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New Animal Model of Shigellosis in the Guinea Pig: Its Usefulness for Protective Efficacy Studies

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It has been difficult to evaluate the protective efficacy of vaccine candidates against shigellosis, a major form of bacillary dysentery caused by Shigella spp. infection, because of the lack of suitable animal models. To develop a proper animal model representing human bacillary dysentery, guinea pigs were challenged with virulent Shigella flexneri serotype 2a (strains 2457T or YSH6000) or S. flexneri 5a (strain M90T) by the intrarectal (i.r.) route. Interestingly, all guinea pigs administered these Shigella strains developed severe and acute rectocolitis. They lost ~20% of their body weight and developed tenesmus by 24 h after Shigella infection. Shigella invasion and colonization of the distal colon were seen at 24 h but disappeared by 48 h following i.r. infection. Histopathological approaches demonstrated significant damage and destruction of mucosal and submucosal layers, thickened intestinal wall, edema, infiltration of neutrophils, and depletion of goblet cells in the distal colon. Furthermore, robust expression of IL-8, IL-1β, and inducible NO synthase mRNA was detected in the colon from 6 to 24 h following Shigella infection. Most importantly, in our new shigellosis model, guinea pigs vaccinated with an attenuated S. flexneri 2a SC602 strain possessing high levels of mucosal IgA Abs showed milder symptoms of bacillary dysentery than did animals receiving PBS alone after Shigella infection. In the guinea pig administration of Shigella by i.r. route induces acute inflammation, making this animal model useful for assessing the protective efficacy of Shigella vaccine candidates.


The Shigella spp. causes bacillary dysentery by invading the large intestinal epithelium and promoting a strong inflammatory response in humans and nonhuman primates (1–3). The hallmark of clinical shigellosis is an acute rectocolitis associated with fever, nausea, anorexia, dehydration, mucopurulent and bloody diarrhea, and tenesmus. Shigella infection can be provoked by an extremely small number of bacteria (10–100 organisms) in humans (4). Worldwide, 164.7 million episodes of Shigella infection are seen each year, ~1.1 million of which end in death. There are 163.2 million cases that occur in developing countries as a result of unhygienic conditions. Children younger than 5 years of age accounted for 69% of all Shigella episodes and 61% of all Shigella-related deaths (2, 5).

The characteristic feature of Shigella pathogenesis is the ability to invade various cells in the large intestine, including macrophages, dendritic cells, and epithelial cells. When Shigella reach the colon and rectum, they translocate through the epithelial barrier via M cells that overlie colonic patches and isolated lymphoid follicles (6, 7). Once they reach the sublumen region, Shigella are phagocytosed by resident macrophages before escaping from the phagosome into the cytoplasm. There, the bacteria multiply, inducing cell death through activation of caspase-1, which in turn leads to the maturation and release of IL-1β (3, 8–10). Shigella then escape from killed macrophages and enter the IL-8-secreting (or CXCL8) epithelial cells, thus triggering a strong inflammatory response that leads to the migration of polymorphonuclear cells (PMN)3 such as neutrophils (11, 12). Furthermore, reactive oxygen metabolites and nitrogen species (i.e., NO) released by phagocytes contribute to microbicidal activities and tissue damage (13). The invasion eventually results in extensive inflammation and tissue destruction of the colon and rectum (11).

The World Health Organization has made the development of a safe and effective vaccine against Shigella a high priority because Shigella is showing increasing antibiotic resistance, even to the newest antibiotics (14). To meet these twin goals, a vaccine would have both to be attenuated in its virulence and capable of sustaining colonization and protective efficacy at the preclinical stage. To screen vaccine candidates for such attributes, an animal infection model mimicking human shigellosis would be essential. However, the four Shigella species (i.e., S. flexneri, S. dysenteriae, S. sonnei, and S. boydii) do not produce acute rectocolitis in experimental animals. The lack of a relevant animal model reproducing human bacillary dysentery is one of the major roadblocks to developing a successful Shigella vaccine. To overcome this hurdle, considerable effort over the years has been devoted to establishing a reliable animal model of bacillary dysentery. Several established Shigella infection models have proven to be useful; for instance, the pneumonia model by intranasal challenge in mice, keratoconjunctivitis

3 Abbreviations used in this paper: PMN, polymorphonuclear cell; i.r., intrarectal; PAS, periodic acid-Schiff; iNOS, inducible NO synthase.

