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## Sewage Effluent: Likely Source of *Salmonella enteritidis*, Phage Type 4 Infection in a Commercial Chicken Layer Flock in Southern California

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**SUMMARY.** Following the diagnosis of *Salmonella enteritidis*, phage type 4, infection in a commercial layer flock in southern California, effluent from a nearby sewer treatment plant was investigated as a potential source of infection. Between July 1994 and March 1995, 68 *Salmonella* isolations, comprising 27 serotypes, were made from the inflow (raw sewage) and effluent (treated sewage). Thirty-nine of 68 (57%) isolations yielded six serotypes, which consisted of *S. enteritidis* 12% (8/68), *S. cerro* 10% (7/68), *S. typhimurium* 7.4% (5/68), *S. tennessee* 7.4% (5/68), *S. give* 7.4% (5/68), *S. mbandaka* 7.4% (5/68), and *S. panama* 6% (4/68). The remaining 43% (29/68) isolations were represented by 21 serotypes. Seventeen *S. enteritidis* isolates originating from the effluent (creek water), resident feral animals (rodents, stray cats, skunks), and chickens (organs, eggs) of the affected flock were subjected to plasmid profile and restriction endonuclease analysis. Twelve of the 17 isolates had identical plasmid profile and restriction digestion patterns. Two of 17 isolates showed similar patterns but both differed from the rest; and 1 of 17 did not yield plasmids. Two other isolates were found to be different from each other and from the rest of the group.

**RESUMEN.** Aguas residuales tratadas: Una posible fuente de infección de *Salmonella enteritidis* fago tipo 4 en un lote de ponedoras comerciales al sur de California.

Después del diagnóstico de una infección por *Salmonella enteritidis* fago tipo 4 en un lote de ponedoras comerciales al sur de California, como una posible fuente de infección se investigó el flujo de salida de una cloaca cercana a una planta de tratamiento de aguas residuales. Entre Julio de 1994 y Marzo de 1995, a partir del flujo de entrada (aguas residuales sin tratar) y flujo de salida (aguas residuales tratadas) se aislaron 68 cepas de *Salmonella* clasificadas en 27 serotipos. De las 68 cepas, 39 (57%) correspondían a 6 serotipos representados por *S. enteritidis* (8 de 68, 12%), *S. cerro* (7 de 68, 10%), *S. typhimurium* (5 de 68, 7.4%), *S. tennessee* (5 de 68, 7.4%), *S. mbandaka* (5 de 68, 7.4%) y *S. panama* (4 de 68, 6%). El resto de las cepas aisladas (29 de 68, 43%) correspondió a 21 serotipos. Se sometieron a la prueba de perfil de plásmidos y a un análisis de endonucleasas de restricción a 17 de las cepas de *S. enteritidis* originarias del flujo de entrada de las aguas residuales (agua de quebrada), animales silvestres residentes (roedores, gatos salvajes, mofetas), y pollos (órganos, huevos) del lote afectado. De las 17 cepas, 12 tuvieron idénticos perfiles de plásmidos y patrones de restricción. Dos de las 17 cepas mostraron patrones similares pero ambas difirieron del resto; y solo una de las 17 no mostró plásmidos. Se encontró que otras 2 cepas fueron diferentes entre ellas y del resto del grupo.

Key words: *S. enteritidis*, phage type 4, sewage effluent, layer flock.

Abbreviations: BGN = Brilliant Green with novobiocin; R&A = restriction endonuclease analysis; SE PT4 = *Salmonella enteritidis*, phage type 4; XLT4 = xylose-lysine-tergitol 4

*Salmonella enteritidis*, phage type 4 (SE PT4), infection of chickens in the United States was first reported in May 1994 in a commercial layer flock in southern California (10,15). This finding raised a serious concern to the U.S. poultry industry because SE PT4 was classified as a foreign animal pathogen by the United States Department of Agriculture. *Salmonella Enteritidis* PT4 is a well-recognized pathogen of humans and poultry in Europe, in particular England (1,11,12). In broilers, the disease was associated with a syndrome of high mortality in chicks less than 1-wk-old followed by stunting and unevenness of growth; however, in layers the infection did not manifest significant adverse effects on fertility, hatchability, or egg production (11).

Several outbreaks of *Salmonella* have been reported in cattle and sheep associated with raw sewage, sewage sludge, and sewage outfalls (5,7,9). The objective of this study was to investigate a possible link between SE PT4 infection of the chickens in a ranch and the municipal sewage effluent that flowed in the nearby creek.

