A Virulent Strain of *Salmonella enterica* Serovar London Isolated in Infants with Enteritis Traced by Active Surveillance and Molecular Epidemiological Study*

A total of 74 isolates of *Salmonella enterica* serovar London were collected through the Laboratory-Based Diarrheal Diseases Surveillance in 2000-2001. In order to characterize the isolates and investigate the source of the epidemic, we performed antimicrobial susceptibility tests and *Xba* I Pulsed-field gel electrophoresis (PFGE) of 44 *Salmonella* London isolates. Forty isolates were from feces of infants and four isolates were from adults aged 30, 52, 54, and 59 yr. Two subtypes were identified: a tetracycline-susceptible A 0 PFGE pattern and a tetracycline-resistant A 1 PFGE pattern. Interestingly, the isolates from all infants and one 30-yr-old adult were A 0 PFGE pattern and tetracycline-susceptible. Furthermore, the A 0 PFGE pattern strain was approximately 2 times more virulent than the A 1 PFGE pattern strain, according to the results of in vitro invasion assay using J774A.1 macrophage-like cells. These results indicate that the active surveillance with molecular epidemiological tools would be valuable for promptly finding new epidemic strains. Our results also suggested that the virulent *Salmonella* London strain might infect the infants through a common contaminated source.

Key Words: *Salmonella enterica*; *Salmonellosis*; Infants; Epidemiology; Sentinel Surveillance; Electrophoresis, gel, Pulsed-Field; Biological Assay

INTRODUCTION

*Salmonella enterica* serovar London (*Salmonella* London hereafter) belongs to *Salmonella* serogroup E and is one of 2,400 *Salmonella* serovars that have ever been found worldwide (1). In Korea, more than 2,000 *Salmonella* isolates of various serotypes have been reported to our institute yearly nationwide through the passive surveillance system. However the incidence of *Salmonella* London was very low until 1999 (2). In 2000, for the purpose of finding and controlling pathogens causing diarrheal diseases, our institute organized and launched the Laboratory-Based Diarrheal Diseases Surveillance—an active surveillance system with 17 Research Institutes of Health and Environment from various cities and provinces. In this system, microbiologists at 17 Research Institutes of Health and Environment in cities and provinces visited their regional hospitals periodically to obtain and identify bacterial samples isolated from diarrheal patients. Our institute gathered and stored these bacteria with the related information sheets for further studies. The data was analyzed for understanding the trends of infectious diseases.

A few literature on the infection due to *Salmonella* London has been available. An epidemic in Hungary was reported in 1980 (3), which reported that raw meat and meat products transmitted *Salmonella* London infections and that the bacterial strains collected between 1976 and 1978 were first susceptible then became multiple resistant strains. In recent years, Yu et al. reported that *Salmonella* London was isolated from the intraocular tissues of a 3-month-old infant. The infant suffered from redness, leukocoria in her right eye and a mild fever with bloody diarrhea (4).

In this study, we investigated 44 *Salmonella* London isolates collected in 2000 and 2001, which grew from stools obtained from infant and adult patients via the Laboratory-Based Diarrheal Diseases Surveillance.

MATERIALS AND METHODS

Bacteria and growth conditions

Forty-four epidemiological unrelated isolates were selected...
from 74 *Salmonella* London isolates collected from infant and adult patients in different geographical regions of Korea through the Laboratory-Based Diarrheal Diseases Surveillance in 2000-2001 for characterization of antibiotic resistance patterns and XbaI PFGE (Table 1). Three *Salmonella* London strains and one *Salmonella typhimurium* Definitive Type (DT) 104 were tested for in vitro invasion assay and are listed in Table 2. All isolates were identified and confirmed by biochemical and serological tests using API 20E (bioMerieux, Durham, NC, U.S.A.) kit and antisera from Difco (Detroit Michigan, U.S.A.), respectively. Bacterial strains were maintained in Tryptic soy agar (TSA, Difco, U.S.A.) and cultured in Luria-Bertani (LB) medium for in vitro invasion assay.