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FIGURE 1. Establishment of bacillary dysentery models by virulent S. flexneri strains in guinea pigs. The guinea pigs were i.r. challenged with 10^6 CFU of several virulent strains such as S. flexneri 2a 2457T, S. flexneri 2a YSH6000, and S. flexneri 5a M90T and observed for physiological (A) and macroscopic (B) changes. All guinea pigs infected with the S. flexneri 2a 2457T strain developed bacillary dysentery characterized by weight loss, tenesmus, and liquid stool mixed with mucus and blood within 24 h. Noninvasive S. flexneri 2a 2457T-vp strain was used for negative control. The incidents of tenesmus occurring in a 1-h period were counted by 24 h after challenge. **, p < 0.01, when compared with initial body weight.

### Materials and Methods

#### Animals

Female specific pathogen-free Hartley guinea pigs ranging in weight from 120 to 250 g were purchased from the Charles River Laboratories. For the duration of the experiments, the animals were maintained in the animal care facilities of the International Vaccine Institute (Seoul, Korea) under specific pathogen-free conditions and received sterilized food (Certified Diet MF; Orient Company) and water ad libitum.

#### Shigella strains

The virulent S. flexneri serotype 2a strains 2457T and YSH6000 (8), as well as the S. flexneri serotype 5a strain M90T (20), were used to develop shigellosis in the guinea pig. The noninvasive S. flexneri 2a 2457T-vp strain was used for control, and the avirulent attenuated S. flexneri 2a strain SC602 (6) was used for vaccination. The Shigella strains were grown routinely in Soybean-Casein Digest broth (Difco) or Luria-Bertani broth (LB).

#### Vaccination and bacterial infection

Guinea pigs were anesthetized using a mixture of ketamine (100 mg/kg of body weight; Yuhan) and xylazine hydrochloride (10 mg/kg of body weight; Bayer) before being inoculated via an intrarectal (i.r.) route with 10^9 CFU of each wild-type Shigella strain in 100 μl of PBS. Guinea pigs were i.r. vaccinated with 5 × 10^9 CFU of attenuated Shigella SC602 strain in 100 μl of PBS under anesthesia and received a boost 2 wk after the first immunization.

#### Histopathological analysis

Randomly selected distal regions of the colon were washed with PBS and fixed in 4% formaldehyde for 1 h at 4°C. The tissues were dehydrated by gradually soaking in alcohol and xylene and then embedded in paraffin (21). The paraffin-embedded specimens were cut into 5-μm sections and stained with H&E or Alcian blue-periodic acid-Schiff (PAS; Merck) according to the manufacturer’s instructions. To detect invasion of Shigella into the colon, dehydrated frozen tissue sections were stained with rabbit anti-S. flexneri 2a-derived LPS Ab (Denka Seiken) and then visualized using FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich). Mouse anti-guinea pig macrophages (clone name MR-1) were used to recognize macrophages, whereas mouse anti-guinea pig T cell Abs (clone name CT5; Serotec) were used for T cells. DAPI (4′,6′-diamidino-2-phenylindole; Molecular Probes) was used to stain the nucleus. The sections were mounted with PermaFluor mounting medium (Thermo) and viewed under a confocal scanning laser microscope (Zeiss).

#### cDNA synthesis and real-time PCR

Samples of the distal regions of the colons of guinea pigs were taken at 0, 2, 6, 12, 24, and 48 h following Shigella infection and then homogenized after washing with nuclease-free water. Total RNA was extracted with TRIzol reagent (Invitrogen Life Technologies) and 5 μg of RNA was converted into cDNA using Superscript II Reverse Transcriptase (Invitrogen Life Technologies). The products were used as a template for each cytokine-specific real-time PCR set for the amplification of β-actin, IL-8, IL-1β, and inducible NO synthase (iNOS). TaqMan primer-probe sets for each cytokine were designed for gene expression assays (Applied Biosystems). The amplification reactions were performed with 100–200 ng of cDNA (TaqMan Universal PCR Master mix; Applied Biosystems) and each of the designated probes (Applied Biosystems). Then gene expression quantification was performed using an ABI PRISM Sequence Detection System Instrument (Applied Biosystems). The levels of mRNA expression were displayed as the expression units of each target gene relative to the expression units of expressed β-actin.