## MATERIALS AND METHODS

**Salmonella isolation.** The modified Moore swab was prepared as previously described (6,19). The swab was wrapped at the mid-section of a 16-gauge wire and secured by using staples, then appropriately folded in a brown paper and sterilized. Three swabs were placed in the city's effluent at three different sites along the creek and upstream from the infected ranch (Fig. 1) on a biweekly basis between July 1994 and January 1995. Subsequently, in February and March of 1995 additional swabs were obtained from the premises of the treatment plant from the inflow (raw sewage) and at the post-anaerobic digestion stage just prior chlorination. The swabs were placed just below the surface of the water and the free ends of the wire were secured to fence posts on each side of the stream. Following the exposure for 2 wk in the stream (effluent) and raw sewage, swabs were collected aseptically using gloves and placed in sterile plastic bags and immediately transported to the laboratory. Initially, each swab from each site was divided into two portions which were incubated either in tetrathionate or selenite cystine broth (Difco Laboratories, Detroit, Mich.). Later, the use of selenite

cystine broth was discontinued because of the slightly reduced recovery rate of *Salmonella* when compared to tetrathionate broth. The swabs were placed in 10 times the volume of tetrathionate broth and incubated at 41.5 C for 24 hr. Following the incubation, the broth was streaked onto Brilliant Green with novobiocin (BGN) and xylose-lysine-tergitol 4 (XLT4) agar plates (Remel Laboratories, Lenexa, Kans.). In addition, 1 ml of tetrathionate broth culture was transferred to a fresh tube containing 9 ml of the same medium daily for 5 days of continued incubation to enhance recovery on streaked plates. The broth cultures were incubated at 41.5 C for 24 hr and streaked onto BGN and XLT4 plates. The plates were incubated at 37 C for 24 hr and examined for *Salmonella*-suspect colonies. At least three colonies were picked from each plate into triple sugar iron and lysine iron agar slants (Remel). Following the overnight incubation at 37 C, the cultures were serogrouped using conventional *Salmonella* grouping sera (Difco) and further confirmed by biochemical tests. *Salmonella* serotyping was performed according to procedures described previously (4). Phage typing of *S. enteritidis* was performed at the National Veterinary Services Laboratory, Ames, Iowa.

**Plasmid and restriction endonuclease analysis (REA).** Seventeen isolates of *S. enteritidis* from the effluent, raw sewage, and from a previous investigation of SE PT4 infection in the layer flock (10,15) were selected for plasmid analysis and REA. These included: four isolates from internal organs of chickens, one from chicken intestines, one from eggs, one from cat intestines, two from mouse livers, one from a skunk liver, four from effluents, and three from raw sewage. Of the 17 *S. enteritidis* isolates, 13 were phage type 4, 1 was phage type 7, 1 was phage type 1, and 2 were untypeable (did not conform to any known phage types). The plasmids from isolates of *S. enteritidis* were extracted by the alkaline lysis method as previously described (18). Briefly, each isolate of *S. enteritidis* was grown overnight at 37 C in tryptic soy broth (Difco) enriched with yeast extract (Difco). Following the incubation, broth culture was centrifuged at 8000 rpm for 5 min at 4 C. The pelleted cells were suspended in GTE buffer (50 mM glucose, 25 mM Tris, pH 8.0, and 10 mM EDTA) containing lysozyme (1 mg/ml) (Sigma Chemical Co., St. Louis, Mo.) and incubated at room temperature for 5 min. The bacterial cells were then lysed by adding a lysis solution (0.2 N NaOH, 1% SDS in distilled water) and then treated with 5 M potassium acetate (pH 4.8) (Sigma). The cell lysate was

