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# Molecular Typing by Random Amplification of Polymorphic DNA (RAPD) and Detection of Virulence Genes of *Salmonella enterica* subspecies *enterica* serovar Gallinarum biovar Gallinarum

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ABSTRACT. *Salmonella enterica* subspecies *enterica* serovar Gallinarum biovar Gallinarum is the causative agent of fowl typhoid in chickens, outbreaks of which have devastated poultry populations in Korea since 1992. In order to identify genetic differences among *S.* Gallinarum isolates, bacteria were examined using the random amplified polymorphic DNA (RAPD) method. Of 13 arbitrary primers screened initially, the primer designated as universal rice primer-6 (URP-6) was selected for subsequent typing assays because it produced a distinctive and reproducible DNA fingerprint for a *S.* Gallinarum reference strain. URP-6-based RAPD analysis assigned 30 *S.* Gallinarum isolates into 6 types, with 26 isolates (86.6%) belonging to 2 major RAPD types. The distribution of virulence genes in *S.* Gallinarum isolates was examined by Southern hybridization. All tested isolates had the invasion gene, *invA*, the virulence plasmid gene, *spvB*, and the *S.* Enteritidis fimbrial gene, *sefC*. The distribution of virulence genes among *S.* Gallinarum isolates did not correlate with any specific RAPD type.

KEY WORDS: RAPD, *Salmonella* Gallinarum, universal rice primers, virulence gene.

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*Salmonella enterica* subspecies *enterica* serovar Gallinarum biovar Gallinarum is a host-adapted bacterial pathogen responsible for fowl typhoid. Infected chickens have a high mortality rate, succumbing to septicemia, enteritis and hemolytic anemia [23]. In most countries of Europe and in North America and Australia, fowl typhoid has almost disappeared, a result of improved surveillance and slaughter practices [6, 8]. However, in South Korea a considerable increase in outbreaks of fowl typhoid has been observed since 1992 and threatens the poultry industry.

Random amplified polymorphic DNA (RAPD) analysis has been widely used for studying genetic diversity and phylogenetic relationships of bacteria [9, 16, 25, 26]. The method generates distinctive DNA fingerprints by using a single, short (10–25mer) synthetic primer in a polymerase chain reaction (PCR). RAPD is faster, simpler, and more economical than other genetic typing assays, including multilocus enzyme electrophoresis [22], ribotyping [8], and restriction fragment length polymorphism (RFLP) analysis [19]. A further advantage of RAPD is that knowledge of the DNA sequence of the target organism is not required, thus a very large array of arbitrary oligonucleotides can be tested to identify suitable primers. However, reproducibility can be a problem under certain RAPD reaction conditions. This is largely due to the low annealing temperature required for PCRs that use arbitrary primers of very short length. Recently, universal rice primers (URPs) developed from a repetitive sequence of the rice genome were successfully used in a RAPD assay to genotype bacterial species, including strains of *Escherichia coli*, *Salmonella*, and *Brucella abortus* [13].

*Salmonella* use diverse virulence factors to aid in colonization on and invasion into host tissue; however, the virulence factors of *S.* Gallinarum and their mechanisms of action are poorly understood [12]. These include *Salmonella* plasmid virulence (*spv*) factor, invasion genes (*invA-H*), plasmid encoded fimbria (*pef*) and *S.* Enteritidis fimbriae (*sef*) [1, 3–5, 7, 10, 15]. It was previously reported that 85% of *S.* Pullorum isolates in the USA had *spvB*, *invA*, and *sefC* [9].

We previously reported on the invasiveness, plasmid profiles and antibiotic susceptibilities of 30 field isolates of *S.* Gallinarum. All of the isolates had higher invasion ability than that of the standard *S.* Gallinarum (ATCC 9184), although no correlation was observed between plasmid profile and invasion ability [14].

In the present study, we analyzed 30 *S.* Gallinarum isolates by RAPD assay to characterize the level of genetic diversity. To optimize the DNA fingerprinting, 13 arbitrary primers, including URPs, were evaluated and appropriate primers were selected and compared. Finally, Southern hybridization was used to detect the presence of *Salmonella* virulence genes, and the association of these virulence factors with the RAPD types was evaluated.