#### ELISA

The microtiter plates (BD Falcon) were coated with S. flexneri 2a-derived LPS (1 μg/ml) in 50 mM sodium bicarbonate (pH 9.4) and incubated overnight at 4°C. After blocking with 1% BSA in PBS, 2-fold serially diluted samples (starting from 1/32 for serum, 1/2 for fecal extracts) were applied to plates and incubated for 2 h at 37°C. The HRP-conjugated goat anti-guinea pig IgG Ab (Bethyl Laboratories) or rabbit anti-guinea pig IgA Ab (Bethyl Laboratories) and biotin-conjugated goat anti-rabbit IgG Abs (BD Pharmingen) and HRP-streptavidin (Vector Laboratories) were used for detection of IgG and IgA, respectively. After tetramethylbenzidine (Moss) solution as a substrate was added to each well and stopped with 0.5 N HCl, plates were measured at 450 nm on an ELISA reader ( Molecular Devices). Endpoint titers of LPS-specific Abs were expressed as reciprocal log₂ titers.

#### Disease score

Using a blinded test slightly modified from previous studies, we assessed the level of destruction in the mucosal and submucosal layers, as well as the erosion, crypt distortion, and PMN infiltration into the inflammation site to arrive at disease scores (11, 16).
Statistics
The data were expressed as the mean ± SD and compared by Student’s t test using SigmaPlot software. Each experiment was repeated at least three times.

Results
Establishment of Shigella-induced bacillary dysentery in the guinea pig

To develop a new animal model reproducing human shigellosis, we tested several different strains of Shigella using guinea pigs. Guinea pigs received S. flexneri 2a (strains 2457T or YSH6000) or S. flexneri 5a (strain M90T) by the i.r. route and were then monitored for signs of clinical dysentery at different time points. We have tested four different doses of each strain of bacteria (10^8, 10^9, 10^10, or 10^11 CFU), finding that all doses confer significant signs of bacillary dysentery (data not shown). To ensure the induction of intense inflammation, we have chosen 10^9 CFU of bacteria for further study. In addition, we did not starve the guinea pigs or subject them to antibiotic treatment to maintain the natural conditions of the resident microflora in the gut. Within 24 h following challenge, all guinea pigs infected with invasive wild-type Shigella strains developed bacillary dysentery characterized by weight loss, tenesmus, and liquid stool mixed with mucus and blood, whereas the control groups infected with PBS or a noninvasive Shigella strain did not develop symptoms (Fig. 1). Tenesmus, characterized by a sudden cramp that causes the body to rise up for an instant (supplemental data),^4^ frequently begins to occur at around 24 h postinfection and is observable up to 48 h after infection. Furthermore, the body temperatures were increased by ~2°C at 24 h postinfection compared with the noninfected group (data not shown). Among the three wild-type Shigella strains, the S. flexneri 2a 2457T strain, which is common in developing countries, was chosen for further analysis. Macroscopically, the distal region of the large intestine of guinea pigs i.r. challenged with S. flexneri 2a 2457T showed inflammation and internal hemorrhage at 24 h postinfection (Fig. 1B). To our knowledge, we are the first to report a guinea pig model that successfully mimics human bacillary dysentery without the need for starvation and/or antibiotic treatment.

^4^ The online version of this article contains supplemental material.
Established infection model in the guinea pig represents histological characteristics of shigellosis in humans

To examine the histological changes, the distal region of the colon in the guinea pig was observed for tissue destruction and inflammation at different time points following i.r. challenge with *S. flexneri* 2a 2457T. At 2 h following infection with *S. flexneri* 2a 2457T, the infected group showed no significant difference from the PBS-treated control; however, at 6 h, animals in this group began to exhibit inflammatory signs (data not shown). At time points extending beyond 48 h postinfection, inflammation caused thickened intestinal walls, complete destruction of mucosal and submucosal layers, edema, erosion, crypt distortion, and infiltration of mononuclear cells (Fig. 2, D–I). Interestingly, diffuse inflammatory infiltrates of the muscularis mucosae and submucosa of the colon were observed (Fig. 2, D and E). To further characterize the signs of inflammation, we stained mucin-secreting goblet cells, known to play a role in the maintenance of gut homeostasis, with Alcian blue-PAS staining method. Interestingly, goblet cells were completely depleted at 48 h following i.r. challenge, in sharp contrast to the PBS group (Fig. 2, C, F, and I). High magnification revealed that a high proportion of PMN was recruited into the submucosal and lamina propria regions at an early stage of infection (Fig. 2, J and K). To better characterize the lesions and introduce grading, we counted the number of crypts and PMNs per 100 μm² on the distal region of large intestine isolated from either the PBS-treated or *Shigella*-infected groups (Fig. 2L). As expected, we found significantly fewer crypts but a higher rate of PMN infiltration in *Shigella*-infected than PBS-treated guinea pig.

