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A Brief History of *Shigella*

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Abstract

The history of *Shigella*, the causative agent of bacillary dysentery, is a long and fascinating one. This brief historical account starts with descriptions of the disease and its impact on human health from ancient time to the present. Our story of the bacterium starts just before the identification of the dysentery bacillus by Kiyoshi Shiga in 1898 and follows the scientific discoveries and principal scientists who contributed to the elucidation of *Shigella* pathogenesis in the first 100 years. Over the past century, *Shigella* has proved to be an outstanding model of an invasive bacterial pathogen and has served as a paradigm for the study of other bacterial pathogens. In addition to invasion of epithelial cells, some of those shared virulence traits include toxin production, multiple-antibiotic resistance, virulence genes encoded on plasmids and bacteriophages, global regulation of virulence genes, pathogenicity islands, intracellular motility, remodeling of host cytoskeleton, inflammation/polymorphonuclear leukocyte signaling, apoptosis induction/inhibition, and “black holes” and antivirulence genes. While there is still much to learn from studying *Shigella* pathogenesis, what we have learned so far has also contributed greatly to our broader understanding of bacterial pathogenesis.

INTRODUCTION

Shi.gel' la. M.L. dim. *-ella* ending; M.L. fem. n. *Shigella* named after K. Shiga, the Japanese bacteriologist who first discovered the dysentery bacillus (1)

...soldiers have rarely won wars. They more often wipe up after the barrage of epidemics. And typhus, with its brothers and sisters – plague, cholera, typhoid, dysentery – has decided more campaigns than Caesar, Hannibal, Napoleon, and

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all the inspector generals of history. The epidemics get the blame for defeat, the generals the credit for victory. It ought to be the other way around.

H. Zinsser, in *Rats, Lice and History*, 1935 (2)

It is appropriate to begin this brief history of *Shigella* (Fig. 1) by reminding our readers of the prominent role that bacillary dysentery, the disease caused by *Shigella*, played, and continues to play, in military operations and troop deployments. Indeed, its importance is reflected in the significant number of seminal discoveries in the field of *Shigella* pathogenesis that came from scientists working at the Walter Reed Army Institute of Research and the continued efforts of the U.S. Army in pursuit of a vaccine against shigellosis.

However, the history of *Shigella* is deeper and richer than just the disease's influence on military operations. The impact of dysentery on public health continues to be felt more than 100 years after Shiga's discovery of the bacterial agent. The importance of *Shigella* in medical history is singular. The history of *Shigella* research also provides a timeline of the advances in techniques and knowledge of bacterial infectious diseases and the evolving paradigms in thinking about these pathogens over the past century. The close genetic relatedness of *Shigella* with *Escherichia coli* allowed the early application of the genetic tools used in *E. coli* to study pathogenic mechanisms of *Shigella*. The themes in *Shigella* pathogenesis are echoed by a multitude of other bacterial pathogens. The discovery of these shared pathogenic mechanisms has accelerated our knowledge and confirmed one of the fundamental concepts of evolution: when an organism finds a path to survival/adaptation in a new environment, natural selection will drive other organisms along the same or a similar path.

Because researchers today tend to cite reviews and do not generally read the primary literature, we decided to focus this review on the early history of *Shigella* and the personalities involved in major discoveries. This review begins with a brief description of the disease caused by *Shigella* and then covers the discovery of the bacterium's role in disease. We then discuss the impact of dysentery on human health over the years. The remainder of the review focuses on the study of *Shigella* the organism, its importance in medical history, and the significant discoveries of the late 20th to early 21st century as powerful molecular biology and genetics tools were applied to the study of *Shigella* pathogenesis. It goes without saying that this overview of the history of *Shigella* is not comprehensive. We refer the readers to other reviews on the different aspects of *Shigella* within the text of the review.

DYSENTERY – CLINICAL SYMPTOMS AND TRANSMISSION

Bacillary dysentery (shigellosis) as a disease has been recognized since biblical times. Hippocrates introduced the term dysentery (*δυσεντερία*) (bowel trouble) to describe a malady characterized by the passage of bloody and mucus-containing stools accompanied by straining and tenesmus (3). Today's clinical definition of dysentery is not much different. The disease ranges from mild diarrhea to frank dysentery, which is marked by fever, abdominal pain and cramps, tenesmus, and the passage of bloody, mucoid stools (4).

These symptoms are manifestations of the ability of *Shigella* to invade, and multiply within, epithelial cells of the large intestine, spread to adjacent cells, and ultimately kill the infected cells. Although ulcerative damage to the intestinal epithelium leads to an intense inflammatory response, systemic spread of *Shigella* is rare. In an otherwise healthy individual, the disease is self-limiting and resolves even without antibiotic treatment within 2 weeks. Children (<5 years of age) and the elderly, however, are at greatest risk of negative outcomes from these infections. Hemolytic uremic syndrome (HUS), for Shiga toxin-producing strains, is a possible sequela of infection. Asymptomatic carriage of *Shigella* has been reported, but the actual prevalence and length of carriage has not been established.

Transmission is by the fecal-oral route. There are many examples of transmission via fecally contaminated food and water, but flies, fingers, and fomites are also known means of transmission. One feature that makes *Shigella* such a potent and successful human pathogen is the low infectious dose needed to cause illness. This dose has been calculated to be as few as 100 organisms in volunteer challenge studies (5). While animal models exist for many bacterial pathogens that infect humans, studies on *Shigella* are limited to nonhuman primates, which are the only animals that faithfully reproduce dysentery when challenged by *Shigella*. Thus, although it is closely related to and has evolved from *E. coli*, *Shigella* is a highly host-adapted bacterial pathogen with no known animal or environmental reservoir.

DISCOVERY OF THE DYSENTERY BACILLUS

Four species comprise the genus *Shigella*: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. Each one has shared and unique pathogenic properties, and each has an interesting story behind its discovery (for more in-depth treatments of these discoveries, the reader is referred to two excellent historical accounts by Bensted [6] and by Hardy and Kohler [7]). Before the dysentery bacillus could be discovered, bacillary dysentery had to be differentiated from amoebic dysentery. Although Losch described the amoeba of dysentery in 1875, it was not until the work of Councilman and Lafleur in 1891 and Kruse and Pasquale in 1893 that these were demonstrated to be separate diseases (described in reference 6). The Japanese physician Kiyoshi Shiga (Fig. 2) is generally credited with the discovery of the dysentery bacillus in Japan in 1898 (8) and, indeed, the genus bears his name. However, 10 years earlier, André Chantemesse (a physician-scientist who tended to both Louis Pasteur and Gustave Mahler during the last days of the lives of these two great men) and Fernand Widal (of Widal test fame, a still widely used diagnostic test for typhoid fever), working in Paris, described an organism from postmortems of soldiers returned from French Indochina, i.e., Vietnam (9). While their descriptions were incomplete and not entirely accurate, the original cultures of Chantemesse and Widal later proved to be identical to the organism discovered by Shiga (10). Shiga cultured his bacillus from the feces of a patient during a dysentery epidemic in Japan. Epidemic dysentery was common in Japan in the late 19th century, and the outbreak of 1897 had a mortality rate of 25% and killed over 22,000 people. Shiga himself called dysentery “the most dreaded disease of children from its fulminating course and high mortality” (11). It was during this epidemic that Shiga isolated the same bacillus from the feces of 34 patients and the bowel wall of two postmortem patients. Given the absence of any selective medium at the time, isolation of an

enteric pathogen from feces was quite a feat and Shiga's use of alkaline agar medium surely aided his discovery. In addition to culture of the organism, the innovation that was critical to Shiga's discovery was his use of patient serum to agglutinate the dysentery bacillus cultured from the same patient's stools. This serum also agglutinated similar organisms (in pure culture) isolated from the stools of other dysentery patients, while sera from healthy individuals failed to agglutinate the dysentery bacillus. Ironically, just 2 years earlier in 1896, Widal had published his observations on the sero-diagnosis of typhoid fever and the agglutination of the typhoid bacillus with serum from vaccinated animals (12). Thus, Widal's method was a key to Shiga's success. Shiga's discovery was first published in Japanese and shortly thereafter in an international journal (8, 13). This strain, which Shiga called *Bacillus dysenteriae*, we now call *Shigella dysenteriae* serotype 1.

In 1899, Simon Flexner (whose younger brother Abraham wrote the famous *Flexner Report* that sparked the reform of medical education in the United States and Canada) set sail from Johns Hopkins University in Baltimore for the Philippine Islands, newly annexed by the United States. As a member of the Johns Hopkins Commission in Manila, Flexner was concerned that enteric disease, and dysentery in particular, was the most frequent and important cause of disability and mortality in the U.S. Army. On the way to Manila, the Commission visited Tokyo where Flexner heard of Shiga's work on dysentery. Flexner followed Shiga's methods and cultured two types of organisms from feces and postmortem tissues of dysentery patients among U.S. Army soldiers.

Flexner thought that he had confirmed Shiga's findings by isolating the "type I bacillus." He had, indeed, but time subsequently showed that the two organisms were dissimilar. The new organism came to be known as the Flexner bacillus and is now known as *Shigella flexneri*. Meanwhile, other scientists working in the Philippines and in Germany also isolated Shiga's bacillus from dysentery patients. Lieutenant Richard Strong of the U.S. Army Pathological Laboratory and William Musgrave, an Army medical colleague in Manila, dealt with over 1,300 cases of dysentery in the Philippines and published their findings in 1900 (14). Walther Kruse, investigating a large dysentery outbreak north of Bonn in Germany, also isolated Shiga's bacillus (15). In 1901, Kruse published a study of dysentery from patients in an asylum (16). This study and subsequent ones from Great Britain dispelled the notion that dysentery was only a disease of the tropics and war. In fact, a significant burden of dysentery in England emanated from insane asylums, and the disease there was also referred to as asylum dysentery or asylum colitis.