## MATERIALS AND METHODS

*Salmonella strains and isolates*: All 30 *S.* Gallinarum isolates used in this study are listed in Table 1. All were

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Table 1. Properties of the *S*. Gallinarum isolates used in the study

| Strain or isolate | Year of isolation | Province | RAPD Type | | *Salmonella* virulence genes | | |
|---|---|---|---|---|---|---|---|
| | | | URP-6 | 1290 | *invA* | *spvB* | *sefC* |
| B1001 | 1992 | Kyonggido | I | A | + | + | + |
| B1002 | 1997 | Kyonggido | I | A | + | + | + |
| B1003 | 1998 | Kyonggido | I | A | + | + | + |
| B1004 | 1999 | Kyonggido | I | A | + | + | + |
| B1005 | 1999 | Kyonggido | I | A | + | + | + |
| B1006 | 1999 | Kyonggido | I | A | + | + | + |
| B1007 | 1999 | Unknown | I | A | + | + | + |
| B1008 | 1999 | Kyonggido | IV | A | + | + | + |
| B1009 | 1999 | Chungcheongdo | I | A | + | + | + |
| B1010 | 1999 | Kyonggido | I | A | + | + | + |
| B1011 | 1999 | Kyonggido | I | A | + | + | + |
| B1012 | 1999 | Kyonggido | I | A | + | + | + |
| B1013 | 1999 | Kyonggido | IV | A | + | + | + |
| B1014 | 1999 | Kyonggido | IV | A | + | + | + |
| B1015 | 1999 | Kyonggido | I | A | + | + | + |
| B1016 | 1997 | Chungcheongdo | V | A | + | + | + |
| B1017 | 1997 | Chungcheongdo | III | A | + | + | + |
| B1018 | 1997 | Chungcheongdo | I | A | + | + | + |
| B1019 | 1997 | Chungcheongdo | IV | A | + | + | + |
| B1020 | 1997 | Chungcheongdo | IV | A | + | + | + |
| B1021 | 1997 | Chungcheongdo | IV | A | + | + | + |
| B1022 | 1997 | Chungcheongdo | IV | A | + | + | + |
| B1023 | 1997 | Chungcheongdo | VI | B | + | + | + |
| B1024 | 1994 | Kyonggido | I | A | + | + | + |
| B1025 | 1994 | Chungcheongdo | I | A | + | + | + |
| B1026 | 1998 | Kyonggido | II | A | + | + | + |
| B1027 | 1998 | Kyonggido | I | A | + | + | + |
| B1028 | 1998 | Kyonggido | I | A | + | + | + |
| B1029 | 1998 | Kyonggido | I | A | + | + | + |
| B1030 | 1998 | Cheollado | I | A | + | + | + |

*S.* Gallinarum (ATCC 9184) and *S.* Gallinarum 9R vaccine strain belong to IV and VII RAPD type with URP-6, respectively.

obtained from layers and identified using a Vitek system, which tested for basic biochemical markers including oxidase and catalase activities. *S.* Gallinarum strain (ATCC No. 9184) was used as a reference strain. *Salmonella enterica* serovars Enteritidis, Paratyphi A, Paratyphi B, Choleraesuis, Typhimurium, Typhi and London were used to screen for suitable RAPD primers. All bacterial strains were stored at –70°C until used.

*Extraction of chromosomal DNA*: Bacterial chromosomal DNA was prepared as described previously [21] with minor modifications. Briefly, the bacteria were cultured in LB broth overnight and the cells from a culture were harvested by centrifugation at 12,000 × g for 1 min. The cell pellet was resuspended in 567 $\mu l$ of TE buffer [10 mM Tris-HCl, 1mM EDTA, (pH8.0)], 30 $\mu l$ of 20% sodium dodecyl sulfate (SDS) and 3 $\mu l$ of proteinase K (2 mg/m*l*) and the mixture was incubated at 37°C for 2 hr. After 2 extractions with phenol:chlorform:isoamyl alcohol (25:24:1), the aqueous phase of DNA was combined with 0.1 volume of 3 M sodium acetate and 0.6 volume of isopropanol. The DNA was incubated at –20°C for 2 hr and precipitated by centrifugation at 12,000 × g for 10 min at 4°C. The DNA pellet was washed with 70% ethanol, air-dried and was dissolved in TE buffer. The concentration of DNA was measured by spectrophotometery at an absorbance of 260 nm and the DNA was stored at 4°C until use.

*RAPD fingerprinting*: Twelve universal rice primers (URPs; Seoulin Bioscience, South Korea) were tested on genomic DNA isolated from the *S.* Gallinarum reference strain (ATCC 9184). URP-6 (5'-GGCAAGCTGGTGGGAGGTAC-3') yielded informative fingerprints and was therefore used to evaluate the *S*. Gallinarum isolates. Primer 1290 (5'-GTGGATGCGA-3'), used previously for S. Gallinarum RAPD [9], was also tested and compared with URP-6.