*Shigella* rapidly invade and colonize the distal region of the colon

To assess whether *S. flexneri* are able to invade and colonize the distal colon, bacteria were visualized by rabbit Abs specific for *Shigella* LPS at different times following i.r. challenge with *S. flexneri* 2a 2457T. One of the interesting findings is the speed with which *Shigella* colonization and replication follow infection. As shown in Fig. 3A, *Shigella* bacteria invaded and colonized tissues as early as 12–24 h postinfection, before gradually declining and finally disappearing at 48 h. However, at 2–6 h postinfection, very few *Shigella* had invaded the epithelium. At 24 h following challenge, bacteria remained in both lamina propria and submucosal regions. To further characterize which cell subsets were affected by i.r. challenge with *S. flexneri* 2a 2457T, Abs specific for macrophages and T cells of guinea pig were adopted for immunohistochemical study (Fig. 3B). Interestingly, at 48 h after infection, a greater number of macrophages was observed at the inflamed site of the colon, but no significant change in T cell levels was noted. These results suggest that this new guinea pig model allows for a successful bacterial colonization of the colonic mucosa by i.r. challenge with virulent *S. flexneri* 2a without altering the gut microflora. They also demonstrate that macrophages play a crucial role in the development of shigellosis.
High expression of proinflammatory cytokines and iNOS in response to Shigella infection

To further characterize immunopathological changes in this model, the gene expression of proinflammatory cytokines and iNOS were analyzed in the distal region of the large intestine at different time points after S. flexneri 2a 2457T infection. The colonic tissues were homogenized, and the levels of mRNA for IL-8, IL-1β, and iNOS, which might be most critically involved in the development of the Shigella infection, were measured. The mRNA levels of IL-8 and IL-1β gradually increased following infection and were significantly higher from 6 to 24 h than those of the PBS group (Fig. 4). The expression of iNOS mRNA, which reflects the production of NO, was also significantly increased at 24 h after S. flexneri 2a infection (Fig. 4). Predominant expression levels of iNOS could be expressed by the large number of neutrophils recruited into the large intestine following i.r. challenge (Fig. 2). Overall, the high transcription levels of IL-8, IL-1β, and iNOS mRNA in the distal region of the colon suggest that these molecules contribute to the immunogenesis of rectocolitis caused by S. flexneri 2a in this guinea pig model.

Newly developed guinea pig infection model can be useful for protection assays

To clarify the utility of this guinea pig model for evaluation of the protective efficacy of vaccine candidates, groups of guinea pigs were vaccinated with 5 × 10⁸ CFU of the attenuated strain S. flexneri 2a SC602 (6, 22) or PBS twice by the i.r. route. This attenuate strain was constructed by deleting the virulent gene for dissemination (icsA) and survival (icsC) of bacteria within the colonic mucosa. One week after the second vaccination, Shigella-derived LPS-specific IgG and IgA Abs were measured in the serum and fecal extracts, respectively. As shown in Fig. 5B, significantly higher levels of LPS-specific IgG and IgA Abs were elicited in both serum and fecal extracts of vaccinated guinea pigs than were found in extracts of controls. Next, groups of guinea pig were i.r. challenged with 10⁷ CFU of wild-type S. flexneri 2a 2457T so that the onset and severity of shigellosis could be studied. Interestingly, the group vaccinated with the S. flexneri 2a SC602 strain did not suffer from weight loss and tenesmus (Fig. 5A). Then, the levels of mucosal tissue destruction, erosion, and neutrophil infiltration were clinically scored. Following Shigella challenge, the i.r.-vaccinated group revealed a significantly lower clinical score than that of the PBS group (Fig. 5C). Additionally, a greater reduction in the number of Shigella bacteria localized in the distal region of the large intestine was seen in the vaccinated group than in the PBS group (Fig. 5D). These results strongly suggest that this guinea pig model will prove reliable and useful in protection assays designed to select promising Shigella vaccine candidates.