**Table 1.** Number of *Salmonella* London isolated in different areas, 2000-2001

| Year | Area | Feces | 10^5 | 10^6 | 10^7 | 10^10 | 10^11 | 10^12 | 10^13 | 10^14 | Total |
|------|------|-------|------|------|------|-------|-------|-------|-------|-------|-------|
| 2001 | SE   | 0/0   | 0/0  | 0/0  | 0/0  | 0/0   | 0/0   | 0/0   | 0/0   | 0/0   | 0/0   |
| 2000 | GJ   | 0/0   | 0/0  | 0/0  | 0/0  | 0/0   | 0/0   | 0/0   | 0/0   | 0/0   | 0/0   |

*Area abbreviation SE, Seoul; GG, Gyeonggi-Do; IC, Incheon; GW, Gangwon-Do; CN, Chungcheongnam-Do; GB, Gyeongsangbuk-Do; JB, Jeolla-buk-Do; JN, Jeollanam-Do; DJ, Daejeon. Denominators represent total number of *Salmonella* London isolates and numerators stand for number of *Salmonella* London isolates from infant patients.

**Table 2.** Origin, antibiogram and PFGE patterns of *Salmonella* London isolates from different area

| Area | Year | Source | No. of isolates | Age (y) | Antibiogram | PFGE pattern |
|------|------|--------|-----------------|---------|-------------|-------------|
| IC   | 2000 | Feces  | 1               | <2      | Susceptible | A0          |
| GW   | 2000 | Feces  | 23              | <2      | Susceptible | A0          |
| GW   | 2000 | Feces  | 1               | 30      | Susceptible | A0          |
| JN   | 2000 | Feces  | 6               | <2      | Susceptible | A0          |
| DJ   | 2000 | Feces  | 1               | <2      | Susceptible | A0          |
| DJ   | 2000 | Feces  | 2               | <2      | Susceptible | A0          |
| CN   | 2000-2001 | Feces | 7               | <2      | Susceptible | A0          |
| CN   | 2000 | Feces  | 3               | 52, 54, 59 | Tetracycline | A1          |

*Area abbreviations: IC, Incheon; GW, Gangwon-Do; JN, Jeollanam-Do; DJ, Daejeon; GB, Gwangju; CN, Chungcheongnam-Do. *Salmonella* London isolates from different area patterned as recommended by the manufacturer, except that the intermediate and sensitive isolates were grouped together. 

**Antimicrobial susceptibility test**

The strains were tested for their antibiotic susceptibility on Mueller-Hinton agar plates by the disk diffusion method (5). The media and disks were purchased from BBL (Becton Dickinson Microbiology Systems, Cockeysville, MD, U.S.A.). Resistance to the following antibiotics was tested with disks containing: ampicillin 10 μg, chloramphenicol 30 μg, gentamicin 10 μg, streptomycin 10 μg, tetracycline 30 μg, nalidixic acid 30 μg, ciprofloxacin 5 μg, ceftriaxone 30 μg, cefoxitin 10 g, kanamycin 30 g, sulfamethoxazole/trimethoprim 23.75 μg/1.25 μg, ampicillin/sulbactam 20 μg, ticarcillin 75 μg, cefotaxime 30 μg, amoxicillin/clavulanic acid 30 μg, and amikacin 30 μg. The inhibition zones were interpreted as recommended by the manufacturer, except that the intermediate and sensitive isolates were grouped together. *Escherichia coli* ATCC 25922 was used as a reference strain for quality control.

**Pulsed field gel electrophoresis (PFGE)**

The preparation of genomic DNA blocks and digestion with a restriction enzyme were carried out, as described by Gautom (6). All *Salmonella* London isolates were analysed by using restriction enzymes XbaI, NcoI or SfiI (New England Biolabs, MA, U.S.A.). Typing by Pulsed field gel electrophoresis (PFGE) of genomic DNA digested with XbaI was carried out in a CHEF Mapper system (Bio-Rad Laboratories, CA, U.S.A.).

**Susceptibility to reactive oxygen species**

The resistance of bacteria to reactive oxygen species was assayed as described by Groote et al. (7) and Allen et al. (8) with some modifications. The direct broths of *Salmonella* colonies selected from LB plates incubated overnight were made to 0.5 McFarland turbidity (approx. 2×10^6 cfu/mL) using VITEK colorimeter (Hach Co., Colorado, U.S.A.). Then the broths were plated on M9 minimal plates containing 0.2% glucose using cotton swabs. Fifteen microliters of 3% hydrogen peroxide was spotted onto 6-mm-diameter 3M paper disks, and the discs were placed onto the bacterial lawn and incubated overnight at 37 °C. The diameter of inhibition zone was measured, averaged, and plotted. Three independent experiments were performed.