In the next few years after Shiga's discovery, over a hundred different strains of the dysentery bacillus were reported. Hiss and Russell added some clarity to the field by classifying these strains on the basis of fermentation reactions (17, 18). More strains of the "Flexner group" were isolated from the stools of dysentery patients. Then in 1915, Carl Sonne isolated a lactose late-fermenter (both Shiga's bacillus and Flexner's bacillus were lactose nonfermenters) and found it to be the main causative agent of dysentery in Denmark. This new strain resembled a Flexner group bacillus, but it did not agglutinate in anti-Flexner antiserum, nor did it agglutinate in polyvalent antidysentery antiserum (19). Sonne noted that most cases of dysentery caused by the Sonne bacillus were of a mild diarrheal nature. Felix d'Herelle also found this atypical organism associated with cases of dysentery in

France (20) as did Theodor Thjøtta in Norway (21). Sonne proposed that his new bacillus be called group III to distinguish it from group I (Bacillus of Shiga) and group II (Flexner, Y and Strong types).

In the 1930s, another new species of *Shigella* was discovered. In 1929, Major J.S.K. Boyd of the British Royal Army Medical Corps was working in Bangalore, India on the so-called “inagglutinable Flexner group.” This group consisted of organisms that had the characteristics of the Flexner bacillus but did not agglutinate in any of the antisera prepared against this strain. Boyd’s project resulted in an impressive collection of several thousand cultures from dysentery cases and allowed him to establish the first comprehensive classification of the dysentery bacilli. His ability to differentiate group- and type-specific antigens allowed Boyd to propose six Flexner groups and six Boyd groups (22, 23). His classification has held up to this day with only minor additions of rare subgroups.

The Congress of the International Association of Microbiologists in 1950 recommended that the name *Shigella* be used as the generic name for the group of organisms commonly known as the dysentery bacilli. Subgroup A is composed of *S. dysenteriae*; subgroup B, *S. flexneri*; subgroup C, *S. boydii*; and subgroup D, *S. sonnei*.

Theodor Escherich, the discoverer of *E. coli*, just missed being one of the first to describe *Shigella*. Escherich was searching for the bacterial cause of childhood dysentery and had probably isolated *Shigella* in several cultures but discarded them because they failed to produce gas in carbohydrate-containing media (see description in reference 24).

Five years after Shiga’s discovery of *B. dysenteriae*, Conradi reported that culture extracts of Shiga’s bacillus had neurotoxic effects when injected into rabbits (25). Neisser and Shiga reported similar findings that same year (26). This toxin came to be known as Shiga toxin and was one of the first toxins reported to be made and secreted by a Gram-negative bacterium. Charles Todd made the important contributions of showing that cultures of the Shiga bacillus produced a soluble toxin and that antiserum to this substance was neutralizing. In addition, only the mannite-fermenting *B. dysenteriae* produced the toxin. The nonmannite-nonfermenting strains did not produce the toxin (27, 28). The history of Shiga toxin is, in itself, a fascinating tale and the reader is encouraged to read the account written by Keusch (29).

IMPACT OF SHIGELLOSIS ON HUMAN HEALTH OVER THE YEARS

Dysentery and War

The epidemics of the 19th century took their toll on children and inmates of jails, prisons, and asylums. However, epidemics of dysentery have plagued mankind since before the time of Hippocrates. The first book of Samuel of the Old Testament of the Bible contains a description of a “plague” that afflicted the Philistines in 1141 BC after they defeated the Hebrews and brought the Ark of the Covenant back to their Temple. Young and old were stricken with “emerods” (1 Samuel 5:6–16). Scholars have alternatively interpreted these to be rectal prolapses or hemorrhoids due to epidemic dysentery or bubos of the bubonic plague (30, 31).

Considerable information is available on epidemics of dysentery during military campaigns. From ancient Greece, through the Napoleonic Wars, and the American Civil War, dysentery and other infectious diseases took a heavy toll on the combatants, often killing or incapacitating more troops than the actual battle did. Statistics from the American Civil War highlight the significance of dysentery in combat troops in the 19th century. More than 1.7 million patients were admitted to field medical installations and hospitals. The case rate for diarrheas and dysenteries, including gastroenteritis, was 741.2 per year per 1,000. More than one death in every four caused by disease was attributed to diarrheas and dysenteries (32). Dysentery was particularly severe in prisoner-of-war camps. An example is the notorious stockade for Union prisoners of war at Andersonville, Georgia. At least 16,772 cases of diarrhea and dysentery were recorded at this prison, and 4,529 men died. More than one-half of all fatalities were attributed to diarrhea and dysentery (33).

Influenza, dysentery, and typhoid fever were the most important diseases in The Great War. The poor sanitary conditions of World War I trench warfare were particularly conducive to the spread of infectious diseases such as dysentery, with transmission via the fecal-oral route or through contaminated food or water. Dysentery is frequently cited as a main cause of the failure of the Gallipoli campaign, and many of the 120,000 casualties evacuated from the peninsula in mid- to late-1915 were due to dysentery (34). Bacteriologists Ledingham and Penfold at the King George Hospital in Waterloo wrote, "During the past two months (that is, since the middle of August, 1915) this hospital has admitted from the Dardanelles area a considerable number of cases presenting a recent history of acute dysentery with the classical symptoms of tenesmus and frequent evacuations of blood and mucus" (35). It is likely that many of the fatalities were due to misdiagnosis of the disease as amebic dysentery and subsequent treatment with emetine (36). Thus, the Gallipoli experience underscored the importance of accurate and early diagnosis. Despite the high morbidity, fatalities due to dysentery declined relative to the military conflicts of the 19th century. Of historical note, the first bacterial isolate deposited in the United Kingdom National Collection of Type Cultures (NCTC) was a *S. flexneri* serotype 2a (now called NCTC1) isolated from a British soldier in 1915. This isolate is the oldest live strain of *S. flexneri* available today. Its genome was sequenced by a team at the Wellcome Trust Sanger Institute and provides fascinating insight into the evolution of bacillary dysentery during the 100 years since World War I (37).

During World War II bacillary dysentery was known variously as "G.I.s," "Delhi Belly," "Simla Trots," and "Gimpy Tummy" (38). It was the U.S. Army's second biggest disease threat in tropical areas after malaria. However, the decline in deaths due to dysentery observed during World War I continued, and diarrhea and dysentery were primarily causes of morbidity and combat nonreadiness during World War II. Although more than 500,000 cases were recorded, case fatality for all forms of dysentery was 0.07%, considerably less than the rate in World War I. Antibiotics, notably the sulfonamides, also contributed to the decline in mortality from dysentery. The real impact of dysentery on combat readiness of U.S. forces is better reflected in the average of 16 days lost in treatment for bacillary dysentery (32). Perhaps the most notable example of dysentery as a decisive factor in battle is the British Army's defeat of Germany's Field Marshal Erwin Rommel's *Afrikkakorps* in the battle of El Alamein in 1942. Sir Sheldon Dudley wrote, "Montgomery says the Eighth Army won, but Rommel claimed the victory for dysentery" (cited in reference 32).

Between 40% and 50% of the Italian and German units were affected by dysentery because "...hygiene was not energetically studied..." By contrast, the British troops learned their lesson from their earlier retreat in North Africa and "the hygiene officers and other ranks of the Eighth Army, by their enthusiastic application of modern public health principles...in a difficult environment, did good work," resulting in an army physically and mentally fit for combat (39). Even as recently as the two Persian Gulf wars, gastrointestinal diseases and shigellosis continued to be frequent causes of combat nonreadiness among U.S. troops despite strict control of food and water sources (40, 41).

Epidemic Dysentery in the 20th Century

In the early 20th century, improvements in housing, sanitation, and hygiene conditions considerably reduced the incidence of asylum dysentery in the civilian population. The advent of antibiotics and their application to treatment of dysentery also contributed to curtailing transmission. Moreover, Shiga's bacillus, *S. dysenteriae* type 1, had virtually disappeared. However, in 1969, an explosive outbreak of epidemic dysentery occurred in Guatemala (42). It was the first documented regional epidemic due to *S. dysenteriae* 1 in the Western Hemisphere, and it spread rapidly throughout the region. In Guatemala during the period from January to October 1969, deaths from dysentery may have exceeded 10,000 (43). The epidemic was further complicated by the multiantibiotic-resistant nature of the strain, the pathogen being resistant to sulfathiazole, chloramphenicol, and tetracycline. The organism subsequently developed resistance to ampicillin (44). The epidemic was likely spread by contaminated water and population movement. Between 1969 and 1973, an estimated 500,000 cases and 20,000 deaths from dysentery occurred in Central America. All ages were affected, but mortality was higher in children and the elderly. The disease was also imported into the United States by air travelers, leading to a 5-fold increase in the number of *S. dysenteriae* type 1 infections in 1988 (45).

In the late 1970s, an epidemic of *S. dysenteriae* 1 dysentery was spreading in central Africa. It began in northeastern Zaire in 1979 and quickly spread to Rwanda, Burundi, and Tanzania. Initial isolates were resistant to ampicillin, chloramphenicol, sulfisoxazole, and tetracycline and acquired resistance to trimethoprim and nalidixic acid soon after these drugs were introduced as treatment (46). Burundi experienced seasonal peaks through the 1980s, and multidrug-resistant *S. dysenteriae* 1 was responsible for two-thirds of the 1990 seasonal dysentery cases. In 1993 through 1994, civil wars in Burundi and Rwanda and mass genocide in Rwanda led to the displacement of millions of people. By December 1993, an estimated 130,000 persons had become displaced within Burundi and approximately 683,000 persons had fled to Rwanda, Tanzania, or Zaire. In April 1994, 500,000 refugees crossed the border from Rwanda into Tanzania, and another 63,000 refugees fled from Rwanda into Burundi. Dysentery and malaria were the most common causes of morbidity in the refugee camps. During the period December 1, 1993 to January 17, 1994, the mean weekly dysentery attack rate in the Rwanda refugee camps was 3.8 cases per 100 persons and as high as 5.8 cases per 100 for children aged <5 years (47). A review of surveillance data from Médecins sans Frontières concluded that *S. dysenteriae* type 1 was the cause of dysentery outbreaks in 11 refugee camps between 1993 and 1995 (48). Displaced populations in refugee and resettlement camps are often confronted with

insufficient supplies of water, poor sanitation, overcrowding, malnutrition, and limited (if any) access to medical care. All of these conditions favor the rapid spread of infectious disease, especially diarrhea and dysentery. Not surprisingly, outbreaks of dysentery in refugee camps, this time caused by multidrug-resistant strains of *S. flexneri* and *S. sonnei*, continued into the 21st century as a new wave of refugees flowed into Europe from the Middle East (49).

