

# 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*

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## INTRODUCTION

*Salmonella enterica* serovar London (*Salmonella* London hereafter) belongs to *Salmonella* serogroup E and is one of 2,400s *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

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

Year	Area*	SE	GG	IC	GW	CN	GB	JB	JN	GJ	DJ	Total
2001		1/1	6/10	0/0	7/7	5/5	7/7	0/0	0/0	1/2	0/0	27/32 <sup>†</sup>
2000		0/0	0/0	0/1	17/19	1/4	2/2	4/6	7/7	1/2	0/1	32/42 <sup>†</sup>

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

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 *Xba*I 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.

#### 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, cefotaxime 30 µg, kanamycin 30 µg, sulfamethoxazole/trimethoprim 23.75 µg/ 1.25 µg, ampicillin/sulbactam 20 µg, ticarcillin 75 µg, ceftiofur 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 *Xba*I, *Not*I or *Sfi*I (New England Biolabs, MA, U.S.A.). Typing by Pulsed field gel electrophoresis (PFGE) of genomic DNA digested with *Xba*I was carried out in a CHEF Mapper system (Bio-Rad Laboratories, CA, U.S.A.).

**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 <sup>†</sup>	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
GJ	2000	Feces	2	<2	Susceptible	A0
CN	2000-2001	Feces	7	<2	Susceptible	A0
CN	2000	Feces	3	52, 54, 59	Tetracycline <sup>‡</sup>	A1

\*Area abbreviations: IC, Incheon; GW, Gangwon-Do; JN, Jeollanam-Do; DJ, Daejeon; GJ, Gwangju; CN, Chungcheongnam-Do. †susceptible to all antibiotics tested. ‡tetracycline resistant.

The pulsing and running conditions of PFGE were an initial 2.2 sec to a final 63.8 sec for 16 hr and 6 Volts/cm at 14 °C. Lambda ladder (New England Biolabs, MA, U.S.A.) was used as a molecular size marker. After electrophoresis, the gels were stained with ethidium bromide for 20 min and were photographed using Gel Doc 2000 (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.  $2 \times 10^8$  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% CO<sub>2</sub>. J774A.1 cells were seeded at  $4 \times 10^5$  cells/well (24-well dishes, Corning Inc., NY, U.S.A.) and incubated overnight in 5% CO<sub>2</sub> 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 ob-

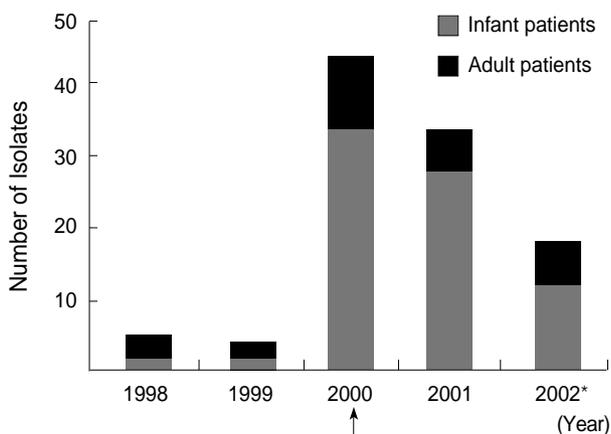


Fig. 1. The number of *Salmonella* London isolates in Korea, 1998-2002. The black bar represents the number of isolates obtained from adults and shaded bar stands for the number of isolates obtained from infants. The arrow indicates the initial year of the Laboratory-Based Diarrheal Diseases Surveillance. \*Data until June 2002.

tained 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, *Xba*I. The PFGE pattern of every isolate obtained from infant patients and the 30-yr-old woman were 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 *Not*I and *Sfi*I. For sub-typing the *Salmonella* London isolates by PFGE, *Xba*I restriction enzyme was more suitable than *Not*I and *Sfi*I because the band patterns of PFGE by *Not*I or *Sfi*I 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

