Shigella sonnei Does Not Use Amoebae as Protective Hosts

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ABSTRACT Shigella flexneri and Shigella sonnei bacteria cause the majority of all shigellosis cases worldwide. However, their distributions differ, with S. sonnei predominating in middle- and high-income countries and S. flexneri predominating in low-income countries. One proposed explanation for the continued range expansion of S. sonnei is that it can survive in amoebae, which could provide a protective environment for the bacteria. In this study, we demonstrate that while both S. sonnei and S. flexneri can survive coculture with the free-living amoebae Acanthamoebae castellanii, bacterial growth is predominantly extracellular. All isolates of Shigella were degraded following phagocytosis by A. castellanii, unlike those of Legionella pneumophila, which can replicate intracellularly. Our data suggest that S. sonnei is not able to use amoebae as a protective host to enhance environmental survival. Therefore, alternative explanations for S. sonnei emergence need to be considered.

IMPORTANCE The distribution of Shigella species closely mirrors a country’s socio-economic conditions. With the transition of many populous nations from low- to middle-income countries, S. sonnei infections have emerged as a major public health issue. Understanding why S. sonnei infections are resistant to improvements in living conditions is key to developing methods to reduce exposure to this pathogen. We show that free-living amoebae are not likely to be environmental hosts of S. sonnei, as all Shigella strains tested were phagocytosed and degraded by amoebae. Therefore, alternative scenarios are required to explain the emergence and persistence of S. sonnei infections.

KEYWORDS amoeba, Shigella sonnei, intracellular survival

Shigella is a genus of Gram-negative enteric pathogens comprised of four species. All species can cause severe diarrhea, and Shigella is estimated to cause 165 million infections and 120,000 deaths annually, accounting for 10% of deaths due to diarrheal disease worldwide (1, 2). Shigella flexneri and Shigella sonnei cause the majority of infections, but the ratio of species dominance is highly dependent on the socio-economic conditions of the area. In countries with a low per capita income, including those of sub-Saharan Africa and some countries in Asia, S. flexneri is the dominant cause of shigellosis, responsible for over 60% of infections. However, in areas with a high human development index, such as Europe and North America, S. sonnei causes around 80% of shigellosis cases (3). Transitioning countries currently undergoing socioeconomic improvements are experiencing a shift in the dominant species causing infections, from S. flexneri to S. sonnei. From 2001 to 2008 the prevalence of S. flexneri in Bangladesh decreased from 65.7% to 47%, while the prevalence of S. sonnei increased from 7.2% to 25% (4). During this time, Bangladesh underwent significant improvements in the nutritional status of children, health care, and water sanitation (5, 6). Other countries, such as China, Vietnam, and Brazil, have experienced a similar trend (7–9).

The reason for the rising dominance of S. sonnei in areas where the S. flexneri infection rate is decreasing is unclear. One hypothesis is that S. sonnei can use amoebae
as environmental hosts to protect it from water sanitation measures that are implemented in transitional countries (10). Amoebae are free-living organisms found in a variety of water sources, such as swimming pools and lakes, as well as in soil and dust. Importantly, they have even been found in chlorinated public water sources in developed countries (11). They are able to tolerate harsh and changing conditions, making them a good host for a variety of bacteria (12). *Legionella pneumophila* is the most well-known bacterium known to utilize amoebae as protective hosts, but *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium, and *Vibrio cholerae* have also been shown to survive intracellularly in amoebae (13–15, 16). However, some bacteria which were initially described as surviving in amoebae have later been shown to grow extracellularly, potentially through saprophytic growth on dead amoebae or amoeba waste (17–20).

Previous work has suggested that *S. sonnei* can survive in amoebae for extended periods of time. *S. sonnei*, *Shigella dysenteriae*, and *S. flexneri* were all found to be phagocytosed by *Acanthamoeba castellanii*; however, only *S. sonnei* and *S. dysenteriae* appeared to survive and replicate in the cytosol (21).

Here, we explore the hypothesis that amoebae can act as an environmental reservoir for *S. sonnei*. Although *S. sonnei* is phagocytosed by amoebae, we found no evidence that *S. sonnei* is able to survive and replicate in the cytosol of *A. castellanii*.