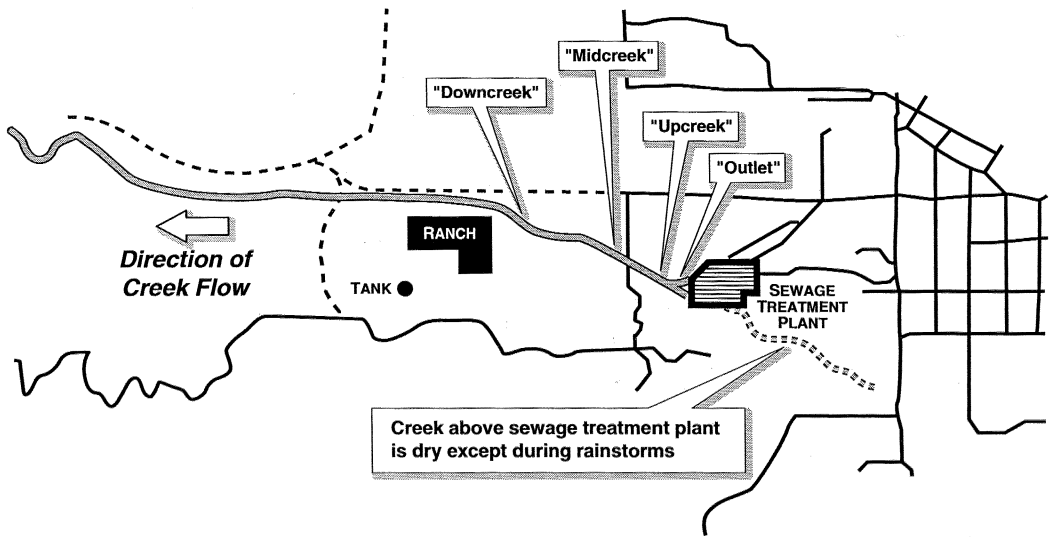


Fig. 1. Sketch map showing creek in relation to ranch and sewage treatment plant.

centrifuged at 15,000 rpm for 10 min at 4 C, and the nucleic acid strings in the supernatant was precipitated using isopropanol (Sigma). The precipitate containing the nucleic acid was resuspended in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA) and was treated with RNAase-A. The plasmid DNA was extracted using phenol:chloroform:isoamyl alcohol (25:24:1). The DNA in the aqueous phase was precipitated with absolute ethanol and 3 M sodium acetate (Sigma). The precipitate was washed with 70% ethanol, air dried, and resuspended in TE buffer. A 5- $\mu$ l aliquot was analyzed on 0.7% agarose gel in 1 $\times$  TAE (40 mM TRIS-acetate and 1 mM EDTA) buffer. The plasmid preparation from each isolate was digested with EcoRI enzyme (GIBCO BRL; Life Technologies, Grand Island, N.Y.) for 3 hr at 37 C. Following the digestion, the DNA fragments were

analyzed on 0.7% agarose gel in 1 $\times$  TAE buffer as previously described (17).

## RESULTS

**Salmonella isolation.** Sixty-eight *Salmonella* isolations were made from the creek water (effluent) and comprised 27 different serotypes. The most frequently isolated serotypes were *S. enteritidis* (12%); *S. cerro* (10%); *S. typhimurium*, *S. tennessee*, *S. give*, and *S. mbandaka* (7.4% each); and *S. panama* (6%). The remaining 43% of the isolations represented 21 serotypes.

**Plasmid profiles and REA.** The plasmid profiles of isolates from two chicken livers (2, 3), two mouse livers (4, 16), effluents (6, 11, 12, 13), skunk liver (7), chicken egg (8), and raw sewage (17) were identical. Two plasmids were present in each of these isolates. The plasmid profiles of isolates 6, 7, 8, 9, 11, 13, 16, and 17 are shown in Fig. 2. The isolates from the cat intestine (1), chicken ovary (5), and raw sewage (14, 15) had only one plasmid (not shown). The isolate from chicken intestine (10) did not have any plasmid.

The restriction endonuclease digestion of plasmids from isolates 2, 3, 6, 7, 8, 9, 11, 12, 13, 16, and 17 revealed identical migration patterns (Fig. 3). The plasmids from the isolates 1 and 5 had similar REA patterns but differed from the rest of the isolates (not shown). The

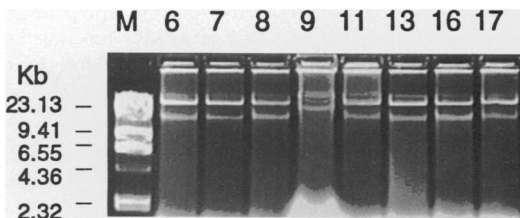


Fig. 2. Plasmid profile of *Salmonella enteritidis* isolates. Lanes: 6—creek water (upstream); 7—skunk liver; 8—chicken egg; 9—chicken liver; 11—creek water (midstream); 13—creek water (downstream); 16—mouse liver; 17—raw sewage inflow; M—molecular weight marker ( $\lambda$  DNA-HindIII digest).