**In vitro invasion assay using J774A.1 macrophage-like cells**

Bacterial infection of macrophage was performed with a modified version of the assays, as described by Tang et al. (9) and Rathman et al. (10). J774A.1 cells (ATCC no. TIB-67) which are BALB/c mouse macrophage-like cell line, were grown in Dulbecco’s minimal essential medium (DMEM, Gibco Life Technologies, NY, U.S.A.) containing 10% fetal bovine serum (FBS) in 5% CO₂. J774A.1 cells were seeded at 4×10^6 cells/well (24-well dishes, Corning Inc., NY, U.S.A.) and incubated overnight in 5% CO₂ at 37 °C. *Salmonella* strains (Table 3) were prepared for the infection, which were grown overnight in 3 mL of LB broth with shaking at 200 rpm, subcultured...
at a 1:20 dilution in new 3 mL of LB broth for additional 1 hr. The J774A.1 cells were infected for 1 hr with Salmonella London strain KJ3320 (from adult), KJ3132 (from infant), Salmonella London ATCC 8389 or Salmonella typhimurium DT104 (strain #54) at a multiplicity of infection (MOI) of 10 bacteria to one macrophage. Salmonella typhimurium DT104 human isolate and Salmonella London ATCC 8389 were used as control strains. Thereafter, the cells were washed with PBS and incubated for 1 hr in DMEM containing 100 μg/mL gentamicin, which killed any extracellular Salmonella remaining in the DMEM after the washes (11). Finally, the J774A.1 cells were lysed with 1% Triton X-100 for 5 min, and the lysates were 10 fold serial diluted and spread on LB plates. The LB plates were incubated in 37°C overnight. Viable Salmonella were counted by plating for colony-forming units (cfu) on LB agar medium. The experiment was performed at least in duplicate.

RESULTS

Surveillance

After the surveillance, Salmonella isolates belonging to serogroup E appeared approximately 10 times more frequently than in the past two years (Fig. 1). The serotype of the isolates was Salmonella London, and surprisingly, most of these isolates were obtained from the stools of infant patients in hospitals (Table 1). Most patients were under two years of age and suffered from fever, diarrhea and vomiting.

Antimicrobial susceptibility

Forty-four Salmonella London isolates were tested for identifying antimicrobial susceptibility pattern. The isolates obtained from infant patients and a 30-yr-old woman were all susceptible to 16 antibiotics, but the isolates from three old patients in Chungnam province were resistant to tetracycline (Table 2). Each tetracycline resistant isolate had an approximately 11 kb-size plasmid, but the remaining isolates did not show any discrete DNA band on agarose gel when we performed plasmid isolation. When the DH5α, an Escherichia coli strain, was transformed with this plasmid, the transformants were resistant to tetracycline (data not shown).

PFGE

In order to compare genetic clonality and subtype the Salmonella London isolates, we performed PFGE with the restriction enzyme, XbaI. The PFGE pattern of every isolate obtained from infant patients and the 30-yr-old woman was A 0, but the pattern of the isolates from the three old patients was A 1 (Fig. 2, Table 2). Similar results were observed with the restriction enzymes NotI and SfiI. For sub-typing the Salmonella London isolates by PFGE, XbaI restriction enzyme was more suitable than NotI and SfiI because the band patterns of PFGE by NotI or SfiI were too obscure to compare the similarity (data not shown).

Susceptibility to reactive oxygen species

Salmonella typhimurium DT104 #54 and Salmonella London ATCC 8389 were used as control experiments (Table 3). Salmonella typhimurium DT104, which is now frequently
isolated worldwide as well as in Korea (12-14), was found to be multidrug-resistant (15, 16). Salmonella London strains isolated from an infant and an adult did not show enhanced resistance to hydrogen peroxide compared with Salmonella London ATCC 8389 and S. typhimurium DT104 #54 (Fig. 3).

