Outbreak of Keratoconjunctivitis due to *Salmonella weltevreden* in a Guinea Pig Colony

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The purpose of this report is to demonstrate that the ability to produce keratoconjunctivitis (KC) is a property found in *Salmonella weltevreden*. This observation is contrary to previous reports that *Salmonella* spp. do not produce KC. An outbreak of KC due to *S. weltevreden* occurred in a guinea pig colony, and the animals carried the organism in the intestinal tract. The same *Salmonella* serotype that caused an epidemic of diarrhea in humans and a routine laboratory isolate also possessed the ability to induce KC. Unlike *Shigella* spp. (the prototype organisms positive for KC), *S. weltevreden* induced KC and bound Congo red dye even when grown at 30°C. It invaded HeLa cells in culture but did not hybridize with a DNA probe for invasiveness of *Shigella* spp. and enteroinvasive *Escherichia coli* even though it harbored plasmids. It was susceptible to all the antibiotics tested, was hydrophobic, and showed mannose-sensitive hemagglutination. It did not have enterotoxic or cytotopic activities.

On inoculation into the conjunctival sac of guinea pigs, members of the genus *Shigella* and the closely related enteroinvasive *Escherichia coli* produce keratoconjunctivitis (KC) (3, 8, 18, 28, 40). The KC test was introduced by Sereny and is known as the Sereny test (36). Although other enteric pathogens such as strains of *Salmonella*, *Campylobacter*, *Aeromonas*, and *Yersinia* also produce invasive diarrhea, reports to date suggest that all except *Yersinia* spp. are uniformly negative in the Sereny test (8, 10, 11, 19, 28, 40). *Yersinia enterocolitica*, however, produces only conjunctivitis and not KC (35). We report here an outbreak of naturally occurring KC due to *Salmonella weltevreden* in the guinea pig colony of the animal house of the International Centre for Diarrhoeal Disease Research, Dhaka, Bangladesh.

MATERIALS AND METHODS

Reference bacterial strains. One *S. weltevreden* strain (MY8863) implicated in an outbreak of diarrhea in a Bangladeshi village (unpublished data); one *S. weltevreden* strain (D21411) and one *Shigella flexneri* 2a strain (611), both obtained from the routine clinical laboratory; and the non-pathogenic *E. coli* K-12 were included for comparison.

Guinea pig colony. The guinea pig colony has approximately 150 animals housed in a separate room of the animal house. Between March and June 1990, 13 animals developed spontaneous KC.

Bacteriological investigation. The affected eyes of the animals were swabbed and cultured on blood agar and MacConkey agar. Gut contents of two animals that developed KC and three consecutive stool samples of all five animal handlers were cultured for enteric bacterial pathogens by standard methods (42). *Salmonella* organisms were serotyped by O and H antisera (Pasteur Institute, Paris, France) by standard methods (7).

Sereny test. The organisms were grown on Trypticase soy agar (BBL) overnight either at 37 or at 30°C. The growth was suspended in normal saline to 10⁹ organisms per ml, and 0.02 ml of the suspension was dropped into the left eye of each guinea pig. Each organism was tested in two guinea pigs, and the animals were observed for up to 7 days for the development of KC.

Histology. After development of KC, all animals were sacrificed. The eyeball and eyelid from one animal inoculated with *S. weltevreden* and those from another inoculated with *S. flexneri* 2a were removed and placed in neutral buffered formalin. Hematoxylin- and eosin-stained sections were viewed under a light microscope for histopathologic changes. Negative controls included the uninoculated right eyes from these animals.

HeLa cell invasion assay. Invasion assays were performed as described previously, with some modifications (39). Instead of HEp-2 cells, HeLa cells were used. Bacterial cells were grown to mid-exponential phase in Trypticase soy broth (BBL) at 37°C. Approximately 10¹⁰ to 2 × 10¹⁰ CFU was added to a HeLa cell monolayer (1 × 10⁴ cells in a 6-ml vial [Kimble, Toledo, Ohio] containing Eagle’s minimum essential medium), centrifuged at 850 × g for 10 min, and then incubated at 37°C for 3 h in a 5% CO₂–95% air atmosphere. The plates were then washed 10 times with phosphate-buffered saline (PBS) and incubated for an additional 2 h in minimum essential medium containing 100 μg of gentamicin per ml to kill extracellular bacteria. After this, the monolayer was washed three times with PBS, and internalized bacteria were released by lysis of the monolayer with a solution containing 0.25% trypsin and 0.5% sodium deoxycholate in distilled water and quantified by plate count. The positive and negative controls included were *S. flexneri* 2a and *E. coli* K-12, respectively. Each strain was tested in duplicate thrice, and the values were averaged.