The epidemic nature of *S. dysenteriae* 1 remains a puzzle. Epidemics are periodic, and it is not clear where the strain resides between epidemics.

SHIGELLOSIS TODAY – CONTINUING AND EMERGING THREATS AND NEW CHALLENGES

Contaminated Food and Water

In 1987, James Smith at the U.S. Department of Agriculture brought to the forefront the concept that *Shigella* species should be considered as a significant foodborne pathogen, in addition to the conventional belief at the time that water was the major vehicle of transmission (50). His foresight was supported by a 1990 report from the Centers for Disease Control and Prevention (CDC) that showed that *Shigella* spp. were responsible for 3.7% (7 of 127) of the total confirmed foodborne outbreaks in the United States due to bacterial pathogens from 1983 to 1987 and were second with 25.2% of the total cases (1,993 of 7,082) (51). Since then, *Shigella* spp., predominantly *S. sonnei* and *S. flexneri* in the United States, have continued to be a major etiological agent of foodborne illnesses (52).

Humans are the primary host for *Shigella*, although some reports have noted that nonhuman primates can also be infected. In one case, several staff members in a primate research unit became ill with diarrheal disease. *S. flexneri* 1b was isolated from three employees, whereas the same serovar was isolated from two monkeys and *S. flexneri* Y was recovered from the other two monkeys (53). Although the authors speculated that the research staff became ill through contact with contaminated monkey fecal material, an ill employee may have been the original source of the pathogen. In another study, fecal samples were collected from mountain gorillas from two national parks in Uganda. Of the 62 samples, 6% of the gorillas were infected with three species of *Shigella*, i.e., *S. sonnei*, *S. flexneri*, and *S. boydii* (54). Again, it was not established if the gorillas, albeit in a human-populated environment, were natural carriers of the pathogens or if they were infected via crossing or grooming in surface water contaminated by human defecation.

The major mode of transmission is the fecal-oral route, and *Shigella* spp. have a relatively low infectious dose, approximately 100 organisms (5). Spread of this pathogen occurs through contaminated water and the five F's: food, feces, fingers, flies, and fomites. Food handlers with poor personal hygiene have frequently been implicated as a contributing source for spreading *Shigella*. In addition, since *Shigella* spp. are not associated with any specific food commodity, increase in the number of foodborne illnesses can be linked to a myriad of environmental parameters, such as improper food handling, storage, and preparation. The latter ranges from the consumers' homes, to small-town gatherings and

picnics, up to large-scale food service operations. What exacerbates these situations is that *Shigella* can easily and rapidly spread from person to person, which can lead to larger numbers of people being infected.

Foodborne outbreaks due to *Shigella* spp. have no specific patterns. Illnesses can be within a small local population or extend to hundreds or even thousands in multiple states or countries. Common sources of contaminated foods are fresh produce or foods handled by preparers who were ill and who transfer sufficient numbers of pathogen to cause illness. Several cases serve to exemplify the different types of outbreaks of shigellosis. The first example is a multistate outbreak of shigellosis that was linked to a five-layer bean dip where the actual implicated component was the cheese layer, prepared by an ill person (55). Another small outbreak involved contaminated homemade moose soup that was prepared by one infected individual that eventually caused shigellosis in 25 people in a small town (56). A large outbreak of shigellosis affected 3,175 women who had attended a music festival. There were approximately 2,000 food handlers and, although the origin of the outbreak was unknown, it was determined that this incident was a point source outbreak, and one food preparer reported having diarrhea prior to the event. It should be noted that, although *S. sonnei* was the etiological agent in this outbreak, not all attendees became ill due to food poisoning but rather as a result of secondary attack (57).

As for the impact of international trade with products contaminated with *Shigella*, Norway had two outbreaks due to imported snow peas (58) and fresh basil in 2011 (59).

A sobering reminder of the potential for rapid, international transmission of dysentery is an outbreak linked to consumption of food served by a Minnesota-based airline. This outbreak involved probable or confirmed shigellosis in 240 passengers on 219 flights to 24 states, the District of Columbia, and four countries between September 14 and October 13, 1988. The likely cause was food items that were contaminated during processing by one or more food handlers who acquired *S. sonnei* infection in the community. The outbreak may have gone undetected were it not for the fact that 32% of 65 players and staff of a “professional football team based in Minneapolis-St. Paul” had onset of confirmed or probable symptoms of dysentery during the first week in October. The ensuing local media attention prompted the extensive state health department investigation (60).

Natural disasters, such as earthquakes and flooding, can have a pronounced health effect on affected populations. In these situations, there is an increased risk to public health if parts of a community’s infrastructure, specifically drinking water sources and distribution systems as well as sewage treatment plants, are damaged. *Shigella* spp. are spread by the fecal-oral route; therefore, water contaminated with *Shigella* (or any enteric pathogen), can be transmitted to humans through direct consumption. Contaminated irrigation water may be deposited on vegetation that may be consumed in the near future. Therefore, a means to reduce the incidence of illnesses and provide a safe water supply requires an adequate sanitation infrastructure.

In the United States and other developed countries, shigellosis outbreaks due to contaminated water are rare. A recent survey (61) provided the number of deaths in the

United States associated with contaminated water during the years 2003 to 2009 for 13 pathogens, including *Shigella*. During this period, there were 6,939 reported deaths, 7% (494) attributed to seven pathogens, not including *Shigella*. *Shigella* spp. were responsible for fewer than 11 deaths per year, and strikingly, had the lowest median age (35 years old) of people who died. Most deaths from environmental sources, including water, were linked to *Pseudomonas* pneumonia, hepatitis A, *Salmonella*, and *Legionella*, and nontuberculous mycobacteria.

Shigellosis in the 21st Century

Dysentery and diarrheal disease continue to be significant public health threats in the 21st century. The Global Enteric Multicenter Study (GEMS) was a 3-year case-control study that examined the burden of pediatric diarrheal disease in sub-Saharan Africa and south Asia (62). The investigators found that *Shigella* was one of the four leading causes of moderate to severe diarrheal disease in children less than 5 years of age. Reassessment of the data by Liu et al. using quantitative molecular diagnostic methods found the incidence of *Shigella* to be at least two times higher than initially measured by classical bacteriology, thereby making *Shigella* the most attributable pathogen in the study (63). The Global Burden of Disease (GBD) Project from the Institute for Health Metrics and Evaluation at the University of Washington in Seattle, WA recently analyzed the global burden of diarrheal diseases over the past 25 years. The GBD study included a systematic reanalysis of the GEMS and assessed cases, deaths, and etiologies as well as diarrhea disability-adjusted life-years (DALYs). The study found that, in 2015, diarrhea was a leading cause of death among all ages. Rotavirus was the leading cause of diarrhea deaths followed by *Shigella* spp. and *Salmonella* spp. Among children less than 5 years old, the three etiologies responsible for the most deaths were rotavirus, *Cryptosporidium* spp., and *Shigella* spp. Of note was the finding that *Shigella* spp. were also the leading cause of death among adults aged 15 to 99 years (64). A more detailed analysis of the *Shigella* serotypes from the GEMS study showed that *S. flexneri* comprised the majority of the isolates (65.9%) followed by *S. sonnei* (23.7%). *S. dysenteriae* and *S. boydii* accounted for 5.0% and 5.4%, respectively (65). This analysis underscores the need for a broad-spectrum *Shigella* vaccine designed to protect against *S. sonnei* and up to 15 different serotypes and subserotypes of *S. flexneri* to afford coverage against the most common strains.

Shigella as a Sexually Transmitted Infection among Men Who Have Sex with Men

A trend that was first observed in the late 1980s has now reached epidemic levels. “Gay bowel syndrome” was a term used in the pre-HIV/AIDS era 1970s to describe a clinical pattern of anorectal and colon diseases frequently observed in homosexual patients, i.e., men who have sex with men (MSM). Disease transmission was presumed to occur via two routes: anal sex and oro-anal contact. *Shigella* was included along with all the known sexually transmitted disease agents as being responsible for this syndrome (66). Perhaps the earliest report of shigellosis as a sexually transmitted infection was in the gay community in San Francisco in 1974. “Oral sexual practices” was listed as the most probable form of disease transmission by a strain of *S. flexneri* 2a (67). Subsequently, an outbreak of shigellosis caused by *S. flexneri* and *S. sonnei* was reported among gay men in the Seattle, WA area over an 18-month period in 1975 to 1976 (68). A case-control study of shigellosis in adults

in San Francisco in 1999 confirmed that shigellosis is a sexually transmitted disease among MSM and that specific sexual activities and HIV status are risk factors (69).

While the term “gay bowel syndrome” (derogatory and pejorative) has, thankfully, become obsolete (the CDC dropped use of the term in 2005), the sexual behaviors and microbial pathogens that cause these diseases are still very much present today (70). Since the early observations in North America, shigellosis among MSM has been reported from Asia, Europe, and Oceania. Of deeper concern is the emergence of multidrug resistance in strains of *Shigella* in the MSM community. Among 32 clusters of shigellosis in the United States between 2011 and 2015, antibiotic resistance was observed in all seven clusters among MSM, but in only two of the other 25 clusters (71). Macrolide-resistant and extended-spectrum β -lactamase-producing strains of *S. sonnei* have been associated with shigellosis in MSM in England (72). Furthermore, outbreaks are no longer local. Intercontinental spread of azithromycin-resistant shigellosis via sexual transmission has been reported (73).

Many questions on the host-pathogen interactions in sexually transmitted shigellosis remain. Determination of host immune status, behavioral, and other correlates that contribute to susceptibility to sexual transmission of shigellosis requires additional study. Importantly, whether these strains of *Shigella* possess any unique adaptive mutations that favor their spread by certain sexual practices in the MSM population remains an open question.