Virulence in vitro

When the invasiveness of Salmonella typhimurium DT104 #54 was set 100%, Salmonella London KJ3320 was able to invade and survive in J774A.1 cells at similar levels to those of Salmonella typhimurium DT104 #54. On the contrary, Salmonella London KJ3132 was approximately 2 times more virulent than Salmonella typhimurium DT104 #54 and KJ3320 (Fig. 4). The invasiveness of Salmonella London ATCC 8389 was very low—no more than one-tenth of KJ3320 (data not shown). This result may be due to the fact that Salmonella London ATCC 8389 has not been a recent clinical isolate. Furthermore, since it was stored for a long time in laboratory freezers, its invasiveness might not have been expressed (17).

DISCUSSION

As shown in Fig. 1 and Table 1, most Salmonella London bacteria were isolated from infants nationwide after the year 2000. Salmonella sero-group B (S. typhimurium, S. derby, S. agona, etc.) and D (S. enteritidis, S. typhi, S. dublin, etc.) were major sero-groups in Korea, while sero-group E was rare (2). In 2000 and 2001 Korea National Institute of Health and Research Institutes of Health & Environment in provinces and cities collected and serotyped unexpectedly Salmonella E group bacteria. The serotype for all these salmonella was Salmonella KJ3320. When Salmonella infection occurs sporadically, the origin of occurrence is obscure but usually food-related. It is strongly assumed that specific foods or milk consumed by babies were contaminated with Salmonella London. Salmonella anatum infections through powdered milk have been reported in England and France (18). Furthermore, dairy product-related Salmonella infections have been reported (19-21).

Possibility of infection by Salmonella is increased if the patient is an infant, an anacidity patient, or a patient whose stomach has been resected due to low gastric acid levels (22). All patients in this study suffered from the typical symptoms of enteritis, such as fever, diarrhea, and vomiting. No deaths was recognized. As mentioned earlier, Yu et al. reported that Salmonella London was isolated from the intraocular tissues of a 3-month-old infant being ill with endogenous endo-phthalmitis (4). Although we did not confirm whether this Salmonella London strain is identical with isolates obtained from this study’s infants, it is assumed that Salmonella London exposed to infants since 2000 was a dangerous bacterium to infants’ health.

According to PFGE and antimicrobial susceptibility test results, the isolates obtained from infants had different genetic
clonality from the isolates obtained from the three old adults. These results suggested that the 
Salmonella London infant epidemic was independent from adult cases, and might have been caused by a common contaminated source. The isolate obtained from the 30-yr-old woman had the same PFGE pattern as that of the infants. It was assumed that she was either a mother or a relative of a certain infant patient, but we could not confirm this.

Resistance to reactive oxygen species produced by macrophage is an important factor for bacterial virulence (23, 24). However, we could not observe any difference between 
Salmonella London and the control bacteria, while invasion rate into macrophage showed a difference. For the purpose of the assessment of relative virulence in vitro, we performed the in vitro invasion assay using J774A.1 macrophage-like cells because the ability of 
Salmonella to survive and replicate within macrophage cells is an essential virulence mechanism (25). To avoid variations of 
Salmonella invasiveness due to bacterial growth state (26), we infected the J774A.1 cells with the 
Salmonella of log-phase state in every in vitro invasion assay. Although we used only the J774A.1 macrophage-like cells for in vitro invasion assay, our results suggested that the 
Salmonella London strain isolated from the infants was a more virulent enteric bacterium than the strain isolated from adults and the prevailing pathogen, 
Salmonella typhimurium DT104.

Our findings in this study deserve closer attention. First, active surveillance with molecular epidemiological study was an efficient strategy in finding new epidemic strain promptly. Many microbiologists in provincial health institutes were able to isolate more bacteria than the passive surveillance system by collecting the samples from patients stools in regional hospitals. PFGE and other sub-typing methods differentiated 
Salmonella into the clonal lineages. These will help us trace the origin of infection. Secondly, the virulent 
Salmonella London strain was determined to be a dangerous factor to infants health. Powdered milk and dairy products fell under suspicion. Therefore, more circumstantial epidemiological investigation is needed. In order to prevent and control infectious diseases efficiently, a closer cooperation system (hospital-laboratory-government) must be constructed.

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