**RESULTS**

**Shigella cells survive extended coculture with amoebae.** Consistent with previous research, we observed that strains of *S. sonnei* and *S. flexneri* were able to survive in coculture with *A. castellanii* over 18 days at 22°C. We used two *S. flexneri* serotypes (strain M90T, serotype 5a, and strain 2457T, serotype 2a) and two *S. sonnei* isolates (the commonly used 53G strain and a recent clinical isolate, H140860381, here referred to as 381). All strains remained culturable at $10^6$ to $10^7$ CFU/ml over the 18 days (Fig. 1A). The amoebae were also maintained at ca. $5 \times 10^4$ cells/ml throughout this time period (Fig. 1B). These data indicate that *Shigella* species can survive extended coculture in the
presence of amoebae but give no information as to whether the bacteria are residing within amoebae and potentially utilizing the amoebae as an environmental reservoir.

We determined the intracellular bacterial numbers by taking samples at the indicated time points, treating with gentamicin to kill extracellular bacteria, and lysing the amoebae prior to CFU determination (Fig. 1C). This analysis revealed that all bacterial strains could be recovered intracellularly at all time points. However, fewer intracellular bacteria were recovered at the later time points. No difference was observed in intracellular bacterial numbers between the _S. flexneri_ and _S. sonnei_ strains at any time point.

The intracellular bacteria observed in this assay could be recently phagocytosed bacteria that had not yet been degraded, or bacteria that had established an intracellular niche and were surviving and/or replicating. We sought to examine these possibilities further.

**All Shigella strains are phagocytosed by A. castellanii.** To determine the efficiency of phagocytosis of _Shigella_ strains, amoebae incubated in low-nutrient medium (Page’s modified Neff’s amoeba saline [PAS]) at 22°C were allowed to phagocytose bacteria for 1 h, which was followed by 1 h gentamicin treatment to kill extracellular bacteria. The numbers of total bacteria (prior to gentamicin treatment) were similar for all strains and indicated that, on average, $5 \times 10^6$ CFU/ml, or approximately 50 bacteria/amoeba, were present (Fig. 2A). Following gentamicin treatment, recoverable CFU decreased by 4 log, indicating that the majority of the bacteria were extracellular or were rapidly degraded by the amoebae during the gentamicin incubation (Fig. 2B). There was no significant difference in the numbers of amoeba-associated bacteria or...

**FIG 2** _Shigella_ and _E. coli_ show similar levels of amoeba association and phagocytosis at 22°C. _A. castellanii_ was incubated with the indicated bacterial strains for 1 h, washed, and then either (A) followed by determination of cell-associated bacteria or (B and C) treated with gentamicin for 1 h to kill extracellular bacteria to determine the number of phagocytosed bacteria. (A and B) Experiments were conducted in low-nutrient medium or (C) high-nutrient medium. One-way analysis of variance (ANOVA) indicated no statistically significant differences between any bacterial strains. Mean and a standard deviation of 5 repeats are shown. (D) _S. sonnei_ 53G and _S. flexneri_ M90T strains expressing GFP were incubated with _A. castellanii_ and washed, and extracellular bacteria were detected with specific antibodies prior to visualization. Individual fluorescence channels for total and extracellular bacteria are shown in the top panels. The bottom panel represents merged transmitted-light images (to visualize the amoebae) and fluorescence images (green, total bacteria; red, extracellular bacteria).
phagocytosed bacteria between nonpathogenic *Escherichia coli* strain MG1655 and the *Shigella* strains, or between the *Shigella* species.

We repeated the experiments in high-nutrient medium (peptone-yeast-glucose medium [PYG]) to increase the rate of phagocytosis by amoebae. As anticipated, significantly higher numbers of phagocytosed bacteria could be enumerated than in low-nutrient medium (Fig. 2C). On average, there were 0.5 intracellular bacteria/amoeba. However, we again saw no difference between the phagocytosis rates for *S. flexneri* or *S. sonnei* strains. All *Shigella* strains showed a small, nonsignificant trend of increased phagocytosis by amoebae compared to that for nonpathogenic *E. coli*. Microscopic analysis of differentially stained bacteria confirmed the presence of intracellular bacteria for both *S. sonnei* and *S. flexneri* (Fig. 2D). Extracellular bacteria can be seen adhered to the plastic, rather than to the amoebae, suggesting that the amoebae efficiently phagocytose all bacteria contacted.

**Shigella does not survive intracellularly in *A. castellanii***. We tested the intracellular survival of *Shigella* by CFU determination at 1 h, 4 h, and 20 h. Cell counts for all strains decreased over this time frame similarly to those of the negative control of nonpathogenic *E. coli*, indicating they were efficiently digested by *A. castellanii*. The same trend of reduced intracellular numbers over time was observed in both high- (Fig. 3A) and low-nutrient media (Fig. 3B), with a 2-log decrease between 1 h and 20 h. Due to the low number of phagocytosed bacteria in low-nutrient medium, by 20 h all strains were below the limit of detection, unlike in high-nutrient medium, where approximately 1,000 bacteria/sample were still recoverable.