Emergence of non-*S. dysenteriae* 1 Strains That Encode and Synthesize Shiga Toxin

As mentioned earlier, strains of *S. dysenteriae* 1 produce Shiga toxin, but Shiga toxins are also produced by certain strains of *E. coli*, notably the strains of enterohemorrhagic *E. coli* such as *E. coli* O157:H7 (74). However, there have been rare reports of the presence of Shiga toxin genes in clinical isolates of non-*S. dysenteriae* type 1 *Shigella* species (75, 76). Recently, we have seen the emergence of multiple strains of *S. flexneri* and *S. dysenteriae* 4 that produce Shiga toxin. Unlike *S. dysenteriae* 1, where the Shiga toxin genes are chromosomally encoded, the toxin genes in these strains are encoded on a lysogenic bacteriophage. Moreover, there is a strong epidemiological link of these strains with travel to Haiti and the Dominican Republic (77, 78). These strains were isolated from travelers returning to the United States and France from Haiti or the Dominican Republic. Confirmation that Shiga toxin-producing *Shigella* are circulating in Haiti came from the isolation of Shiga toxin-producing strains of *S. flexneri* that contain the toxin-encoding bacteriophage from Haitian patients with diarrhea in a region just west of Port-au-Prince (79). In addition, it was demonstrated that the toxin-encoding bacteriophage is capable of lysogenizing Shiga toxin-negative *Shigella* species isolated from Haitian patients, indicating that this toxin-encoding bacteriophage has the ability to infect other *Shigella* strains circulating within Haiti. While it is not surprising that Shiga toxin-encoding bacteriophages have spread to other species of *Shigella*, it is still not clear why these strains are emerging now and why their emergence seems to be centered on Hispaniola, the island of Haiti and the Dominican Republic. The clinical impact of these new strains remains to be determined and will only become clearer as more cases are identified and described. We may just be seeing the tip of a potentially global emergence of these strains.

***Shigella* and Biological Weapons**

Finally, mention should be made of the potential and actual use of *Shigella* as a bioweapon. Because it is relatively easy to grow and it has a low infectious dose, *Shigella* has the characteristics for use as a bioweapon, although obviously it is more suited as an incapacitating agent than a lethal one. The only reported case of deliberate use of *Shigella* as a bioweapon was an intentional contamination of laboratory workers at a large medical center in 1996 (80). Twelve laboratory workers (attack rate of 100%) developed severe acute diarrhea after eating muffins or doughnuts likely deliberately contaminated with the laboratory's stock culture of *S. dysenteriae* type 2. Although a criminal investigation was launched, the motive and method of contamination remain unknown.

IMPORTANCE OF *SHIGELLA* IN MEDICAL HISTORY

Bacteriophage Therapy

French-Canadian microbiologist Félix d'Herelle is credited with discovering bacteriophages in 1917 at the Institut Pasteur in Paris, France. d'Herelle was sent to study an outbreak of severe hemorrhagic dysentery among French troops stationed at Maisons-Laffitte, northwest of Paris, in July 1915. When he added filtrates of feces from patients recovering from dysentery to a broth culture of the original strain, the culture cleared. Similarly, addition of drops of the filtrate to agar plates covered with bacterial culture led to formation of clear spots. These results only were found in filtrates from convalescent patients and not from patients with acute dysentery. Thus, d'Herelle had found “un microbe invisible antagoniste des bacilles dysentériques” (81), “an invisible microbe endowed with antagonistic effects toward the Shiga bacillus” (82). His discovery was followed by the first therapeutic use of bacteriophages by d'Herelle to treat dysentery at the Hôpital des Enfants-Malades, Paris in 1919. Four patients were injected with preparations of the antidysentery phage. Within 24 hours, all four patients were recovering from symptoms (see reference 83). The question of whether d'Herelle was indeed the first person to discover bacteriophages or whether the English microbiologist F.W. Twort discovered bacteriophages 2 years earlier in 1915 has been much debated (84). Nevertheless, it is clear that d'Herelle was the first person to recognize and attempt to demonstrate the therapeutic value of bacteriophages by treating patients with dysentery. His discovery led to several decades of the study and application of bacteriophage for the treatment of dysentery (see reference 83 for review of these early years).

While d'Herelle's seminal work with *Shigella* marked the birth of the bacteriophage therapy era, the introduction of antibiotics in clinical practice in the 1940s pushed the development of bacteriophage therapy into near obscurity. However, the emergence of multiple-antibiotic-resistant bacteria over the past 50 years and the paucity of new antibiotics in the development pipeline have breathed new life into research on bacteriophages as therapeutic agents. Ironically, *Shigella* also played a key role in the history of multiple-antibiotic resistance as described in the next section.

Transmissible Multiple-Antibiotic Resistance

Shigella has the distinction of being one of the first bacterial pathogens to be reported to be resistant to multiple antibiotics. The arrival of the antibiotic era in the 1940s provided powerful new drugs for the treatment of dysentery. Sulfonamides were the most effective drugs against the outbreaks of dysentery in postwar Japan. Unfortunately, the efficacy of these drugs lasted only a few years, and, by 1952, more than 80% of the *Shigella* isolates were resistant to sulfonamides. Isolates resistant to streptomycin, tetracycline, and chloramphenicol emerged in Japan soon after their introduction. At first, these isolates showed resistance to just a single antibiotic. In 1956, Kitamoto et al. reported an isolate of *S. flexneri* 4a that was resistant to multiple antibiotics: tetracycline, chloramphenicol, streptomycin, and sulfonamides (85). To researchers at the time, the appearance of multiple-drug resistance was difficult to explain by single mutational events alone. Selection of multiple resistance through multiple rounds of mutation was one possibility. However, while mutations to drug resistance are often accompanied by growth defects, these new mutants grew similarly to the drug-sensitive strains. Since resistance to these antibiotics mapped to separate genes, single-step mutation to multiple-drug resistance seemed unlikely. Spontaneous mutation to resistance to one drug followed by spontaneous resistance to a second and then a third drug was also mathematically unattractive as a model. The fact that multiple-drug-resistant *Shigella* strains of different serotypes were emerging in the late 1950s further made the model of spontaneous multiple-drug resistance less likely. Thus, the emergence in Japan of *Shigella* strains that were resistant to multiple antibiotics could not easily be explained by simple random mutation and selection. In fact, the underlying mechanism was something simpler and more sinister: transmissible antibiotic resistance (for a more complete background, see references 86 and 87). This discovery was to have a far-reaching impact on medicine and molecular genetics.

The antibiotic resistance patterns of *Shigella* strains in Japan displayed unusual features. Patients treated with a single antibiotic excreted strains that were resistant to that antibiotic as well as other antibiotics that had not been used in the patient. Multiple-drug-resistant strains were isolated from patients who had excreted sensitive strains at the onset of dysentery. Strains of *E. coli* were also isolated that displayed the same pattern of multiple-antibiotic resistance as the *Shigella* strains. Tomoichiro Akiba at the University of Tokyo was the first to suggest that multiple resistance might be transferred from a drug-resistant *E. coli* to *Shigella* and presented experimental evidence for such transfer (88). Independently, Kunitaro Ochiai at the Nagoya City Higashi Hospital reported similar results (89). Both groups showed transfer of multiple-antibiotic resistance *in vitro* between multiply drug-resistant *E. coli* and sensitive *Shigella*. Kagiwada provided support for this model by demonstrating *in vivo* transfer of resistance between multiply drug-resistant *E. coli* and sensitive *Shigella* (90). Susumu Mitsuhashi, Tsutomu Watanabe, and Rintaro Nakaya extended these observations and confirmed that transmission was via conjugation, direct cell-to-cell mediated horizontal genetic exchange between bacteria. Mitsuhashi coined the term R-factor (for resistance transfer factor) for the genetic elements that encode multiple resistance (91–93). Soon R-factor transfer of multiple-antibiotic resistance was being reported in *Salmonella* and pathogenic *E. coli* worldwide. Once again, *Shigella* was at the birth of a revolution in clinical microbiology and bacterial genetics.

Relatedness to *E. coli* – *Shigella* Is an *E. coli* with Attitude

Joshua Lederberg at the University of Wisconsin, Madison, and his graduate student, Norton Zinder, were the first to demonstrate generalized transduction using phage P22 in *Salmonella* in 1952 (94), a discovery that provided one of the most powerful tools in the field of bacterial genetics in the pre-recombinant DNA era. However, P22 does not infect *E. coli*, the workhorse of bacterial genetics. A year earlier, Giuseppe Bertani, working in the laboratory of Salvador Luria at the University of Illinois, Urbana, isolated the generalized transducing phage P1 from a strain of *E. coli* and showed that P1 infected both *E. coli* and *Shigella* (95). This paper also contained the formula for LB medium. LB is often, but apparently incorrectly, referred to as Luria broth, Lennox broth, or Luria-Bertani medium. In a 2004 review, Bertani set the historical record straight by stating that “the abbreviation was intended to stand for ‘lysogeny broth’” (96). Subsequently, Ed Lennox, a physicist and postdoc with Luria, worked with Bertani to show that P1 is capable of transducing a large variety of genetic markers between *E. coli* and *S. dysenteriae* (97). Among the markers transferred from *E. coli* into *S. dysenteriae* were the ability to utilize lactose and arabinose. A key conclusion from Lennox’s study was that the transfer of markers between *E. coli* and *S. dysenteriae* “indicate at least limited genetic homologies between these organisms” and that “a detailed comparison of the ordered genomes should provide some interesting insight into the evolutionary history of this group of bacteria” (97).