FIGURE 4. Expression levels of mRNA for IL-8, IL-1β, and iNOS in the Shigella-induced dysentery model. Tissue samples were taken randomly from the inflamed region of the colon of the guinea pigs at different time points following i.r. challenge with S. flexneri 2a 2457T. mRNA expression levels were measured by the real-time PCR method using IL-8, IL-1β, and iNOS primer sets designed by Applied Biosystems. The levels of mRNA expression were displayed as the expression units of each target gene relative to the units of expressed β-actin. The results are expressed as the mean ± SD from three different experiments. ***, p < 0.001 when compared with PBS.

FIGURE 5. Evaluation of guinea pig models for protection assays. Groups of guinea pigs were vaccinated with 5 × 10⁸ CFU of the avirulent S. flexneri 2a SC602 strain or with PBS alone by the i.r. route. At 7 days after the second vaccination, both vaccinated groups were challenged with S. flexneri 2a 2457T. A. Physiological changes were monitored at 24 h after challenge. The frequency of tenesmus was determined from 24 to 25 h after i.r. challenge. *, p < 0.05, when compared with initial body weight. B. The levels of LPS-specific IgG Ab in the serum (□) and IgA Ab in fecal extract (●) were measured, respectively. *, p < 0.05 and ***, p < 0.001, when compared with the PBS group. C. Disease scores were graded in the PBS group or S. flexneri 2a SC602-vaccinated groups at 24 h following i.r. challenge. Scoring was based on levels of destruction of the mucosal and submucosal layers, erosion, crypt distortion, and neutrophil infiltration into the inflammation sites using a blinded test. *, p < 0.05, when compared with the PBS group. D. Localization of bacteria in the distal region of the colon was detected by rabbit anti-Shigella LPS Ab and FITC-conjugated goat anti-rabbit IgG Ab. The results are expressed as the mean ± SD from several experiments. Scale bar, 50 μm.
Discussion

In this study, we sought to establish a new bacillary dysentery model in the guinea pig by inoculating Shigella through the i.r. route. An i.r. challenge with three different strains of S. flexneri, i.e., 2a 2457T, 2a YSH6000, or 5a M90T, induced severe and acute bacillary dysentery that mimicked human shigellosis in terms of loss of body weight, fever, tenesmus, severe damage to the colonic mucosa, enhanced expression of proinflammatory cytokines such as IL-8, and predominant recruitment of neutrophils. Most interestingly, this novel model is reproducible and so should prove to be a useful and reliable tool for analyzing the safety and protective efficacy of vaccine candidates.

A previous study using rectal biopsy specimens from acute Shigella dysentery patients identified the histopathological symptoms that characterize human bacillary dysentery (11). The majority of shigellosis patients show marked damage of the epithelial barrier, cellular infiltration (lymphocytes, plasma cells, and neutrophils), dramatic tissue injury (erosion, crypt alterations, necrosis, and edema), goblet cell depletion, and watery diarrhea (11, 23). General histological studies of rectal biopsies of Shigella-infected humans revealed epithelial hyperplasia, tissue destruction, and penetration of crypts into the submucosa (24). We found the same symptoms and histology in this guinea pig model, confirming that it successfully mimics shigellosis symptoms in humans.

Another interesting finding of this study concerns the entry site for the bacteria. Our results show that Shigella do not require entry through organized lymphoid follicles (i.e., colonic patches and/or isolated lymphoid follicles) because the location of invasion and colonization by Shigella is not a superimposed organized lymphoid follicle (Fig. 3). Similar results were previously demonstrated in human intestinal xenograft mice lacking both Peyer’s patches and M cells (25). Our own previous study revealed the existence of M cells in regions of the intestinal epithelium that are distant from the Peyer’s patches in the mouse (26). However, our experiments did not provide conclusive evidence that M cells are directly taken up by bacteria in the epithelial region of the distal colon away from lymphoid follicles. Alternatively, certain specified epithelial cells or dendritic cells in the lamina propria region might be involved in this event (27). Further studies are needed to clarify these issues.