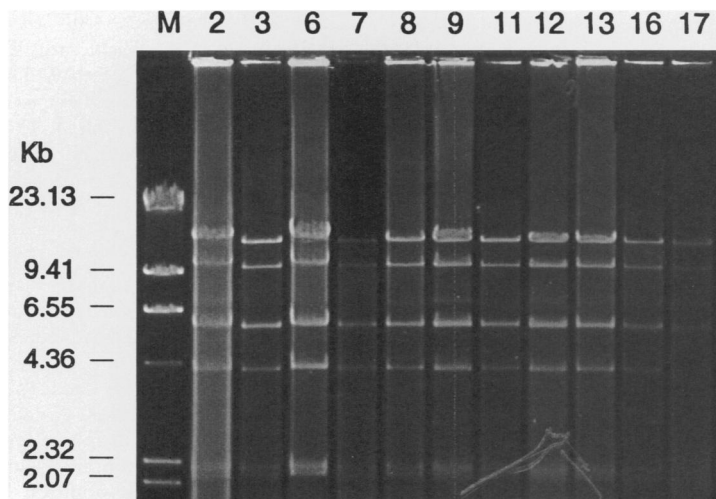


Fig. 3. Plasmid restriction digestion patterns of *Salmonella enteritidis* isolates. Lanes: 2—chicken liver; 3—chicken liver; 6—creek water (upstream); 7—skunk liver; 8—chicken egg; 9—chicken liver; 11—creek water (midstream); 12—creek water (upstream); 13—creek water (downstream); 16—mouse liver; 17—raw sewage inflow; M—molecular weight marker (lambda DNA-HindIII digest).

plasmids from isolates 14 and 15 differed from each other and from rest of the isolates in their restriction digestion pattern (not shown).

### DISCUSSION

Recently, there has been an upsurge of *S. enteritidis* cases in humans in southern California (S. Mack, pers. comm.). Although some of these cases have been reported to be associated with the consumption of table eggs or egg products, no confirmation has been made linking eggs to human *S. enteritidis* infection in California.

There has been no convincing explanation made for the regional (14) or global (16) increase of egg-associated *S. enteritidis* isolation from human cases of food poisoning. Human infection due to SE PT4 has been reported in California since the early 1990s (S. Abbot, pers. comm.). However, the infection in the layer flock presents the first documented case in the U.S.A. (10,15). This led to the investigation of the creek water that originates from a city municipal sewage treatment plant. The plant is located half a mile upstream and passes within 200 ft of the infected ranch to eventually join the Santa Ana River in the city of San Bernardino. The creek is entirely composed of sewage effluent and provides the only source of drink-

ing water for resident feral animals, including rodents, skunks, and stray cats, especially in the summer months.

The treated sewage effluent in the creek was cloudy and foamy and occasionally produced a fecal odor, suggesting inadequate treatment process. These observations prompted us to investigate the creek water as a possible source of the SE PT4 infection in chickens. The release of inadequately treated effluent or sludge in the environment, in particular in orchards, grazing pastures, golf courses, and other public entertainment parks, presents potential health risks (18). The effectiveness of disinfection with chlorine depends upon a range of factors, including the presence of organic material, or certain industrial wastes and pH (2).

The epidemiologic evaluation of SE PT4 infection, supported by the plasmid profile and REA, provides information on the most probable source of infection (human sewage) in the layer flock of this study. Investigations of human *S. enteritidis* infection often conclude that the ingestion of contaminated food, usually egg products, was the source of infection. This case is an example of how a contaminated environment may be the source of infection for susceptible animals. It is also conceivable that this contaminated environment could be the source of human salmonellosis, either directly through

contact with contaminated water or indirectly through biological vectors such as rodents, which, in turn, could contaminate a variety of human foods, in particular those prepared in restaurants.

There was no evidence of infection in the breeder flock, replacement pullets, feed, and water from the ranch well (10). Stool samples from ranch employees were also negative for *Salmonella* (Riverside County Public Health, Riverside, Calif.). *Salmonella enteritidis* is prevalent in mice on contaminated poultry farms (13) and could account for up to 75% of the *Salmonella* isolated in mice (8). A recent study showed that once mice are naturally infected with SE PT4, the organism could be excreted intermittently for up to 19 wk (3). A single pellet of mouse feces may contain up to  $2.3 \times 10^5$  *S. enteritidis* (8). The identical plasmid analysis profile and the REA of the SE PT4 isolates from the effluent, internal organs of chickens, and mice suggest that mice were an important biological vector of SE PT4 to the layer flock. Mice could have contaminated feed bins in the layer houses, hence infection of hens resulted by the fecal-oral route.

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