Amplification reactions were performed in a 30 $\mu l$ PCR mixture containing 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.8), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.2 mM each dNTP, 20 pM primer, 150 *n*g genomic DNA as a template, and 1 unit of *Taq* Plus DNA polymerase (Bionics, South Korea). PCR amplification with the URPs was carried out using the following profile: one cycle of 94°C for 4 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; one cycle of a final extension at 72°C for 10 min. PCR amplification with primer 1290 was similar except that the annealing temperature was 42°C [9]. After PCR, 10 $\mu l$ of amplified PCR products were electrophoresed in a 1.8% agarose gel in 0.5 × TBE buffer (90 mM Tris-borate, 2 mM

EDTA) and visualized by ethidium bromide staining. The DNA fingerprint patterns were scanned and transformed into binary code and analyzed by TREECON (ver. 1.3b, Belgium) [18].

To test for reproducibility of the RAPD fingerprints produced by URP-6 and primer 1290, RAPD analyses were performed on *S.* Gallinarum (ATCC 9184) and 7 other *Salmonella* strains with different serotypes.

*Detection of virulence genes by Southern hybridization*: Nucleotide probes for *Salmonella* virulence genes, *invA, spvB* and *sefC* were generated by PCR as described previously [9]. PCR products were purified with a Gel Extraction Kit (Qiagen, Germany) and the purified DNAs were labeled by random-primed incorporation of digoxigenin (DIG)-labeled dUTP by Klenow enzyme according to the manufacturer's instructions (Roche, Germany). Southern hybridization was conducted as previously described [11]. Chromosomal DNA preparations were digested with *Eco*RI, and electrophoresed in 1.0% agarose gels in TBE buffer. After transfer of DNA to the membrane, hybridization was carried out at 68°C overnight in hybridization buffer containing DIG-labeled probe. Hybridization was detected using alkaline phosphatase-conjugated anti-DIG antibodies and X-phosphate + NBT according to the manufacturer's instructions (Roche).

## RESULTS

*Selection of primers*: To select suitable primers for RAPD analysis, 12 URPs and primer 1290 were tested in reactions using genomic DNA of *S.* Gallinarum (ATCC 9184) as a template (Fig. 1). The resulting DNA fingerprints contain bands ranging in size from 150 bp to approximately 4 kb and include both weak and intense bands. The number of bands in a fingerprint varies from 5 to 12. RAPD PCR analysis in triplicate revealed indistinguishable banding patterns for all identical samples (data not shown).

RAPD analyses with URP-6 and primer 1290 were also performed on 8 *Salmonella* serotypes, including *S.* Paratyphi A, *S.* Paratyphi B, *S.* Typhimurium, *S.* Choleraesuis, *S.* Enteritidis, *S.* Typhi, *S.* Gallinarum, and *S.* London. Both primers produced a unique DNA fingerprint for each of the *Salmonella* serotypes (Fig. 2). Phylogenetic analysis of these RAPD patterns revealed that *S.* Gallinarum forms a distinct branch in the dendrogram compared with other *Salmonella* serotypes (data not shown). For further analyses, URP-6 and primer 1290 were chosen because both yielded reproducible patterns and relatively numerous bands. In addition, the URP-6 primer seemed to be the most promising because it showed totally different DNA fingerprints for Standard *Salmonella* serotypes (Fig. 2).

*RAPD analysis with URP-6 and primer 1290*: DNA fingerprints obtained using URP-6 for the 30 *S.* Gallinarum isolates are shown in Fig. 3. Each fingerprint contains 9 to 12 DNA bands varying in size from 0.15 to 3.5 kb and can be classified into one of 6 different RAPD types (I to VI) (Table 1 & Fig. 3). RAPD types I and IV are the most common; 19 of the 30 *S.* Gallinarum isolates (63.3%) belong to RAPD type I while 7 of the 30 isolates (23.3%) belong to RAPD type IV. Single isolates (3.3%) comprise RAPD types II, III, VI and VII. Two different RAPD types were produced with primer 1290 (Table 1 & Fig. 4). The most prevalent is RAPD type A, to which 29 *S.* Gallinarum isolates (96.7%) belong.