Plasmid analysis. Bacterial plasmids were extracted by the method of Birnboim and Doly (2), separated by agarose gel electrophoresis, and stained by ethidium bromide as described previously (27).

Plasmid curing. Bacteria were treated with novobiocin (26) and sodium dodecyl sulfate (14), and colonies were examined for plasmids as described above.

DNA hybridization. The DNA probe used was constructed

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from the invasive plasmid of *S. flexneri* 5 (M90T) and consisted of a 17-kb *EcoRI* digestion fragment of pRM17 (37). The appropriate restriction fragment was purified as described by Moseley et al. (29). The probe was labeled by nick translation (22) with [γ-32P]dCTP (Amersham International plc., Aylesbury, Buckinghamshire, United Kingdom) and a nick translation kit (BRL). Colony blots were prepared, processed, and hybridized under stringent conditions as described by Echeverria et al. (6).

**Enterotoxin and cytotoxin production.** The organisms were grown in Trypticase soy broth at 37°C for 20 h, and the supernatants were tested for heat-labile enterotoxin in Y-1 adrenal tumor cells (33), heat-stable enterotoxin in suckling mouse (4), and cytotoxin in HeLa cells (9).

**Congo red binding.** Congo red binding of the organisms was determined by streaking the organisms on Congo red agar and observing for development of pigmented colonies as described previously (31).

**Salt aggregation test.** Fresh colonies of strains obtained after growth on Trypticase soy agar for 18 h at 37°C were used to determine cell surface hydrophobicity by using different concentrations of ammonium sulfate (31). As described previously (31).

**Hemagglutination test.** Bacteria grown in Trypticase soy broth were used for agglutination of a 2% suspension of guinea pig erythrocytes (30). Inhibition of hemagglutination was tested by using 1% solutions of mannose, glucose, galactose, and *N*-acetyl neuraminic acid.

**Antibiogram.** The antibiotic susceptibilities of bacteria were tested by the disk diffusion method (1).

**RESULTS**

From the eyes of all 13 animals which developed spontaneous KC, a *Salmonella* sp. was isolated. Serotyping of the isolates revealed that all of them possessed an O antigen 3, 10; a phase 1 H antigen, r; and a phase 2 H antigen, Z6. This antigenic composition is identical with that of *S. weltevreden*, which belongs to serologic group E.

The KC test was carried out with two guinea pig isolates, GP-1 and GP-2, the human epidemic diarrheal isolate MY8863, the clinical laboratory isolate D21411, and *S. flexneri* 2a after the bacteria were grown at 37°C. The *Shigella* isolate produced conjunctivitis at 48 h and keratitis at 72 h. All the *Salmonella* isolates produced conjunctivitis at 48 h and keratitis at between 72 and 96 h. The KC produced by strain GP-2 is shown in Fig. 1. When the KC test was repeated with GP-2 and *S. flexneri* 2a grown at 30°C, GP-2 still produced KC, but *S. flexneri* 2a did not.

Histology of the eyes of the animals sacrificed 72 h after inoculation with *Salmonella* spp. and *Shigella* sp. (after development of KC) showed a similar picture. The eyelids and palpebral conjunctivas showed severe acute inflammation with polymorphonuclear cell infiltration, edema, and hyperemia. A similar picture was seen in the corneas.

*Shigella* sp. produced a much more severe inflammatory reaction in the cornea than did *Salmonella* spp. The histology of *S. weltevreden* KC is shown in Fig. 2.

All four *Salmonella* strains and the *Shigella* strain invaded HeLa cells. The survival of salmonellae in the gentamicin-HeLa cell assay ranged from 2×10^3 to 4×10^5 organisms per ml, whereas that of *Shigella* sp. was 4×10^4 organisms per ml. The survival rate of *E. coli* K-12 was 0.