IL-8 can be detected in epithelial cells in rectal biopsy specimens from humans with shigellosis (28). Previous reports have also shown that IL-8 expression correlates with the severity of ulcerative colitis, the inflammatory signs of which resemble those observed in human shigellosis (29, 30). The main function of IL-8, which can be produced by epithelial cells, is to recruit and activate neutrophils, which in turn activate NO and trigger the release of granular contents, i.e., elastase (31), lactoferrin (18), and myeloperoxidase (32). In addition, IL-8 appears to be a major mediator of the recruitment of neutrophils in the human (22) and the rabbit-ligated ileal loop model (33). Mice receiving IL-8 together with invasive Shigella developed diseases resembling human shigellosis (20).

IL-1 is another cytokine produced following Shigella infection in both animal and human models, and blockade of IL-1 inhibits tissue damage in the rabbit-ligated ileal loop and murine lung infection models (34, 35). Interestingly, a previous study demonstrated that macrophages undergoing apoptosis induced by S. flexneri infection released IL-1 (10). Other studies using rectal biopsies of patients during the acute phase of shigellosis revealed enhanced levels of iNOS expression in the surface epithelium (13, 23). We found that following i.r. challenge with wild-type S. flexneri 2a, the large intestine of guinea pigs expressed increasing mRNA level of IL-8, IL-1β, and iNOS. Although our results were obtained by real-time PCR amplification using homogenized whole colon tissue samples, these findings strongly suggest that guinea pigs that were i.r. challenged with virulent S. flexneri 2a undergo typical Shigella pathogenesis (36).

Many approaches to using mice, guinea pigs, rabbits, and macaques as possible animal models of bacillary dysentery have been explored. In mice, the model of pulmonary pneumonia, which involves intranasal inoculation with Shigella (37), can be used to assess virulence attenuation, to run immunization experiments, and to determine protection against infection (38). However, the major drawback of this model is the lack of clinical relevance to the infection site. Recently, Sansonetti and colleagues (39) have been able to develop a model of intragastric infection in newborn mice using massive amounts of Shigella (10⁹ CFU). This murine infection model led to inflammatory destruction of the mucosa and significant infiltration of PMNs into the gut, but it cannot be used to evaluate protective immunity because the model must be used within a narrow window of time, i.e., 4–5 days following birth. In a unique approach developed by another group, the human intestine xenograft model in SCID mice resulted in extremely high levels of IL-1β and IL-8 production, as well as in the marked infiltration of neutrophils following direct injection of bacteria into the lumen of the intestinal xenograft (25). Although this model is valuable for studying the interaction between Shigella and the human intestine, it is too artificial and laborious for the purpose of assessing protective efficacy. In guinea pigs, the keratoconjunctivitis assay (known as the Sereny test) is considered the gold standard assay for protective immunity; however, it too has drawbacks, including the difficulty of quantitatively describing the inflammatory response and the irrelevance of the target organ (40). Additionally, a previous report demonstrated that guinea pigs that were administered wild-type S. flexneri 2a and then starved for 4 days and treated with opium developed fatal enteric infections (41). However, because this challenge method induced fatal effects at an early stage of infection, it cannot be adopted for the purpose of screening vaccine candidates. In rabbits, the ileal-ligated loop assay is well known as a model that can cause bacterial invasion and inflammatory rupture of the epithelial cells at ∼18–24 h after infection (7, 42). In addition, adult rabbits subjected to direct colonic intubation by cecal bypass also developed shigellosis (16). Although both rabbit shigellosis models are quite sensitive when it comes to reflecting virulence attenuation, it has not yet been established whether they are suitable for protection studies. Finally, rhesus monkeys are the only animals in which typical bacillary dysentery can be induced by oral infection with S. flexneri without starvation and/or pretreatment with antibiotics (9, 43). However, the cost of using these animals can be prohibitive, especially in the developing countries, and this model also raises ethical issues. The most interesting finding of our current study is that our newly developed guinea pig model successfully represents typical bacillary dysentery under natural conditions and can also be used to reliably predict the protective immunity of vaccine candidates.

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Disclosures

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