In 1953 Lederberg and Edward Tatum (Stanford University) first discovered genetic exchange by bacterial conjugation in *E. coli* (98). In 1957, Luria and Jeanne Burrous, also at the University of Illinois, Urbana, extended these observations and demonstrated conjugation between *E. coli* donors and *S. dysenteriae*, *S. flexneri*, and *S. boydii* recipients (99). *E. coli* and *Shigella* already were known to have enough characteristics in common to suggest a close evolutionary relationship. Luria and Burrous’ results showed that these strains could mate and exchange stably inherited genetic traits and that the genetic linkage between loci was similar in *Shigella* and *E. coli*. Thus, conjugation allowed the authors to create what they referred to as “monstrosities from the stand-point of traditional bacterial classification, such as strains of *S. dysenteriae* that promptly ferment lactose” (99). More importantly, they concluded that “the results suggest a possible role for hybridization occurring in nature in the evolution of the *Enterobacteriaceae* and in the origin of aberrant and intermediate strains of enteric bacteria.” They presciently warned that genetic recombination could pose problems for procedures used to identify enteric pathogens (99). Thus, the work of Lennox and Luria and Burrous provided strong evidence of the close genetic relatedness of *E. coli* and *Shigella* and gave researchers two powerful new genetic tools for studying *Shigella*. In addition, Luria and Burrous established a guiding principle for an understanding of how bacterial pathogens evolve: horizontal gene transfer between species.

The next key to unraveling the relatedness of *E. coli* and *Shigella* came from studies conducted by Don Brenner, Stanley Falkow, and colleagues at the Walter Reed Army Institute of Research and Georgetown University in Washington, DC. They measured DNA-DNA reassociation kinetics by using genomic DNA from different members of the *Enterobacteriaceae*. The underlying principle of this method is that after strand separation

(melting out of DNA-DNA double-strand duplexes) the ability of DNA from different organisms to form stable, specific DNA duplexes is a measure of their genetic relatedness. In reactions that used *E. coli* and *S. flexneri* DNA, the stable reannealed duplexes that formed suggested <15% divergence between the two species, i.e., “relatively little evolutionary divergence in these organisms” (100).

Additional genetic analyses over the next 30 years contributed to the growing body of evidence that *Shigella* is a metabolically inactive biotype of *E. coli* ([101] and briefly summarized in reference 1). In 2000, Peter Reeves’ group at the University of Sydney in Australia used DNA sequence analyses of housekeeping genes to build on their earlier multilocus enzyme electrophoretic studies to propose that *Shigella* did not simply evolve from *E. coli* as a single event but that it emerged as many as seven separate times (102, 103). They further estimated that the three main *Shigella* clusters evolved within the last 35,000 to 270,000 years. In 2003, Fred Blattner’s group at the University of Wisconsin, Madison, published the first whole-genome sequence of a *Shigella* strain, the prototype *S. flexneri* 2a, strain 2457T (see Box 1). The authors concluded that their data “...are consistent with *Shigella* being phylogenetically indistinguishable from *E. coli*” (104). Since then, multiple strains representing all four species of *Shigella* have been sequenced. The whole-genome sequence of the oldest known isolate of *Shigella* is that of a strain of *S. flexneri* isolated from a British soldier in France in 1915 during World War I (37). The strain NCTC1 was resistant to penicillin and erythro-mycin, even though these antibiotics would not be discovered or enter the clinical arena until decades later. Comparison with the reference genome of strain 2457T showed the *S. flexneri* 2a genome to be relatively stable over time. However, it also showed that the “modern” *S. flexneri* 2a genome has gained drug resistance, virulence, and serotype conversion islands.

The question of the classification of *Shigella* has frequently arisen over the past 100 years. In the first lines of the chapter on *Shigella* in the third edition of *Topley and Wilson’s Principles of Bacteriology and Immunity* in 1946, the authors refer to the “vexed question of classification” (105). The section on classification begins “Space does not permit of a description of the numerous attempts that have been made to afford a satisfactory classification of the dysentery bacilli.” DNA and genetic evidence of the past 40 years has contributed to the debate and, as pointed out by Peter Reeves, “These data provide compelling support for the inclusion of *Shigella* species within *E. coli*; indeed, it is not possible to discuss sensibly on any other basis” (102). In the latest edition of *Bergey’s Manual of Systematic Bacteriology*, Nancy Strockbine and Tony Maurelli acknowledged the weight of the phylogenetic evidence and argued for maintaining separate genera in stating, “...widespread adoption [of new nomenclature] will happen only when it is important to users to communicate these relationships through the bacterial names. Until that time, the medically useful link between the genus epithet *Shigella* and shigellosis, the term for the disease caused by these bacteria, will sustain the current nomenclature” (1). Or, as argued by Wilson and Miles in addressing the earlier debate on *Shigella* nomenclature, “The primary purpose of nomenclature is utility, and to insist on an inapt and uninformative specific name merely on the grounds of botanical convention is to forget that the Sabbath was made for man and not man for the Sabbath” (105).

MOLECULAR BIOLOGY AND GENETICS IN THE LATE 20TH TO EARLY 21ST CENTURY

Pathogenesis Themes in *Shigella* Shared by Other Bacterial Pathogens

Over the course of the past 50 or more years of research on *Shigella* pathogenesis, discoveries have been made that revealed common themes that *Shigella* shares with other bacterial pathogens. Among them are toxin production, multiple-antibiotic resistance, virulence genes encoded on plasmids and bacteriophages, invasion of epithelial cells, global regulation of virulence genes, pathogenicity islands, intracellular motility, remodeling of host cytoskeleton, inflammation/polymorphonuclear leukocyte (PMN) signaling, apoptosis induction/inhibition, and “black holes” and antivirulence genes. In many cases, these shared pathogenic features were first described in *Shigella*.

Characterization of the Shiga Toxin

For 50 years after Conradi, Neisser, and Shiga first described Shiga toxin, separation of neurotoxic activity from endotoxic activity was difficult since toxin preparations were contaminated with lipopolysaccharide. After years of effort, Shiga himself concluded that “dysentery toxin by its nature belongs to the endotoxins and is a constituent of the bacillary body... dysentery toxin produces antitoxin. This contradicts the idea we had hitherto held in regard to an endotoxin. Therefore we must accept the view that there are some endotoxins which produce antitoxins” (11). A deeper understanding of the toxin produced by Shiga’s bacillus thus began with its partial purification by van Heyningen and Gladstone at the University of Oxford, England in 1953 (106). The toxin was still referred to as a neurotoxin because parenteral inoculation into rabbits led to paralysis, cerebral and spinal cord hemorrhages, and death. Although purified Shiga toxin was now available and the toxin recognized to be a protein distinct from endotoxin, research was slow. Since the toxin was only made by one species of *Shigella* and the “neurotoxin” activity was not obviously relevant to clinical dysentery, researchers were not drawn to study Shiga toxin.

The next breakthrough in Shiga toxin research came from the laboratory of Gerald Keusch at The New England Medical Center (see references 29 and 74 for a review of the history of Shiga toxin). Keusch was an infectious disease fellow in 1969 and had the opportunity to visit patients in Guatemala City during the Central America epidemic. He noticed that many dysentery patients presented with watery diarrhea. Prompted by his own experience studying cholera, Keusch postulated that a *Shigella* “enterotoxin” might be involved. Together with Leonardo Mata at the Instituto de Centro America y Panama in Guatemala City, he showed that cell-free spent medium from a clinical isolate of *S. dysenteriae* 1 caused fluid accumulation in ligated rabbit ileal loops (107). This result fulfilled the definition of an “enterotoxin,” and the Keusch laboratory went on to demonstrate that the partially purified enterotoxin causes inflammatory enteritis in rabbits (108).

The question of a role for Shiga toxin in the pathogenesis of dysentery was addressed by Peter Gemski in the laboratory of Sam Formal at the Walter Reed Army Institute of Research in 1972. Gemski and colleagues found that a Shiga toxin-producing but noninvasive strain of *S. dysenteriae* 1 (strain 3818O; see Box 1) failed to cause clinical

disease in several animal models. Similarly, a mutant that neither invaded nor produced Shiga toxin was avirulent (109). Their results suggested that the ability of *Shigella* to invade and multiply within the colonic mucosa is more important to disease than toxin production.

These results were extended by Annick Fontaine and Josette Arondel in the laboratory of Philippe Sansonetti at the Institut Pasteur in Paris in 1988 (110). They constructed a mutant of *S. dysenteriae* 1 in which the Shiga toxin genes had been insertionally inactivated and examined the mutant in tissue culture invasion and the macaque monkey challenge models. The absence of toxin production had no effect on the invasion of tissue culture cells, intracellular growth, or killing of invaded cells. In the monkey model, the most striking difference was the absence of blood in dysenteric stools of animals infected with the mutant strain. The characteristic vascular damage and destruction of the capillary loops within the chorion in the sigmoid colon which appeared in monkeys infected with the toxin-producing parent strain were absent in animals infected with the toxin-negative mutant. Their results suggested that Shiga toxin plays only a limited role when released intracellularly in epithelial and phagocytic cells, but that Shiga toxin released within infected tissues acts predominantly at the level of inducing intestinal vascular damage.

One hundred years after its discovery, the medical impact of the toxin from Shiga's bacillus has expanded well beyond the *Shigella* genus. In 1983, Alison O'Brien, at the Uniformed Services University in Bethesda, MD, reported that the *E. coli* O157:H7 strain that was responsible for an outbreak of hemorrhagic colitis in the United States produced a Shiga-like toxin (111). Eventually, other strains of Shiga toxin-producing *E. coli* (STEC) were discovered worldwide. Today outbreaks of disease caused by STEC strains are more prominent than epidemics of *S. dysenteriae* 1. A more complete history of STEC and the different classes of Shiga toxins that they produce can be found in these excellent reviews (74, 112).