To confirm that the amoebae were capable of facilitating intracellular growth, *L. pneumophila* was used as a positive control. These experiments were conducted at 30°C, as this is the optimal temperature for *L. pneumophila* growth (Fig. 3C). As expected, the numbers of culturable wild-type *L. pneumophila* cells increased over the 20 h of incubation, whereas cell counts for all *Shigella* strains again decreased, in line with those of nonpathogenic *E. coli*.

To investigate the intracellular fate of bacteria, we observed the association of bacteria with amoebae stained with neutral red (Fig. 3D). Neutral red preferentially accumulates in lysosomes, due to their relative acidity (22, 23). *S. flexneri*, *S. sonnei*, and *E. coli* were all observed to have neutral red structures accumulating around the intracellular bacteria and to be infiltrated with neutral red, suggesting they were being digested by the amoebae (24). This provides a visual confirmation of the intracellular killing observed in the preceding assays by bacterial enumeration.

**The T3SS does not alter *Shigella* interaction with amoebae.** Considering the importance of the type 3 secretion system (T3SS) for *Shigella* virulence, the intracellular survival assays were repeated at 37°C, the temperature at which the T3SS is active and effector proteins are translocated (25). *Shigella* bacteria induce different T3SS-dependent outcomes depending on the cell type infected; in epithelial cells, vacuolar escape and intracellular replication, and in macrophages, vacuolar escape and pyroptosis.

If the T3SS facilitated intracellular survival within amoebae, we would expect to see increased intracellular bacterial counts at 4 h and 20 h during incubation at 37°C. Instead, we saw a decrease in viable intracellular bacteria numbers, similar to those at 22°C and 30°C, suggesting that an active T3SS could not facilitate intracellular survival in amoebae (Fig. 4A). To further investigate the involvement of the T3SS, the intracellular survival of T3SS mutants was determined. Again, no difference in intracellular survival between wild-type and T3SS mutants was found (Fig. 4B), indicating that the T3SS was not altering the interaction of *Shigella* with amoebae.

It was previously reported that *S. flexneri* used its T3SS to kill amoebae (21, 26). Having not seen an effect of the T3SS (Fig. 4A and B), or significant amoeba death upon long-term exposure to *S. flexneri* (Fig. 1C), we decided to investigate amoeba cell death further by using a propidium iodide (PI) assay to measure the membrane integrity of the amoebae (Fig. 4C). At all temperatures analyzed, there were no significant differ-
ences in PI levels between amoebae infected with the negative controls (E. coli MG1655 and Shigella T3SS mutants) and any of the wild-type Shigella strains. Therefore, in our assays neither S. flexneri or S. sonnei were able to induce cell death in amoebae.

5. sonnei is not released by amoebae. V. cholerae was recently shown to resist intracellular killing by A. castellanii, and at low frequency it can be released intact by the amoebae (16). While we have seen no evidence of Shigella resisting intracellular killing, we questioned whether a small number of bacteria were being released from the amoebae. Following killing of extracellular bacteria with gentamicin treatment, fresh medium with no gentamicin was added to cells, and the cell supernatant was harvested after 4 h (Fig. 4D). Low numbers of Shigella were recovered from the supernatant, and these could potentially be a source of Shigella for infection. However, there was no significant difference in bacterial release between S. sonnei and S. flexneri strains, and indeed, no significantly increased numbers of bacteria were released compared to those for nonpathogenic E. coli, indicating this is not a Shigella- or S. sonnei-specific mechanism for dispersal.

Intracellular Shigella bacteria are not more infectious. Amoebae have been proposed to act as “training grounds” for intracellular pathogens, adapting them to an intracellular lifestyle (27). While this is considered a long-term adaptation, we ques-
tioned whether it facilitated short-term infectivity as well. We therefore tested whether bacteria harvested from amoebae were more proficient at invading or replicating within mammalian cells. We found the bacteria harvested from amoebae were less able to invade and survive in mammalian cells (Fig. 5A and B). These findings support the previous conclusion that Shigella bacteria are being degraded by the amoebae, rather than adapting and surviving.