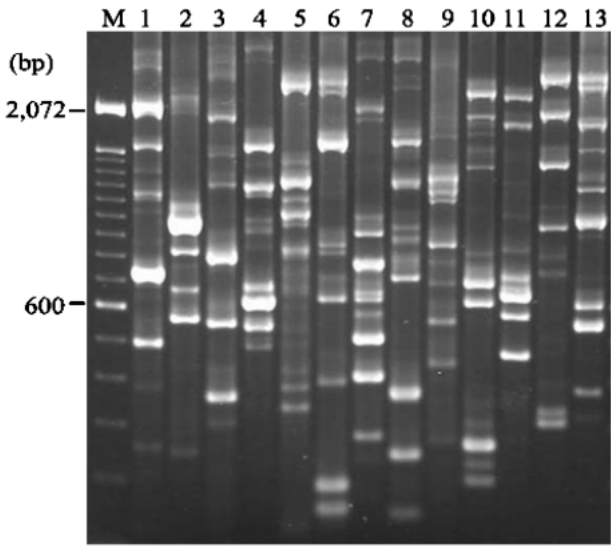


Fig. 1. RAPD fingerprints generated with universal rice primers and primer 1290 for *S.* Gallinarum (ATCC 9184). Lanes M, 100-bp DNA size standard; 1–12, URP-1 to URP-12; 13, primer 1290.

Particular *S.* Gallinarum RAPD types do not appear to be associated with any specific province or geographical location (Table 1).

*Distribution of Salmonella virulence genes*: To assess the potential virulence of *S.* Gallinarum isolates, Southern hybridization was used to detect the presence of *Salmonella* virulence genes. All 30 *S.* Gallinarum isolates were positive for *invA, spvB*, and *sefC* (Table 1). Each gene probe detected a single, specific *Eco*RI fragment of the same size in each *S.* Gallinarum isolate (data not shown).

## DISCUSSION

The RAPD method using arbitrary primers was chosen in this study to evaluate genomic diversity among *S.* Gallinarum isolates. The advantage of RAPD is that it does not require any specific knowledge of the DNA sequence of the target organism; therefore, RAPD can be adapted to any species. However, RAPD assays can suffer from poor reproducibility, including inconsistent band intensities, which complicates interpretation of the fingerprints. This lack of reproducibility is largely due to the low annealing temperatures that are necessary when short random primers are used. To overcome this problem, we used a URP set composed of twelve 20-mer oligonucleotides. Because these longer primers work at higher annealing temperatures

