Comparative Virulence of Blood and Stool Isolates of *Shigella sonnei*

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Received 12 July 1993/Returned for modification 1 September 1993/Accepted 16 December 1993

Shigellemia is rare in developed countries and might result from the emergence of unusually virulent strains. We compared systemic invasiveness markers of isolates from the blood of 3 temporally clustered patients with *Shigella sonnei* bacteremia in Boston with those of 11 unrelated contemporaneous strains from stools of people in New England. We found no difference between the two groups in O-chain length by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, mouse 50% lethal dose, in vivo response to iron, and susceptibility to serum, which varied from moderately susceptible to ultrasusceptible. Mean intraperitoneal 50% lethal doses of smooth form I colonies for mice were equally low ($10^{5.8}$ CFU) in both groups, and the 50% lethal doses were lowered equally further in the two groups by predosing with iron to levels useful in mouse model sepsis studies. *S. sonnei* bacteremia may reflect compromised host defenses, not bacterial virulence.

Shigella bacteremia is rare in industrialized countries. In the United States, patients with AIDS, cancer, and other underlying diseases are at the highest risk of *Shigella* bacteremia (2, 8). Bacteremic shigellosis is more common in developing countries (16) where enteric infections are more prevalent. We detected three cases of *Shigella sonnei* bacteremia in Boston over only 2 years (1985 to 1987). This unusually high frequency suggested the possible emergence of virulent strains able to invade and survive in the bloodstream. To test the hypothesis that our blood isolates were unusually invasive, we compared them with randomly chosen contemporaneous *S. sonnei* isolates from the stools of people in New England for markers of gram-negative septicemic potential.

Classic gram-negative sepsis markers include serum resistance (14), in vivo iron scavenging (4), lipopolysaccharide (LPS) O-chain length (13), and mouse lethality after parenteral inoculation (1). Assessment of *S. sonnei* virulence is complicated by the normal reversion from smooth form I colonies to rougher form II colonies through the spontaneous loss of a 120-MDa plasmid coding for epithelial cell invasion proteins, O-antigen expression, and mouse virulence (3, 7, 9). Although models of human gram-negative septicemia have been hampered by the rarity of strains that are highly lethal to mice, mouse models of *S. sonnei* infection have appeared only in Russia (12).

Isolate BC 3175 was obtained from the blood of a 56-year-old homeless, confused, incontinent, alcoholic white male. He presented at Boston City Hospital with diarrhea, a temperature of 39°C, scleral icterus, hepatosplenomegaly, ascites, and a blood count with 9,200 leukocytes (WBCs) per mm³ of blood and 69,000 platelets per mm³ blood. He had a prolonged prothrombin time, elevated serum creatinine and liver enzyme levels, and a right upper lobe pulmonary infiltrate. Urine, cerebrospinal fluid, and ascitic fluid were acellular and culture negative. The only stool sample that was cultured (profuse watery diarrhea the day after admission) yielded no bacterial

pathogens. No sputum was obtained. Blood obtained for culture at the time of admission to the hospital was negative, but blood taken on hospital day 2 yielded trimethoprim-sulfamethoxazole-susceptible *S. sonnei*. Fever, bacteremia, diarrhea, and infiltrate resolved over 1 month with trimethoprim-sulfamethoxazole therapy.

Strain BC 3048 was isolated from the blood of a 2.5-year-old Hispanic male outpatient at Boston City Hospital after 3 days of vomiting, fever, and diarrhea. His neonatal screen for sickle cell disease was negative; he had no indication of any underlying disease. Stool and blood samples were cultured because of heme-positive diarrhea, a WBC count of 10,300/mm³, and a temperature of 40°C. He was recalled and admitted to the hospital on the next day for observation because a blood culture was positive for a gram-negative organism. However, his fever had fallen to 37.7°C and his WBC count had fallen to 10,000/mm³ without antibiotic therapy. This improvement continued after trimethoprim-sulfamethoxazole treatment was instituted; he was discharged after 4 days. Both blood and stool samples eventually yielded *S. sonnei* isolates susceptible to trimethoprim-sulfamethoxazole, but the stool isolate was not saved by the clinical laboratory.

Strain E 1613 was isolated from the blood of a 32-year-old human immunodeficiency virus-positive Haitian male with chronic hepatitis B virus infection and AIDS. He was admitted to Mt. Auburn Hospital with a temperature of 39.4°C, severe headache and stiff neck, oral thrush, and a WBC count of 6,000/mm³ of blood. His recent history included herpes zoster and oral herpes simplex virus infections, two episodes of diarrhea of unknown etiology, and >20-kg weight loss. Cryptococcal meningitis was confirmed by culture of cerebrospinal fluid and blood. Over the next 5 days his fever decreased with amphotericin B therapy. Morphine and meperidine hydrochloride (Demerol) treatment for headache led to constipation, which was treated with bisacodyl (Dulcolax), resulting in a liquid stool on day 5. This stool sample, like all others during his hospitalization, was not tested for bacterial pathogens. On hospital day 6 his temperature rose abruptly to >40°C, prompting ceftazidime and penicillin therapy after blood culture, which subsequently grew *S. sonnei*. He died from neurologic complications on hospital day 7.