The Invasive Properties of *Shigella* – Tissue Culture and Animal Models

One of the many seminal publications on *Shigella* from the laboratory of Sam Formal and his team at the Walter Reed Army Institute of Research was the description of the invasive properties of *Shigella* in the classic paper by LaBrec et al. (113). This paper was also significant for several other key findings. It was the first description and characterization of a spontaneous avirulent mutant of *Shigella*. Wild-type *S. flexneri* 2a forms translucent colonies on agar plates, but it also produces smooth, opaque colony variants (thus, the designation of the prototype strains 2457T and 2457O; see Box 1). These opaque variants are no longer virulent in the mouse virulence assay, the starved guinea pig model, and the Serény test (see below). LaBrec et al. also showed that, while the wild-type strain 2457T produced diarrheal symptoms and intestinal lesions after oral administration to rhesus monkeys, the colony variant 2457O failed to do so. Histological examination of tissues from monkeys fed 2457T who showed clinical signs of dysentery revealed typical ulcerative lesions in the colon but few lesions in the ileum. These lesions were similar to those observed in humans with dysentery. Frozen sections of these tissues labeled with fluorescent anti-*S. flexneri* 2a antibody showed fluorescing bacteria in the lamina propria, inflammatory cells, and epithelial cells. Finally, the invasive phenotype of the virulent and avirulent strains

was measured in a tissue culture invasion assay in HeLa cells. Numerous bacteria were observed in HeLa cells infected with 2457T, but no bacteria were observed in HeLa cells infected with 2457O.

It is difficult to overstate the importance of the LaBrec et al. study. Up until this time, the only animal models to study *Shigella* were rabbits, mice, and guinea pigs, none of which produced dysentery, death being the end point (summarized in reference 105). LaBrec et al. established the rhesus monkey model as the animal model that faithfully mimics dysentery symptoms seen in humans. We all now take for granted that invasion of colonic epithelial cells is the hallmark feature of *Shigella* pathogenesis. However, the prevailing view at the time of the LaBrec et al. publication was that dysentery lesions in the intestine gradually developed after waves of absorption and excretion of stable bacterial toxins liberated from the bacteria through autolysis (105, 114). The observations of LaBrec et al. plus the still unpublished electron microscopy images of Akio Takeuchi in the Formal laboratory (115) clearly demonstrated the cell invasive capacity of *Shigella*. In typically modest Sam Formal fashion, the LaBrec et al. paper concluded with this statement: "...it is conceivable that epithelial cell penetration and at least limited survival in the lamina propria are the necessary attributes for pathogenicity of dysentery bacilli... Because, to date, there has been no reasonable means to explain the pathogenicity of dysentery bacilli, these possibilities should be considered" (113). In summary, these studies established the ability to penetrate epithelial cells as a key principle of *Shigella* virulence and also validated animal and tissue culture models to measure this phenotype that continue to be used today.

A RING OF DNA RULES IT ALL – DISCOVERY OF A PLASMID IN *SHIGELLA* VIRULENCE

For many years, the Formal laboratory labored to identify chromosomal regions of *Shigella* required for virulence using mating experiments with the laboratory strain of *E. coli* K-12. This strategy was a reasonable one since there was ample evidence of close genetic relatedness between the two organisms. Stanley Falkow, who initially came to Walter Reed as a graduate student in 1960 (see Box 2), began genetic studies with Formal to create hybrid strains by conjugation between a donor *E. coli* K-12 and recipient *S. flexneri* 2a 2457T. These recombinants were then assessed by Falkow and coworkers Herman Schneider and Lou Baron for virulence in guinea pigs to identify chromosomal regions that contribute to *Shigella* virulence, at least in the guinea pig model (116). Replacement of a chromosomal region located between the Rha⁺ and Xyl⁺ genes of the *S. flexneri* recipient with donor *E. coli* K-12 DNA showed that this region of the *S. flexneri* chromosome is essential for virulence. These studies also showed that several chromosomal regions of *E. coli* K-12 and *S. flexneri* 2a are homologous.

Much effort also went into the reverse strategy that aimed to identify regions of the *Shigella* chromosome required for virulence by using conjugation with a donor strain of *S. flexneri* to convert the laboratory strain of *E. coli* K-12 into a strain capable of invading epithelial cells and producing a positive Serény test. A brief report of these efforts summarized the results as follows: "Although deliberate attempts have been made to confer invasive virulence

on *E. coli* strain K-12 by employing classical procedures of recombination with virulent *S. flexneri* donor strains, they have not yet been successful” (117). The explanation for this failure to convert *E. coli* K-12 into an invasive pathogen was not the requirement for inheritance of multiple genetic loci by conjugation, but rather the yet undiscovered role of the invasion plasmid of *Shigella*.

The importance of plasmids in bacterial virulence first took center stage with the discovery of transmissible antibiotic resistance (R factors) discussed above. Plasmids were quickly found to be critical to virulence in enteric pathogens, encoding properties such as toxin production and colonization factors (briefly reviewed in reference 86). The first suggestion of a link between a plasmid and *Shigella* virulence came from the work of Dennis Kopecko, in the Formal laboratory, and Philippe Sansonetti at the Institut Pasteur. Strains of *S. sonnei* form smooth colonies (form I) on agar medium but readily and irreversibly generate colony variants (form II) that lack the form I antigen. These strains are also avirulent. Kopecko and Sansonetti both found that the transition from form I to form II is associated with the loss of a large (~180 kilobase [kb]) plasmid (118, 119). Efforts by Kopecko et al. to transfer the large plasmid from form I to genetically marked form II cells by conjugation were unsuccessful. Shortly after he joined the Formal laboratory at Walter Reed, Sansonetti, along with Kopecko and Formal, established a definitive link between the plasmid and the ability to invade mammalian cells (120). The elegant experimental strategy they used followed principles that were later codified by Falkow in his “Molecular Koch Postulates” (121).

The role of the large plasmid in *S. sonnei* virulence was soon followed by confirmation that *S. flexneri* also carried a large plasmid that was responsible for virulence (122). It is interesting to note that the presence of a large plasmid in *S. flexneri* had already been reported by the Formal laboratory (123). However, the spontaneous transition of *S. flexneri* 2a strain 2457 from virulent, translucent (T) to avirulent, opaque (O) discussed above was not accompanied by loss of the plasmid. Thus, no correlation could be made between the plasmid and virulence at that time. We now know that the transition of *S. flexneri* 2a strain 2457T from T to O is due to spontaneous insertion of an IS element into *virF*, a key positive regulator of the virulence genes on the plasmid (124).

Temperature-Dependent Regulation of Virulence Gene Expression and Congo Red Binding

Another hallmark of *Shigella* pathogenesis is temperature regulation of virulence. Tony Maurelli, working as a graduate student in the laboratory of Roy Curtiss III at the University of Alabama in Birmingham, first reported that the ability of *S. flexneri* 2a to invade tissue culture cells and to cause keratoconjunctivitis in the guinea pig Serény test was inhibited if the culture was grown at 30°C instead of 37°C (125). The inhibition is reversible and full virulence is restored if the bacteria are grown again at 37°C. This mode of regulation is a logical one for a bacterial pathogen that moves between a mammalian host (37°C) and the extrahost environment (<37°C). Gene regulation in response to an environmental indicator that signals to the bacterium entry into the mammalian host allows *Shigella* to conserve energy such that expression of the genes required for invasion are not expressed until they are needed. It also allows for coordinate regulation of multiple unlinked genes (i.e., regulons). Thus, it was not surprising that other bacterial pathogens such as *Bordetella*

pertussis, *Yersinia pestis*, and *Borrelia burgdorferi* were found to regulate virulence genes in response to temperature (126, 127). The molecular mechanism by which *Shigella* regulates its virulence genes is complex and involves two positive transcriptional activators, VirF and VirB, and a repressor, H-NS. The latter acts as a transcriptional silencer of virulence genes in many bacterial pathogens. Several reviews present models for how temperature regulation works in *Shigella* (128, 129).

Another hallmark phenotype of virulent strains of *Shigella* is the ability to bind Congo red. In 1977, Shelley Payne, then a postdoc with Dick Finkelstein at The University of Texas Health Science Center in Dallas, reported that agar medium containing Congo red dye could be used to differentiate virulent and avirulent colonies of *Shigella*, *Vibrio cholerae*, *E. coli*, and *Neisseria meningitidis* (130). Maurelli extended these observations and established a correlation between Congo red binding by *Shigella* and growth temperature and further showed that complete loss or deletions in the virulence plasmid led to loss of Congo red binding and loss of virulence (125, 131). Thus, Congo red binding became a useful tool for rapidly screening for avirulent mutants of *Shigella*.

Claude Parsot, working in the Sansonetti laboratory at the Institut Pasteur, showed that incubation of *Shigella* cultures with Congo red induced secretion of the invasion effector molecules through the type III secretion system (132). This observation, too, provided a useful tool for future molecular and cell biology studies. While Payne and Finkelstein initially linked the ability of bacteria to bind Congo red to iron responsiveness, in *Shigella* at least, the phenotype is not linked to iron. In fact, despite the efforts of many and the publication of several papers, the factor(s) responsible for Congo red binding in *Shigella* have, to date, escaped elucidation. Nevertheless, Congo red binding has proved to be, and continues to be, useful for screening of avirulent mutants of all species of *Shigella* and for inducing secretion of invasion effector proteins.

The next big breakthrough in plasmid-encoded *Shigella* virulence came from the Sansonetti laboratory. After completing his fellowship in the Formal laboratory at Walter Reed, Sansonetti established his own laboratory in the Service des Entérobactéries at the Institut Pasteur in Paris, France. The use of Tn5 insertion mutagenesis identified multiple genes on the invasion plasmid that were essential for invasion, but these genes were not closely linked. Therefore, Sansonetti used the strategy of constructing a cosmid library of large fragments of the partially digested *S. flexneri* 5 invasion plasmid and screening for clones that imparted the invasion phenotype in a plasmidless *S. flexneri*. Maurelli, a new postdoc in the Sansonetti laboratory, devised the screening strategy and, along with Bernadette Baudry and Helene d'Hauteville in the Sansonetti laboratory, and Larry Hale at Walter Reed, they identified a 37-kb minimal region of invasion plasmid DNA that restored invasion in the plasmidless *S. flexneri* mutant (133). The invasion phenotype was temperature regulated as was the expression of proteins that were recognized by convalescent antiserum from monkeys infected with *Shigella*. While the complementing clones restored invasion, they failed to restore the ability to form plaques on confluent monolayers of tissue culture cells, a phenotype that requires intracellular growth and the ability to spread from cell to cell (see below and reference 134). They were also negative in the Serény test. Thus, the

plasmid-encoded region was necessary but not sufficient for full virulence. The reason for this limited pathogenicity was to be revealed a few years later.