The guinea pig strains GP-1 and GP-2 possessed two plasmids with molecular masses of 60 and 2.6 Mda, whereas the isolates MY8863 and D21411 possessed only the large plasmid. None of the strains hybridized with the invasive plasmid probe. Attempts to cure the plasmids were not successful. None of the strains produced enterotoxins or cytotoxin. All of them were susceptible to ampicillin, tetracycline, chloramphenicol, streptomycin, gentamicin, trimethoprim-sulfamethoxazole, nalidixic acid, and furaxone. All strains formed pigmented colonies on Congo red agar at 30 and 37°C. The surface hydrophobicity of the strains as determined by the salt aggregation test was found to be 1.5 M. All strains gave a strong and rapid agglutination of guinea pig erythrocytes which was inhibited by mannose only.

Culture of the gut contents of two animals that developed KC was positive for *S. weltevreden*, and none of the animal handlers carried the organism.

**DISCUSSION**

Although both *Salmonella* spp. and *Shigella* spp. are invasive enteric pathogens, there are differences in the pathogenesis of diarrhea caused by these organisms. *Shigella* invade and multiply within colonic epithelial cells, causing ulceration of the mucosa. The lesion rarely extends beyond submucosa, and bacteremia is relatively uncommon (17). However, non-typhoid salmonellae invade both terminal ileum and colon, and the organisms are transported by
FIG. 2. Histology of the eye of a guinea pig (Fig. 1A) showing severe KC (A) compared with that of the eye of a normal guinea pig (B) (Fig. 1B) (hematoxylin and eosin staining). Magnification, ×66.

macrophages across the mucosa and deeper into the tissue and mesenteric lymph nodes. Bacteremia is more common in salmonellosis than in shigellosis (3). In shigellae, although the ability to cause KC is encoded on both chromosomal and large invasive plasmid genes, invasiveness of HeLa cells is directly correlated with the latter (18). Loss of the plasmid results in loss of the ability to both produce KC and invade HeLa cells (12). Similarly, in Y. enterocolitica, another invasive pathogen, the ability to cause conjunctivitis depends upon both chromosomal and plasmid genes. However, the ability to invade tissue culture cells alone is dependent upon chromosomal genes (21, 32). In many salmonellae, for full pathogenicity the presence of a cryptic plasmid is required, although for invasiveness of tissue culture cells, as in Y. enterocolitica, chromosomal genes alone are sufficient (12). All the S. weltevreden strains had a
common 60-MDa plasmid, and we wanted to determine the role of this plasmid in the induction of KC. However, attempts to cure the strains of this plasmid were not successful. Salmonella spp., for example, *S. typhimurium* do not cause KC yet invade HeLa cells in culture (10). Thus, the abilities to produce KC and to invade HeLa cells are independent characteristics. *S. weltevreden* possessed the abilities to produce KC as well as to invade HeLa cells.

Congo red binding is correlated with the invasiveness of shigelae and enteroinvasive *E. coli* (24, 31). Moreover, in these organisms, the abilities to bind Congo red and to induce KC are temperature dependent; the organisms lose these properties when cultured at less than 37°C (25, 31). However, *S. weltevreden* bound Congo red as well as induced KC when cultured both at 37 and at 30°C, suggesting that in this organism, these abilities are not strictly temperature dependent. A DNA probe constructed from the invasive 140-MDa plasmid of *Shigella* sp. failed to hybridize with *S. weltevreden*, suggesting that the invasive genes in these organisms may not be related. Multidrug resistance in shigelae is a serious problem in Bangladesh (38), but *S. weltevreden* was susceptible to all the antibiotics tested. *S. weltevreden*, like other salmonellae (41), showed mannose-sensitive hemagglutination, suggesting the presence of type I fimbriae. They also agglutinated at a 1.5 M salt concentration, suggesting that they are relatively hydrophobic (20, 31). These attributes strengthen the virulence potential of *S. weltevreden*. There have been reports of salmonellae producing heat-labile enterotoxin (34), heat-stable enterotoxin (15), or cytotoxin (16), but *S. weltevreden* was negative for all these properties.

Investigation of the source of infection suggested that the animals were the carriers of the organism and fecal contamination of the eye resulted in KC. A strain of *S. weltevreden* that caused an epidemic of diarrhea in a Bangladeshi village in 1983 (unpublished data) and a routine clinical isolate also possessed the ability to induce KC. Our unpublished data suggest that intestinal infection with *S. weltevreden* is not uncommon in Bangladesh. This organism is also known to be frequently isolated in Hawaii (23). The potential of this organism to cause KC should be borne in mind.

Thus, contrary to the observation that salmonellae are incapable of causing KC, we have demonstrated that at least *S. weltevreden* is capable of inducing this reaction. It will be interesting to study the molecular basis of this virulence property.

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