DISCUSSION

The frequency of S. sonnei isolation directly correlates with per capita gross domestic product (GDP) (28). The underlying reason(s) for this association is not understood, although a number of hypotheses have been proposed, one of which is that S. sonnei uses amoebae as a protective host (10). We show here that S. sonnei has no survival advantage in amoebae compared to S. flexneri, or indeed, compared to nonpathogenic E. coli. Both Shigella species were able to survive in long term coculture assays in low-nutrient medium suggesting that, like Listeria monocytogenes, Shigella species can utilize amoeba debris for nutritional requirements (17). However, this offers no explanation for why S. flexneri, but not S. sonnei, levels of infection are reduced in areas where living conditions and water sanitation are improved.

Having disproven one hypothesis explaining the emergence of S. sonnei, it remains to experimentally test additional hypotheses. One popular suggestion is that exposure to unsanitized water in developing countries can result in Plesiomonas shigelloides infection and hence in natural immunity against S. sonnei. P. shigelloides serotype O17
has a lipopolysaccharide O-antigen identical to that of *S. sonnei* (10). People living in areas with good water sanitation would therefore have reduced exposure to *P. shigelloides*, and hence reduced cross-protection against *S. sonnei*. This hypothesis is difficult to prove without widespread serological data from countries with high *S. flexneri* versus high *S. sonnei* infection rates. However, it also suggests additional differences regarding the transmission of *S. sonnei*, as the reduced exposure to *P. shigelloides* and *S. flexneri* through improved water quality does not extend to *S. sonnei*. This could be explained by the suggestion that *S. sonnei* is spread directly from person to person or that *S. sonnei* has an increased ability to acquire antibiotic resistance. Acquisition of antimicrobial resistance has clearly aided the spread and establishment of particular *S. sonnei* isolates (29). However, epidemiological data indicate that *S. flexneri* and *S. sonnei* isolates have similar resistance profiles (30, 31), suggesting antibiotic resistance alone does not explain the altered transmission.

*S. sonnei* possesses multiple antibacterial mechanisms. The majority of clinical isolates produce colicins (29, 32, 33), which are effective against a narrow phylogenetic range of bacteria. While *S. flexneri* cells are also reported to produce bacteriocins (34), there are few studies indicating the prevalence or identity of bacteriocins in *S. flexneri* clinical isolates. *S. sonnei* has also recently been shown to have a functional type 6 secretion system (T6SS), which provides a niche-specific competitive advantage for *S. sonnei* over *S. flexneri* (35). Therefore, the success of *S. sonnei* may be explained by a combination of these factors potentially altering colonization dynamics and facilitating person-to-person spread.

We have demonstrated that amoebae are not a protective host for *S. sonnei* and that alternative explanations for the rising rates of *S. sonnei* infection in transitional countries require further investigation. Now that it is well established that *S. sonnei* possesses unique pathogenic traits (35–37), considerable work is required to understand the differences in virulence and transmission of *S. sonnei* in comparison to those of *S. flexneri*.

**MATERIALS AND METHODS**

**Bacterial strains and growth.** Isolates of *Shigella* (Table 1) were plated on trypticase soya agar (TSA) + 0.01% Congo red to identify those with a large virulence plasmid (LVP) (38). Colonies were inoculated in trypticase soya broth (TSB) and incubated overnight at 37°C and 200 rpm. The overnight culture was diluted 1:100 in TSB and incubated until an optical density at 600 nm (OD₆₀₀) of 0.5 was reached. Bacteria were washed in phosphate-buffered saline (PBS), resuspended in the appropriate medium, and added to cells.

*Legionella* cells were plated on buffered charcoal-yeast extract (CYE) agar plates for 3 days at 37°C. Bacterial colonies were diluted to OD₆₀₀ = 0.1 in ACES [N-(2-acetamido)-2-aminoethanesulfonic acid] yeast extract (AYE) broth and incubated for 21 h at 37°C and 200 rpm.

**FIG 5** Intracellular bacteria are not hyperinfectious for epithelial cells. (A) Bacteria harvested from amoebae following 1 h of gentamicin treatment were used to directly infect epithelial cells, in parallel with standard log-phase bacteria grown in TSB at 37°C or 22°C. Following a 30 min infection and 1 h of gentamicin treatment, intracellular bacteria were released, and invasive bacteria was enumerated by colony counting. (B) The intracellular survival of these bacteria was measured after 3 h of gentamicin treatment. The fold change from 1 h to 3 h postinfection was calculated. Solid lines indicate bacteria grown in TSB at 37°C, dashed lines indicate bacteria grown in TSB at 22°C, and dotted lines indicate bacteria harvested from amoebae.
TABLE 1  Bacterial strains