Actin Polymerization and Intracellular Motility

Beyond the ability to invade a mammalian cell, one of the more remarkable feats of bacterial evolution and cellular microbiology that was first demonstrated in *Shigella* is the ability to exploit the cytoskeleton of the host cell for intracellular motility of the invading bacterium. The development of the guinea pig ocular infection model by B. Serény in Budapest, Hungary in 1957 allowed for the measurement of invasion of corneal epithelial cells as well as the cell-to-cell spread of invasive bacteria (135). This test, commonly known as the Serény test, was important in demonstrating that *Shigella* invasion was necessary but not sufficient for full pathogenicity (136). The ability to spread from cell to cell is also required. The discovery of the molecular mechanism underlying this phenotype began with the 1986 observation that, within 30 minutes of invading HeLa cells, *Shigella* lyses the endocytic vacuole. Electron micrographs taken by Antoinette Ryter at the Institut Pasteur clearly showed the bacteria free within the host cell cytoplasm (137). Growth curves also showed a remarkably rapid intracellular rate of growth for *Shigella*. The previous year, Ed Oaks, a National Research Council fellow in the Formal laboratory, developed the plaque assay as a method for measuring the ability of *Shigella* to invade, multiply intracellularly, and move to adjacent cells in a confluent tissue culture monolayer (134). Intercellular mobility and plaque formation is temperature sensitive. Oaks et al. recognized the utility of this new assay and concluded, "...it is conceivable to obtain mutants which appear invasive by light microscopy but do not form plaques."

Shigella does not make flagella and is nonmotile. The plaque assay measures cell-to-cell spread of intracellular *Shigella*, and so it suggested that *Shigella* must express some other means of motility while in the mammalian cytoplasm. More than 20 years earlier, Hidemasa Ogawa at the National Institute of Health in Tokyo, Japan, used phase-contrast video microscopy to show that intracellular *Shigella* do, in fact, display rapid random movements in the host cell cytoplasm. Ogawa noted, "When virulent bacilli entered into the tissue cells, they exhibited an unexpected vigorous movement. This movement was apparently random, not directional, and independent of the movement of intracellular organelle..." (138). This movement frequently led to formation of protrusions of the host cytoplasm. The observation that tetracycline inhibited intracellular bacterial movement also suggested the role of a *Shigella*-specific factor. Ogawa et al. concluded that their observations suggested "...that these bacilli are capable of infecting neighboring uninfected cells via the microfibrillar protrusions or across connecting intercellular bridges..."

In 1986, Masanosuke Yoshikawa's group at the University of Tokyo, Japan reported the identification of a gene (*virG*) encoded on the *Shigella* invasion plasmid that mediated this cell-to-cell spread phenotype (139). The authors did not use the plaque assay in their study but predicted that the *virG* mutant would be negative since this mutant failed to cause conjunctivitis in the Serény test that also was a measure of cell-to-cell spread. While a gene had been identified, the molecular basis for the intercellular spread phenotype came several years later in 1989. Maria Bernardini, a postdoc in the Sansonetti laboratory, used labeling

with the fluorescent dye NBD-phalloidin to demonstrate that intracellular movement of *S. flexneri* occurs through interaction of the bacterium with the host cell F-actin and the cytoskeleton (140). Bernardini et al. then used the Oaks plaque assay to screen for mutants that did not form plaques and identified a locus on the invasion plasmid that they named *icsA*, for intercellular spread. The locus is identical to the *virG* locus identified by Yoshikawa's group (139).

This remarkable exploitation of the host cell cytoskeleton by an intracellular bacterial pathogen was soon described in *Listeria monocytogenes* and other bacteria (and ultimately in Vaccinia virus, as well). While the Sansonetti laboratory was attempting to identify the molecular basis of *L. monocytogenes* cell-to-cell spread, Dan Portnoy and Lew Tilney at the University of Pennsylvania in Philadelphia published their classic study on the subject (141). The Sansonetti laboratory soon followed with their results in *Listeria* several months later (142). Thus, *Listeria* (a Gram-positive organism) and *Shigella* (a Gram-negative organism) became models for intracellular motility phenotypes and pathogen exploitation of the host actin cytoskeleton for movement.

Secretion of Invasion Plasmid Antigens – A Novel and Shared Structure

Another remarkable discovery in *Shigella* was the observation that the bacterium synthesizes proteins that it actively secretes and that interact with the host cell to mediate uptake of the bacterium. Larry Hale in the Formal laboratory identified several invasion plasmid antigens (Ipas) that were plasmid-encoded, temperature-regulated, major immunogenic antigens of *S. flexneri* that were required for invasion (143, 144). Gerry Andrews, a U.S. Army Medical Service Corps officer and graduate student in the Maurelli laboratory, showed that IpaB and IpaC were not just membrane-associated proteins but were truly secreted from *S. flexneri* and found in the culture supernatant (145). They further showed that a large intracellular pool of the antigens is present in the cytoplasm and suggested that “host-specified signals modulate the excretion of preexisting *ipa* proteins once the bacteria enter their epithelial host cells.”

Andrews et al. also identified two invasion plasmid loci, *mxiA* and *mxiB* (for membrane expression of invasion plasmid antigens), as encoding accessory proteins needed for secretion of the Ipa proteins across the outer membrane. *mxiA* was part of an operon that encoded other genes in the transport system. The authors concluded that “the products of this operon may constitute a unique multicomponent protein secretion apparatus involved in the transport of *Shigella* virulence determinants” (145). All these observations are characteristic of what we now know to be a type III secretion system (T3SS), a widely distributed virulence mechanism that has since been characterized in a broad range of Gram-negative pathogens (146). Malabi Venkatesan, along with Jerry Buysse and Ed Oaks at Walter Reed, identified the additional genes of the T3SS, the *spa* (surface expression of invasion plasmid antigens) genes (147), while Chihiro Sasakawa and colleagues in the Yoshikawa laboratory in Tokyo, Japan went on to genetically define the *S. flexneri* T3SS *mxi-spa* operons (148). Many other researchers feature prominently in the elucidation of the *Shigella* T3SS, its structure, its secreted effectors and the role that they play in invasion

and postinvasion phenotypes. The reader is directed toward several excellent reviews on this topic (149, 150).

Intracellular Survival – Apoptosis and Antiapoptosis

Macrophages act as a principal line of host defense against infection by ingesting and destroying foreign agents. Pathogens, in turn, have evolved mechanisms to escape this phagocytic machinery by avoiding uptake, surviving within the macrophage, or killing it. Macrophages ingest *Shigella* but fail to destroy the pathogen. *Shigella* survives within macrophages, and phagocytosis of *Shigella* is toxic for the macrophage. The mechanism underlying this phenotype was described in 1992 by Arturo Zychlinsky, a postdoc in the Sansonetti laboratory in Paris. This study was the first published description of an invasive bacterial pathogen that killed its host cell by inducing the programmed cell death pathway (apoptosis) in the host (151). At the time, apoptosis was generally viewed as a mechanism for an organism to remove unwanted cells (e.g., during fetal development) without inducing an inflammatory response. Thus, the Zychlinsky study showed once again how *Shigella* manipulates the host cell to promote its own survival and suggested that other pathogens may have evolved a similar survival strategy. The authors concluded that the ability of *Shigella* to elicit programmed cell death “...represents a novel pathogenic mechanism of intracellular bacteria” (151). Soon after this paper appeared, apoptosis as an intracellular survival response was described in other bacterial pathogens such as *Salmonella* and *Yersinia* (152). Since these early studies, pyroptosis has been described as another form of noninflammatory cell death induced by bacterial infection (153). The cell death evoked by *Shigella* infection of macrophages is more correctly characterized as pyroptosis (reviewed in reference 154).

The ability to induce apoptosis in macrophages answered one question about *Shigella* pathogenesis (i.e., how it escapes host cell killing), but it presented a paradox: why doesn't *Shigella* also induce apoptosis of epithelial cells? Epithelial cells infected with *Shigella* undergo a stress response but do not die (155). Clearly, the bacteria need to avoid inducing the apoptotic pathway if they are to use this host cell as a niche for their own growth and replication. Christina Clark-Faherty, a graduate student in the Maurelli laboratory, showed that *Shigella* protects the epithelial cells it infects against apoptosis by interfering with caspase 3 activation (156). Thus, the study of *Shigella* revealed two more novel pathogen strategies: induction and inhibition of apoptosis. The latter also soon became an emerging theme in bacterial pathogenesis (157).

Black Holes and Antivirulence Genes

The steps in the evolution of *Shigella* from *E. coli* included acquisition of the plasmid responsible for invasion (discussed above) as well as acquisition of several clusters of chromosomal DNA known as pathogenicity islands (158). Bacterial adaptation by gene loss is the converse of this process. The process of pathoadaptive evolution via gene loss or inactivation in bacterial pathogens was first described in *S. flexneri* in 1998. It began with a simple and well-known biochemical trait: the decarboxylation of lysine to produce cadaverine and CO₂ (159). *Shigella* are uniformly deficient in this trait, while almost 90% of *E. coli* strains can decarboxylate lysine (160). Maurelli and Rey Fernandez determined that

introduction of the *cadA* gene (encoding lysine decarboxylase) from *E. coli* into *S. flexneri* did not alter invasion or plaque formation ability. However, working with Alessio Fasano at the University of Maryland Medical School, they showed that the recombinant *S. flexneri* expressing lysine decarboxylase is deficient in enterotoxin activity as measured in ligated rabbit ileal loops. Moreover, addition of cadaverine (the product of lysine decarboxylation) alone to crude preparations of this enterotoxin also inhibits toxin activity (161). Craig Bloch and Chris Rode at the University of Michigan compared the *S. flexneri* and *E. coli* genomes and determined that the region of the genome where the *cadA* gene is found in *E. coli* is deleted in *S. flexneri*. Subsequent studies by Bill Day, a postdoc in the Maurelli laboratory, extended these genetic observations to show that, in all four species of *Shigella*, the *cadA* gene is either deleted or contains an insertion element that inactivates the gene (162). Taken together, these observations led to the conclusion that, as *Shigella* spp. evolved from *E. coli* to become pathogens, they not only acquired virulence genes on a plasmid but also lost or inactivated genes whose expression is incompatible with virulence, i.e., antivirulence genes. As a broader model of pathoadaptation, Maurelli et al. suggested that “The formation of these ‘black holes,’ deletions of genes that are detrimental to a pathogenic lifestyle, provides an evolutionary pathway that enables a pathogen to enhance virulence” (161). Over the next decade, additional examples of antivirulence genes were reported in *Shigella* and in other bacterial pathogens (163).