Eleven *S. sonnei* strains from stools isolated during the same period as the three strains from blood were obtained from the

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TABLE 1. Lethalities of *S. sonnei* strains administered i.p. to mice with or without iron supplement

Isolate source	Colony type (form)	No iron		0.1 mg of iron		1.0 mg of iron	
		No. of strains tested	LD ₅₀ ^a	No. of strains tested	LD ₅₀	No. of strains tested	LD ₅₀
Blood	I	3	5.8 (5.5–6.0)	3	5.5 (5.3–5.8)	3	4.1 (4.0–4.2)
	II	3	8.8 (8.5–9.0)	3	8.2 (8.1–8.4)	3	8.2 (8.1–8.4)
Stool	I	11	5.8 (5.2–6.4)	3	5.8 (5.7–5.9)	3	4.1 (3.9–4.5)
	II	11 ^b	8.9 (8.4–9.1)	2	8.2 (7.9–8.4)	2	8.2 (7.9–8.4)

^a Geometric mean (range) log₁₀ median lethal dose.^b Means and ranges are based on six strains for which titration endpoints were reached. For five other strains, minimum log₁₀ LD₅₀s ranged from ≥8.4 to ≥8.7.

state health laboratories of Vermont and Massachusetts. Clinical histories were unobtainable for the seven strains from stools of people in Massachusetts. Of the four strains from stools of people in Vermont, three were from children who were outpatients (two afebrile siblings and one unrelated child with a temperature of 39.4°C) and one was from a hospitalized adult with a temperature of 38.3°C. No matching isolates from blood corresponding to the strains from stools at either state health department were available.

All strains were received from Boston City Hospital or the state health laboratories. Although hospital and state laboratories did not record passage histories, all strains retained the form I colony type, indicating that at least plasmid-encoded intestinal virulence factors were not lost during serial in vitro culture. Bacteria were grown and titrated as CFU on blood or brain heart infusion agar. The rate of reversion from form I to form II was similar within strains on the two agars; seed stocks of form I and isogenous form II sister colonies were frozen at –70°C. Working suspensions were made from selected microscopically examined colonies grown overnight. In 50% lethal dose (LD₅₀) assays, serial saline dilutions were inoculated intraperitoneally (i.p.) (0.2 to 0.5 ml) or intravenously (i.v.; 0.2 to 0.3 ml; tail vein) into groups of five female 4- to 7-week-old, viral antibody-free, CF-1 mice (Charles River, Wilmington, Mass.), and simultaneous CFU titrations were performed. Mice were observed for 1 week, but all deaths occurred by 3 days, and the mice had the classical signs compatible with gram-negative septicemia in this species: hunched posture, ruffled fur, and watery eyes. The rate of mortality consistently decreased and the incubation period increased with the use of increasingly dilute inocula. To confirm *S. sonnei* septicemia, blood from a moribund mouse inoculated with the lethal endpoint dilution of each of three isolates (BC 3175 and two isolates from stools of people in Vermont) was cultured on agar medium; blood from all three animals tested yielded *S. sonnei* on culture. Shigellemia was not confirmed for other strains because of consistently identical clinical signs and mortality patterns. In iron utilization tests in mice, iron dextran complex (Tech America, Elwood, Kans.) diluted in saline to contain either 0.1 or 1.0 mg of elemental iron per dose was inoculated i.p. 30 min before the bacteria were.

To measure complement-dependent lysis, we compared the survivals of logarithmic-phase bacteria grown for 4 h at 35°C and then suspended for 2 h at 35°C in either freshly thawed normal human serum or an aliquot of the same serum preheated to 56°C for 30 min (17). The index of serum resistance of Vosti and Randall (17) is 100 times the ratio of the surviving CFU to the inoculum CFU in unheated serum, with heat-inactivated serum serving as the growth and agglutination control. Only form I strains were assayed quantitatively; form II strains were uniformly very highly susceptible to serum by a qualitative screening plaque assay (5).

To visualize LPS, we extracted it from overnight brain heart infusion agar colonies by the hot phenol-water method (18) and resolved the different chain lengths by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10) with a 4% stacking gel and a 14.06% separating gel with 25% urea; this was followed by silver staining (kit 161-0443; Bio-Rad Laboratories, Richmond, Calif.).

The i.p. LD₅₀ of isolates from blood was indistinguishable from that of strains from stools, regardless of the bacterial form or the iron concentration inoculated (Table 1). The lowest LD₅₀ noted was 10^{3.9} CFU for a strain from the stool of an afebrile child in Vermont (form I; i.p.; supplemented with 1.0 mg of iron). By using the form I i.p. average LD₅₀ of 10^{5.8} CFU as a baseline, lethality decreased 2.5-fold by the i.v. route and 1,000-fold when form II was used and increased 50-fold with administration of 1.0 mg of supplemental iron.