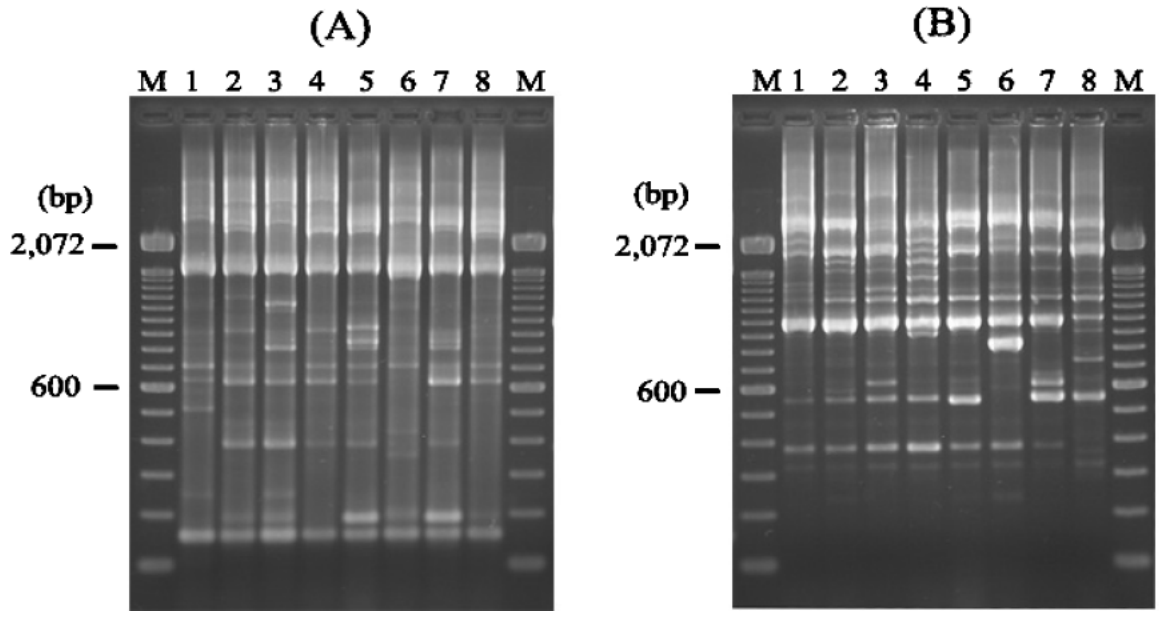


Fig. 2. RAPD fingerprints of eight standard *Salmonella* serotypes obtained by primer URP-6 (A) and primer 1290 (B). Lanes M, 100-bp DNA size standard; 1, *S.* Paratyphi A; 2, *S.* Paratyphi B; 3, *S.* Typhimurium; 4, *S.* Choleraesuis; 5, *S.* Enteritidis; 6, *S.* Typhi; 7, *S.* Gallinarum; 8, *S.* London.

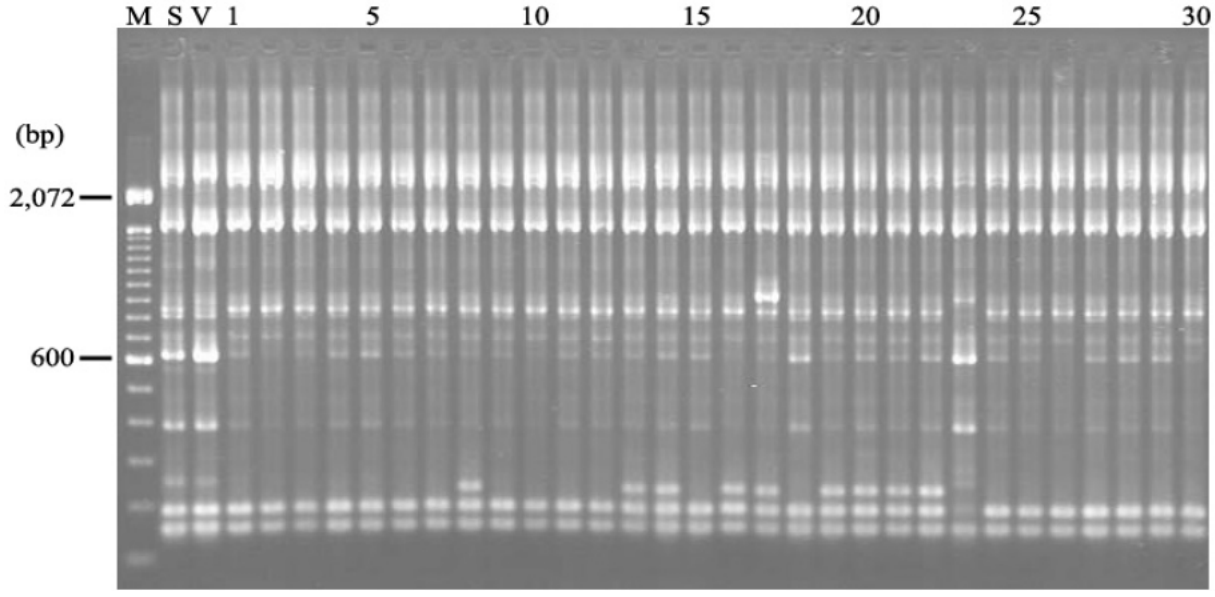


Fig. 3. RAPD fingerprints of *S.* Gallinarum isolates obtained by primer URP-6. Lanes M, 100-bp DNA size standard; S, *S.* Gallinarum (ATCC 9184); V, *S.* Gallinarum 9R vaccine strain; 1–30, *S.* Gallinarum isolates (B1001 to B1030).

(e.g., 55°C), RAPD reactions performed with URP primers are highly reproducible, as shown here and elsewhere [13].

URP-6 and primer 1290 identified 6 and 2 RAPD types, respectively, among 30 *S.* Gallinarum strains examined. Primer 1290 showed less discriminatory power than URP-6 for *S.* Gallinarum isolates, although the former exhibited high discriminatory power for both *S.* Pullorum and avian *Escherchia coli* isolates [9, 17].