CLOSING THOUGHTS

We would like to extend our sincere apologies to those researchers whose contributions we did not cite in this brief history. As we mentioned in the introduction, a “history” is too broad and complex for a single review. We would not have accepted the offer to contribute this review if the editor wished for us to write a comprehensive review of *Shigella*. So we did not, and we arbitrarily chose to limit our focus as we did. Many of the major personalities we highlighted in this review are deceased, retired, or soon to retire. We dedicate this review to those luminaries of *Shigella* research. They sought to understand the genetic and molecular elegance of this deadly agent of human disease, and they sought a vaccine. Alas, an effective vaccine against dysentery is still just beyond our reach. Therefore, we also dedicate this review to the many scientists who continue to carry on the work. Keep the goal in sight. Or as Kiyoshi Shiga told his audience in his 1935 Harvard address, “It is a slow process to bring about the practical application of a scientific achievement. But it is a consolation and pleasure to think that we have devoted our efforts to humanity with an unselfish spirit of service...” (11).

SPECIAL DEDICATION TO SAM FORMAL

Sam Formal passed away on November 18, 2017. This review represents the last of his long list of contributions to the scientific literature. As news of his death spread through the scientific community, many people recalled Sam as a giant in the field, always generous with reagents and advice, and an inspiration to many. Just as importantly, however, Sam is remembered as a humble, kind, and caring person. He always had time to talk, and when it was time to say goodbye, Sam would always tell you to “Stay loose.” We will, Sam.

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Box 1**Origins**

An historical recounting of any story on bacterial pathogens should include the origins of certain key strains. The most well-known of the *Shigella* strains used for research over the past 60 years are listed here:

S. flexneri 2a strain 2457T – Observations in *Salmonella enterica* serovar Typhi showed that the wild-type strain, which expressed the Vi antigen, frequently formed variants on agar medium that failed to agglutinate in anti-Vi antiserum and lacked the Vi antigen. These variants were avirulent. A visual means for distinguishing the two forms was that the virulent Vi-expressing strain formed opaque colonies, while the avirulent Vi antigen negative strain formed translucent colonies. These colony types were referred to as V and W forms, respectively (164). In 1954, Sam Formal and Arthur Abrams were sent to Japan from the Walter Reed Army Institute for Research. Formal and Abrams (the curator of the type culture collection at Walter Reed) wanted to collect and screen strains of *Shigella* for a phenotype similar to what was observed in *S. enterica* serovar Typhi. U.S. Army Medical Service Corps Lieutenant Colonel Oscar Felsenfeld, who was with the 406th Medical General Laboratory, U.S. Army Medical Center, Japan, provided them with a strain of *S. flexneri* 2a. The strain came from a Japanese child at Komagome Hospital in Tokyo in 1954. This strain was designated 2457. In 1957, Cooper, Keller, and Walters, at the University of Cincinnati in Ohio, used oblique illumination microscopy to describe colonies of *Shigella* and their relation to mouse pathogenicity. They observed, “Shigellae highly virulent for mice were obtained from translucent colonies in which soft red-gold or gold-green colors predominated and whose surfaces appeared cross-hatched and irregular. Shigellae of low virulence for mice were obtained from opaque colonies whose colors were dull shades of pink-blue or gray and whose surfaces were smooth and edges slightly lobulated or entire” (165). Thus, the colony appearance that differentiates virulent from avirulent strains in *Shigella* is the opposite of that in *S. enterica* serovar Typhi. As noted in this review, 2457 forms translucent colonies on agar medium and also produces spontaneous opaque colony variants. These variants are avirulent. Since there is no Vi antigen in *Shigella*, another nomenclature was needed. Formal chose the simple designation of T for translucent and O for opaque. The first published use of 2457T and its avirulent opaque variant 2457O was in 1964 (113). M4243, another strain of *S. flexneri* 2a that was used in the Formal laboratory, was isolated during monkey challenge experiment 42 from monkey 43.

S. flexneri 5 strain M90T – While he was working in Sam Formal’s laboratory, Philippe Sansonetti started using *S. flexneri* 5 strain M90T as a reference strain instead of the prototype *S. flexneri* 2a strain 2457T. M90T (originally designated M90) was isolated in Mexico City in 1955 from a child with dysentery. The reason for the switch to M90T was the presence in 2457T of a large, ~160 kb cryptic plasmid that could not be easily separated from the 220 kb invasion plasmid by gel

electrophoresis. This cryptic plasmid is not present in strain M90T, so it greatly facilitated subsequent genetic manipulation and construction of the cosmid library that identified the minimal region of invasion plasmid DNA necessary for invasion (133). The Sansonetti laboratory has continued to use M90T ever since.

S. flexneri 2a strain YSH6000 – This strain of *S. flexneri* 2a is the strain used by Masanosuke Yoshikawa at the Institute of Medical Science, University of Tokyo, Japan, starting in 1986. It was originally isolated from a primate suffering from shigellosis and was provided by Masao Takasaka at the Tsukuba Primate Center for Medical Science, National Institute of Health, Japan (166). It continues to be used by his colleague Chihiro Sasakawa.

S. dysenteriae 1 strain 3818T – This strain was provided to the Formal laboratory by Dr. Leonardo Mata at the Instituto de Centro America y Panama in Guatemala City. It was isolated during the Central America epidemic of 1969. The T and O designations were added by Peter Gemski and coworkers in the Formal laboratory in their study in 1972 (109).

The Maurelli Laboratory BS Collection – Sam Formal generously provided strains and advice to Maurelli when he began working with *Shigella* in the laboratory of Roy Curtiss III in 1979. A new strain designation was needed as Maurelli isolated and constructed new derivatives of the Formal strains. BS is said to stand for “bloody stool” or “bad sh_ _.” The origin has never been clear.

Box 2**Genealogy is important**

It is interesting to trace the lineage of some of the more significant contributors in the field of *Shigella* pathogenesis. Sam Formal received his Ph.D. from Brown University in 1952 under the direction of his mentor, Charles “Doc” Stuart (Fig. 3). In 1961, Stanley Falkow (Fig. 4) also received his Ph.D. in Biology from Brown University under the direction of Doc Stuart (167, 168). Falkow was to be Stuart’s last student. Doc Stuart officially retired from Brown 18 months after Falkow joined his laboratory, so he could not officially mentor a graduate student. Doc Stuart fixed the problem by arranging for Falkow to finish his graduate research under the direction of Lou Baron at Walter Reed Army Institute of Research. Falkow worked on *Salmonella enterica* serovar Typhi with Baron, but Formal convinced Falkow to study *Shigella* and he soon started his conjugation studies with *Escherichia coli* and *S. flexneri*. Falkow took a faculty position in 1966 at Georgetown University, where he continued to work on *Shigella* before moving into different areas of bacterial pathogenesis, first at the University of Washington, Seattle, then at Stanford University, Palo Alto, California.

Alison O’Brien (Fig. 5) came to the Formal laboratory at the Walter Reed Army Institute of Research in 1976 as a National Research Council Research Associate and worked there until 1978. In July 1978, O’Brien moved to a faculty position at the Uniformed Services University in Bethesda, Maryland, and established her own laboratory where she performed her seminal studies on Shiga toxin-producing *E. coli*. She continued her work on Shiga toxin, and over the following 40 years trained dozens of students and postdocs. Shortly after O’Brien left the Formal laboratory, Philippe Sansonetti (Fig. 6) joined the laboratory. After receiving his Ph.D. from the University of Paris, Sansonetti joined the Formal laboratory in 1979 as a National Research Council Fellow. Sansonetti worked on *Shigella* in the Formal laboratory until 1981 when he returned to Paris. There, Sansonetti established his own laboratory at the Institut Pasteur to continue his work on *Shigella*. Tony Maurelli (Fig. 6) was a graduate student in the laboratory of Roy Curtiss III. He was introduced to *Shigella* in 1978 when Maurelli and Curtiss visited the Formal laboratory in Washington, DC to learn about working with *Shigella*. After receiving his Ph.D., Maurelli joined the Sansonetti laboratory in September 1983 as the first of many postdocs who would pass through the Sansonetti laboratory and work on *Shigella* over the next 35 years. Maurelli left the Sansonetti laboratory and Paris to accept a faculty position at the Uniformed Services University in Bethesda in June 1986. He continued working on *Shigella* in Bethesda for the next 30 years before moving to the University of Florida in 2016. While his laboratory continues to study *Shigella* and Chlamydia, Maurelli now also works on public health projects in developing countries.

Figure 3.
Doc Stuart (right) with Sam Formal and Sam's sons (circa 1955).

Figure 4.
Stanley Falkow and Lucy Tompkins, 1998. Reprinted from (171).

Figure 5.
Alison O'Brien and Sam Formal, Bethesda, MD, 2013.

In a way, Doc Stuart was the great-grandfather of *Shigella* researchers. Doc Stuart trained Sam Formal, who trained Stanley Falkow, Alison O'Brien, and Philippe Sansonetti, who trained Tony Maurelli. It is important to note that these *Shigella* researcher descendants of Doc Stuart not only became world-renowned researchers themselves, but they were also mentors to many students and postdocs who themselves went on to study a variety of different bacterial pathogens in their own laboratories.

Figure 6.
Philippe Sansonetti, Sam Formal, and Tony Maurelli, Silver Spring, MD, 2003.

Figure 1. Motility of *Shigella flexneri* 2a inside tissue culture cell.
Reprinted from reference 169.

Figure 2. Kiyoshi Shiga (1871–1957).

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