impeding effector secretion. When T3SS is activated by host contact, MxiC detaches from MxiI and interacts with the chaperone IpgC, opening the gate and allowing cytoplasmic translocator (IpaB and IpaC) secretion. The following step requires MxiC and OspD1 secretion. Free from MxiC, IpgC would bind the transcriptional activator MxiE, which finally stimulates transcription, production, and secretion of the later effectors. The protein that recruits IpgC is coupled to the translocators and presents this complex to the ATPase surface presentation of Ipa antigen 47 (Spa47), forming the soluble Spa13. This function was named as export gate-activator switch. However, Spa13 also acts as a chaperone [97].

Along with MxiC–MxiI interaction, IpaD-IpaB also has an important role in controlling secretion in both earlier and later stages of infection. Site-directed mutagenesis studies [98–100] have been performed to evaluate clinical IpaD variants. This research has demonstrated that the colocalization of IpaD and IpaB regulates the secretion of OspD1 (antiactivator protein) and other late effectors; however, some strains performed less secretory activities [98]. In addition, changes in specific IpaD amino acids strengthened the strains, increasing the levels of translocator secretion, pore formation, and cell entry [100]. All these data reinforce the need for more molecular studies to interpret the effect of natural mutants on the clinical manifestations of *Shigella* infection.

The ability of *Shigella* species to cause inflammatory response through macrophage invasion is well characterized. However, an opposite conflicting process occurs inside epithelial cells. Although macrophages undergo death by apoptosis, the infecting *Shigella* species secretes many effectors that subdue the inflammatory response, in order to keep the epithelial cell alive, and maintain replication and cell-to-cell spreading. This process, as well as the mechanism of each secreted effector related to inflammatory subjugation, has been described in a recent published review [82▪▪].

Shigella species are also known for their production of toxins. The best described is the Shiga toxin 1 from SD1 [92,101]. In addition, five toxins produced by *S. flexneri* have also been studied; *Shigella* enteroxins 1 and 2 (ShET1 and ShET2), protein involved in colonization (Pic), *Shigella* IgA-like protease homolog(SigA), and *Shigella* extracellular protease (SepA). ShET1 and Pic are chromosome-encoded toxins. ShET2 is encoded by *sen* or *ospD3* and it requires T3SS for secretion. However, SepA and SigA are plasmid-encoded toxins, which are not dependent on T3SS for secretion. Further research must be conducted to understand the role of the serine protease autotransporter of the Enterobacteriaceae family of toxins (Pic, SepA, and SigA) in the pathogenesis of *S. flexneri*. However, the contribution of Pic and SepA to the enterotoxic activity of this species has already been confirmed [83].

Although the role of many structured and secreted proteins has been investigated in the pathobiology of shigellosis, few studies have tried to identify the molecular profile of their virulence genes and to associate their presence with clinical symptoms. Some works [18,102,103] have described the molecular profile from species but do not associate them with clinical manifestations. On the other hand, ShET1B subunit was associated with dehydration and ShET2 was related to intestinal injury [17▪▪].

CONCLUSION

The latest data on diarrhea cause show that shigellosis is still a public health problem worldwide. *S. flexneri* remains the most prevalent in developing areas, but evidence has suggested a shift in the dominant infecting species to *S. sonnei* in areas with recent socioeconomic advances. The impact of this change on the pathobiology and outcome of the *Shigella* infection needs further investigation. Despite the various techniques developed and/or adapted for bacterial investigation in the past few years, use of an alternative diagnostic gene, which allows for molecular differentiation between *Shigella* species and EIEC, still represents a challenge. Although the pathobiology of *Shigella* infection has been described in detail, some areas still need further probing, such as the adhesion process and virulence gene coregulation.

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Conflicts of interest

The authors declare no financial conflict in interest, or affiliation with any institution, organization, or company relating to the preparation of the manuscript.

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- ■■ of outstanding interest

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