Isolates from blood were also indistinguishable from a limited number of strains from stools when the isolates were administered by the i.v. route, which is used in anti-LPS immunoprophylaxis research (11). The three strains from stools varied in origin (two from Vermont, one from Massachusetts) and in the relative rate of reversion from form I to form II. The mean (range) log₁₀ LD₅₀s of three *S. sonnei* isolates from blood and stool were 6.2 (6.0 to 6.5) and 6.2 (6.1 to 6.3), respectively. Testing of isolates from stools was curtailed after initial comparisons showed no clear advantage of the use of blood over enteric isolates as potential i.v. challenge organisms in gram-negative sepsis models.

Table 2 shows the variation in resistance to serum between strains, but not between strains from blood and stool. By the criteria of Vosti and Randall (17), no strain was highly resistant (index, >100); one strain from blood and two strains from stools were classified as ultrasusceptible.

The LPS patterns determined by SDS-PAGE appeared to be the same for four isolates from stools and three isolates from blood (Fig. 1). All form I preparations gave strains with similar, typical long smooth "ladders." The LPS patterns of the rougher form II of one isolate from blood and one isolate from

TABLE 2. Susceptibilities of form I *S. sonnei* clinical isolates to serum

Source	No. of strains tested	No. of strains with serum bactericidal index of ^a :			
		0–1	1–9	10–25	40–50
Blood	3	1	1	0	1
Stool	10 ^b	6	1	3	0

^a Index of Vosti and Randall (17).^b Includes only one strain of two strains isolated from simultaneously infected children who were siblings.

FIG. 1. SDS-PAGE patterns of *S. sonnei* LPSs prepared from form I and form II colonies of isolates from blood (BL) and stool (ST).

stool were also identical; they were missing long O-antigen chains, as was expected.

At the Walter Reed Army Institute of Research, isolate BC 3175 from blood and one isolate from the stool of an afebrile child who was an outpatient in Vermont were both positive by the Serény test for guinea pig keratoconjunctivitis and marginally positive for HeLa cell penetration. The strains were judged to be equally typical of normally virulent *S. sonnei* (6). Testing was limited to two strains because these assays correlate with intestinal epithelial cell penetration and diarrhea, not septicemic invasion.

We found no difference in any marker of systemic invasiveness between *S. sonnei* isolates from blood and stools. Instead, the shigellemia in the patients described here may reflect deficient host defenses. One patient had AIDS, which has been associated with *S. sonnei* bacteremia (2); his slowed bowel motility because of the administration of opiates may be another predisposing factor (16). Another patient had severe alcoholic cirrhosis, which inhibits phagocytosis. The pediatric outpatient had no underlying disease, but young age is associated with *S. sonnei* bacteremia in developing countries. Since malnutrition worsens the prognosis (15, 16), his adequate nutrition may explain his spontaneous improvement before antibiotic therapy.

Our conclusions confirm and complement findings in Bangladesh which correlate shigellemia with host factors but not with bacterial virulence markers (15). The Bangladesh study compared isolates from the blood of 13 septicemic patients

with strains from the stools of 26 matched dysenteric but nonbacteremic control patients and included the four major *Shigella* species in both groups. A decreased bactericidal activity in host serum and increased transferrin iron saturation related directly to shigellemia and severe protein malnutrition in Bangladesh. Within a given *Shigella* serotype, tests for bacterial resistance to serum and siderophore type did not distinguish isolates from blood from strains from the stools of controls, and all *S. sonnei* isolates expressed both aerobactin and enterochelin siderophores. The siderophore studies done in Bangladesh provide a molecular explanation for our observations in mice; given hosts of equal nutritional status, the potential bacterial response to iron is equal in isolates from both blood and stools.

Our report extends the studies done in Bangladesh (15) by including endotoxin structure and an in vivo mouse shigellemia model and confirms that resistance to serum cannot distinguish strains from blood from those from stools. AIDS and alcoholism, implicated as shigellemia risk factors in our study and other studies done in the United States, were not identified in the report from Bangladesh (15).

Our work further shows that form I *S. sonnei* is consistently and unusually lethal to mice, qualifying it for a general model of human gram-negative sepsis (1, 3). Since strains of gram-negative bacilli that are highly lethal to mice are rare, the availability of *S. sonnei* as a frequent clinical isolate may prove to be useful.

We thank William R. McCabe for advice and facilitation of the study. We are grateful to Dale Vance and Susan Maker of the Vermont and Massachusetts state health laboratories, respectively, for making bacterial strains available.

This study was supported by grants NIH 2-S07-RR05569-23 and NIH 5 R01-AI 14789-10.

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