| Strain      | Details                                                      | Source and/or reference |
|-------------|--------------------------------------------------------------|-------------------------|
| S. sonnei 53G | Clinical isolate                                             | 36                      |
| S. sonnei 381 | Clinical isolate H140860381                                   | C. Jenkins, PHE°         |
| S. flexneri M90T | Serotype 5a                                                   | 37                      |
| S. flexneri 245TT | Serotype 2a                                                  | 38                      |
| S. flexneri M90TAT3SS | mxID replaced with aphA-3, conferring kanamycin resistance | 39                      |
| S. sonnei 53GAT3SS | mxID replaced with aphA-3, conferring kanamycin resistance | This study               |
| S. sonnei 53G/GFP | Expresses GFP from pUltraGFP-GM                             | This study, 42            |
| S. flexneri M90T/GFP | Expresses GFP from pUltraGFP-GM                             | This study               |
| L. pneumophila 130b | Serotype O1; clinicola                                       | ATCC BAA-74 (41)         |

°GFP, green fluorescent protein.
°PHE, Public Health England.

**Cell culture.** Acanthamoeba castellanii cells (a kind gift from C. Buchrieser, Institut Pasteur) were cultured in ATCC 712 peptone-yeast-glucose medium [PYG] medium 2% protease peptone, 0.1% yeast extract, 0.1 M glucose, 4 mM MgSO4·7H2O, 0.4 mM CaCl2, 0.05 mM Fe(NH4)2(SO4)2·6H2O, 2.5 mM KH2PO4, 2.5 mM Na2HPO4, 16 mM NaCl, and 0.01% Na citrate. Inoculation was carried out in 2% BSA in LF medium for 30 min. Amoebae were washed with ice-cold LF medium, centrifuged briefly (2 min, 1,000 g) at room temperature (RT). Amoebae were washed in LF medium before addition of bacteria harboring GFP and centrifuged briefly (2 min, 1,000 g). Amoebae were overlaid with agarose and immediately imaged on a Zeiss Axio Observer inverted microscope.

**Fluorescence microscopy.** Amoebae seeded on 4-well µ-slides (Ibidi) were infected as above with GFP (green fluorescent protein)-expressing S. sonnei 53G and GFP-expressing S. flexneri M90T. Amoebae were then washed with low-fluorescence (LF) medium (41) and placed on ice, and extracellular bacteria were detected with sera against S. sonnei (Remel agglutinating sera) or S. flexneri 5a (Public Health England [PHE]) in 2% BSA in LF medium for 30 min. Amoebae were washed with ice-cold LF medium, followed by anti-rabbit Cy3 (Jackson ImmunoResearch) in 2% BSA in PAS for 30 min. Amoebae were washed with LF medium before being overlaid with agarose and immediately imaged on a Zeiss Axio Observer inverted microscope.

**PI assay.** A. castellanii was seeded as described previously in 24-well plates. Immediately prior to infection, medium was replaced with 5 µM propidium iodide in PAS. Infection was carried out as described, and cells were incubated at 22°C, 30°C, or 37°C. At 1 h postinfection, gentamicin was added directly to wells to a final concentration of 150 µg/ml for 3 h. For 100% membrane permeabilization control, sodium deoxycholate was added to wells at a final concentration of 0.25% for 10 min. Fluorescence was measured at 530/620 nm on a FLUOstar Omega microplate reader (BMG Labtech).
**HeLa cell infection.** HeLa cells were seeded in 96-well plates at 1 × 10^4 cells/ml 24 h prior to infection. A. castellanii was seeded at 10^5 cells/flask in T75 flasks in PAS medium, infected with Shigella at an MOI of 100, and incubated at 22°C. After 24 h, amoebae were detached, centrifuged at 500 × g for 5 min, and resuspended in 150 μg/ml gentamicin in PAS for 1 h. Amoebae were then washed with PAS and lysed with 0.25% sodium deoxycholate. Released bacteria were centrifuged, washed, and resuspended in DMEM. Broth-cultured Shigella cells were prepared as described above and resuspended in DMEM. Prior to infection, medium was replaced with serum-free DMEM, and HeLa cells then infected at an MOI of 100 with Shigella cells released from amoebae or grown in broth at 37°C or 22°C. Cells were centrifuged at 600 × g for 10 min and incubated for 30 min at 37°C and 5% CO₂. Medium was replaced with 150 μg/ml gentamicin in supplemented DMEM and incubated for a further 1 h or 3 h. At these time points, cells were washed with PBS and lysed with 0.5% Triton X-100. Serial dilutions were performed and plated to calculate intracellular CFU.

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