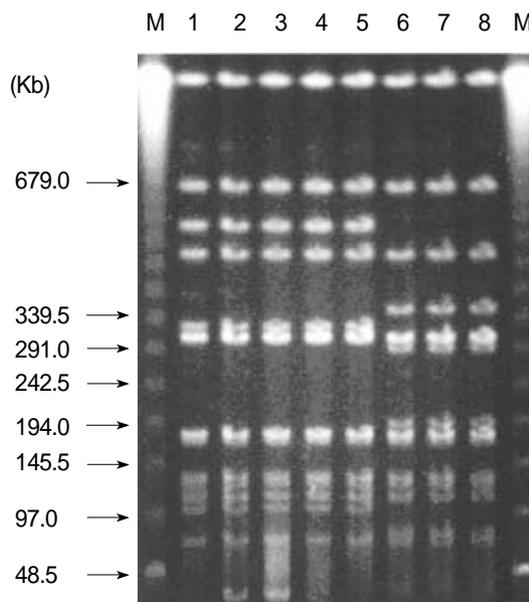


Fig. 2. Representative *Xba*I PFGE patterns of *Salmonella* London isolated from infants and adults. Lane 1-5 shows the pattern of isolates obtained from infants (A 0 pattern). Lane 6-8 shows the pattern of isolates obtained from the three old patients (A 1 pattern). M is a lambda phage DNA size marker.

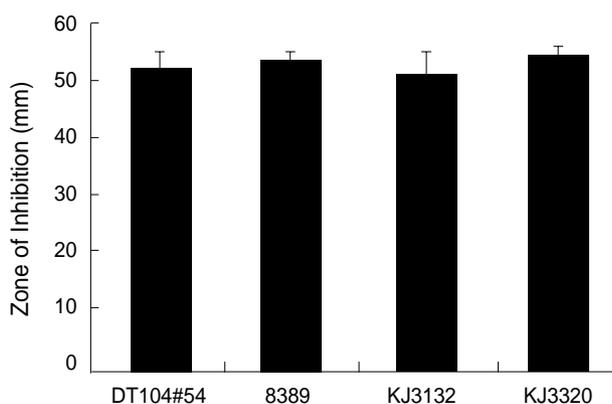


Fig. 3. Susceptibility to reactive oxygen species. DT104 #54 and 8389 are *Salmonella typhimurium* DT104 and *Salmonella* London ATCC 8389 as controls, respectively. KJ3132 is a *Salmonella* London isolate from an adult and KJ3320 is a *Salmonella* London isolate from an infant. Detailed method was described in Materials and Methods. Bars represent means  $\pm$  standard deviations of the results obtained in three experiments.

Table 3. Strains used in the susceptibility test against reactive oxygen species and the in vitro invasion assay

Strain	Information	Source
<i>Salmonella</i> London KJ3320	Isolate from a 54-year-old man lived in Chungcheongnam-Do, 2000.	KNIH*
<i>Salmonella</i> London KJ3132	Isolate from an infant lived in Gwangju, 2000.	KNIH
<i>Salmonella typhimurium</i> DT104 #54	Isolate from a patient, Jeollanam-Do, 1997.	KNIH
<i>Salmonella</i> London ATCC 8389		ATCC <sup>†</sup>

\*Korea National Institute of Health, <sup>†</sup>American Type Culture Collection.

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 *Salmonella 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 those 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

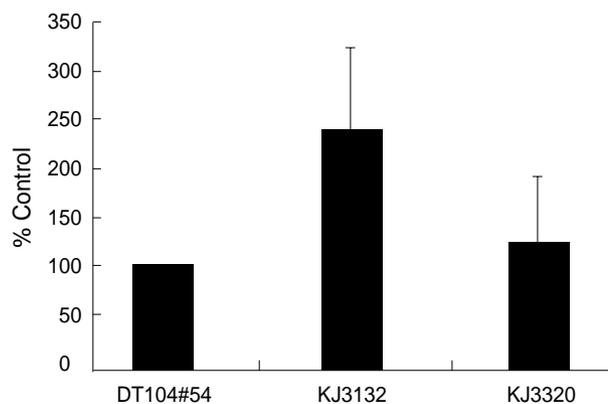


Fig. 4. In vitro invasion assay with J774A.1 macrophage-like cells. The invasiveness of *Salmonella typhimurium* DT104 #54 was set 100%. Bars represent means  $\pm$  standard deviations of the results obtained in three experiments.

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* London.

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 acidity 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 were 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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