Phylogenetic analysis of the *S.* Gallinarum isolates showed them to be nearly homogeneous. Most *S.* Gallinarum isolates were clustered closely together in the phylogenetic dendrogram (data not shown). The largest difference observed among the 30 *S.* Gallinarum isolates tested using URP-6 involved 4 bands. Specific RAPD types

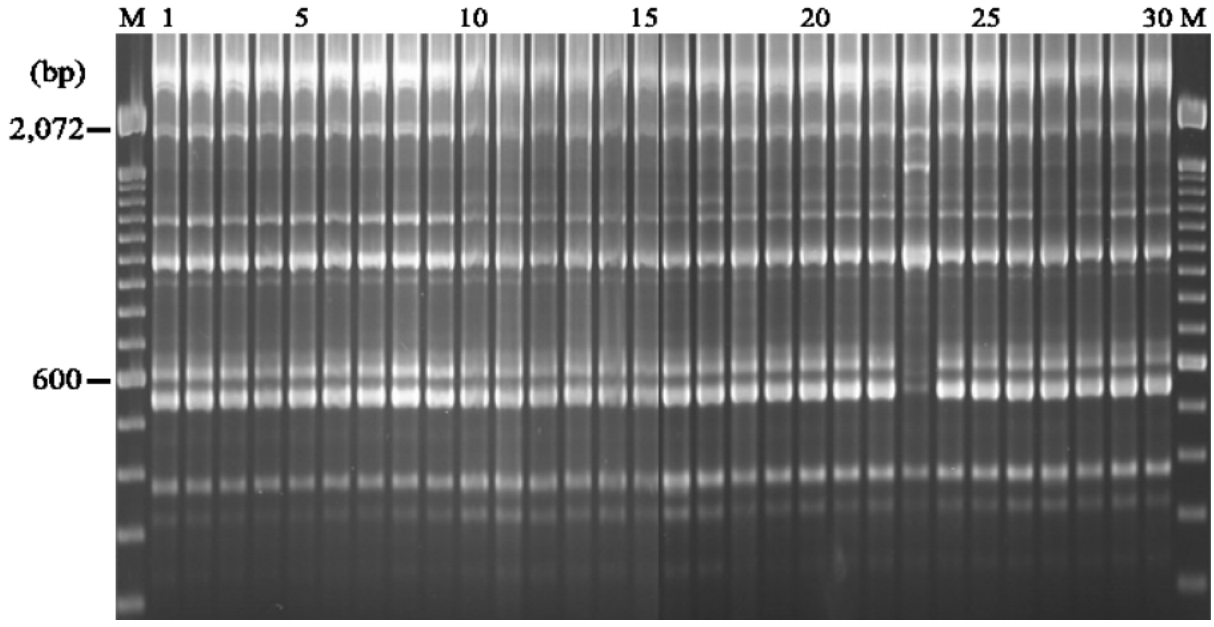


Fig. 4. RAPD fingerprints of *S.* Gallinarum isolates obtained by primer 1290. Lanes M, 100-bp DNA size standard; 1–30, *S.* Gallinarum isolates (B1001 to B1030).

did not appear to be localized to any particular geographical location.

In a previous study, *S.* Gallinarum isolates were examined for plasmid type and divided into 4 plasmid profiles [14]. We compared the RAPD types in the present study with the plasmid profile but the isolates belonging to a specific RAPD type do not belong to a specific plasmid profile.

*Salmonella* virulence is mediated by a number of genes responsible for colonization, invasion, and dissemination within chickens [2, 15, 20, 24]. These bacterial genes also affect the host inflammatory response and eventually the bacterium's fate within its host. All *S.* Gallinarum isolates were positive for *invA* and *spvB*. In addition, all *S.* Gallinarum isolates were positive for the *sefC* fimbrial gene, a marker unique to avian-adapted *Salmonella* species, such as *S.* Enteriditis, *S.* Pullorum and *S.* Gallinarum [5].

We previously examined these same *S.* Gallinarum isolates for phenotypic differences in their ability to invade epithelial cells [14]. The invasion assay revealed moderate (51.8% for isolate No. B1026) to high invasion levels (79.4% for isolate No. B1017) in a Vero cell line. Although there were variable levels of invasion among these *S.* Gallinarum isolates, all potential virulence genes evaluated in this study were present in each isolate. Moreover, there is no correlation between the invasiveness and RAPD types. This indicates that other virulence factors modulating cell invasion might be involved. Further investigations for such virulence factors are needed for a better understanding of the pathogenesis of *S.* Gallinarum.

Fowl typhoid due to *S.* Gallinarum has had a devastating impact on the poultry industry in Korea since 1992. Based on this investigation, most of the isolates responsible for the disease incidents were probably either genetically identical or very similar, despite the differences in timing and geography of the outbreaks. This study also showed that RAPD analysis, when performed with an appropriate primer, such as URP-6, is a simple, rapid and powerful method for typing *S.* Gallinarum.

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