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Genetic Diversity of Multi-resistant *Salmonella enterica* Serotype Typhimurium Isolates from Animals and Humans

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In this study, the genetic diversities of multi-resistant *Salmonella typhimurium* (ST) isolates were analyzed via the application of both pulsed field gel electrophoresis (PFGE) and Polymerase chain reaction (PCR) analysis methods, using 6 kinds of primers (REP, ERIC, SERE, BOX, P-1254 and OPB-17). And their discriminative abilities (DA) were also compared in order to determine the most effective and reliable analysis method. 118 *S. typhimurium* isolates, cultured from diverse animals and human patients in Korea beginning in 1993, were analyzed and subjected to a comparison of Simpson's index of diversity (SID), using both PFGE and PCR methods. PFGE by *Xba*I enzyme digestion allowed for discrimination into 9 pulsotypes, with high SID values (0.991) on the genomic DNA level. This shows that PFGE is a very discriminative genotypic tool, and also that multiple clones of *S. typhimurium* isolates had existed in domestic animals and humans in Korea since 1993. However, we could ultimately not to trace the definitive sources or animal reservoirs of specific *S. typhimurium* isolates examined in this study. Depending on the SID values, the combined method (7 kinds of method) was found to be the most discriminative method, followed by (in order) SERE-PCR, REP-PCR, ERIC-PCR, PFGE & OPB-17 (RAPD), P-1254 (RAPD), and BOX-PCR at the 80% clone cut-off value. This finding suggests that the REP-PCR method (which utilizes 4 primer types) may be an alternative tool to PFGE for the genotyping of *S. typhimurium* isolates, with comparable cost, time, and labor requirement. The establishment of a highly reliable and discriminatory method for epidemiologic analysis is considered necessary in order for researchers to trace the sources of specific pathogens and, consequently, to control and prevent the spread of epidemic *S. typhimurium* isolates to humans.

Keywords: Molecular Epidemiology, Genotyping, PCR, PFGE, *S. typhimurium*

The genetic analysis of *Salmonella* serotypes is a epidemiological tool necessary in order to trace sources of infection, as well as to identify epidemiological links among isolates from animals, humans, and environmental sources, as most of *Salmonella* serotypes have been recognized as zoonotic pathogens (Liebana *et al.*, 2001). In general, *Salmonella* species are known to be genetically similar. Therefore, the genetic characteristics between *Salmonella* serotypes must be analyzed accurately and objectively, using an efficient, reliable, and discriminative genetic analysis technique (Burr *et al.*, 1998; Garaizar *et al.*, 2000; Woo, 2005). Thus far, a number of genetic analysis methods for the genotyping of *Salmonella* serotypes have become available. Since its development by Schwartz and Canto, pulsed-field gel electrophoresis (PFGE) has become the method of choice for the genotyping of *Salmonella,* and PFGE is now the standard method used at the CDC (Centers for Disease Control and Prevention, USA) (Kaufmann and Pitt, 1994; Thong *et al.*, 2002). This technique has proven extremely discriminative as compared to serotyping, ribotyping, or other genetic sub-typing techniques (Liebana *et al.*, 2001; Woo, 2005).

However, repetitive sequence-based PCR (REP-PCR), which utilizes primers for repetitive DNA sequences, has also been used to genetically differentiate between enteric bacteria (Burr *et al.*, 1998; Lim *et al.*, 2002) and has recently been applied to the genetic analysis of *Salmonella* serotypes (Garaizar *et al.*, 2000). REP-PCR has a possible advantage over PFGE, in that it is more rapid and less costly and time-consuming than PFGE. Molecular sub-typing data may prove useful in the analysis of novel epidemic strains from salmonellosis outbreaks (Liebana *et al.*, 2001; Woo, 2005). The primary objective of this study, then, was

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to determine the extent of genetic diversity and clonality in *S. typhimurium* isolates from different regions and origins in Korea, using both PFGE and PCR techniques. We also evaluated and compared the discriminatory abilities (DA) of these methods, on the basis of Simpson's index of diversity (SID), in order to select the most discriminatory technique for the genotyping of *S. typhimurium.*

The MRSTDT 104 strain has already been identified as an epidemic and zoonotic pathogen worldwide. However data from investigations of this specific strain is very rare in Korea (Woo *et al.*, 2000(a); 2001(b); Kim *et al.,* 2004). Therefore, we have attempted to find out the MRSTDT 104 strain among domestic multi-resistant *S. typhimurium* isolates, using the multiplex-PCR (M6-PCR) technique, which was performed to amplify and detect the six antimicrobial drug-resistance genes, simultaneously.

Materials and Methods

Strains and Serotyping

A total of 118 isolates of *S. typhimurium* from humans (blood and feces, n=13), swine (n=69), avians (chicken organs, n=4; chicken meat, n=29; wild birds, n=2), a goat (n=1), cattle (n=3) and MRSTDT 104 (n=7) were collected and typed. Both the isolation and biochemical identification of the organisms were conducted via standard laboratory methods. Serotyping was typed in accordance with the Kauffmann-White scheme, using the slide and tube agglutination method, at the National Veterinary Research and Quarantine Service (NVRQS, Korea) (Woo *et al.*, 2000 (a); 2001 (b)).

Pulsed-field gel electrophoresis (PFGE) and PCR

Intact chromosomal DNA was prepared with a low-melting agarose plug, via a previously described method, with some modifications (Liebana *et al.*, 2001; Woo, 2005). Depending on the previous experiences, one restriction endonuclease, *Xba*I, was selected, and was used to digest the chromosomal DNA of *S. typhimurium* isolates. All *S. typhimurium* isolates were genotyped using a pulsed field gel electrophoresis (PFGE) apparatus (CHEF system, Bio-Rad, USA). PCR, using primers for repetitive extragenic palindromic sequences (REP-PCR), were conducted using a PCR apparatus (Applied-Biosystems, USA). The primer sequences for both REP-PCR and RAPD (random amplified polymorphic of DNA) were as follows: REP (REP 1R-I (5'-IIIICgICgICATCIggC-3') & REP 2R-I (5'-ICgICTTATCIggCCTAC-3')), ERIC motifs (*Enterobacterial* repetitive intergenic consensus; 1R (5'-ATgTAAgCTCCTggggATTCAC-3') & 2R (5'-AAg TAAgTgACTggggTgAgCg-3')), BOX (*Streptococcal* box motif; 5'-CTACggCAAggCgACgCTgACg-3'), SERE (*Salmonella enteritidis* repetitive extragenic; 5'-gTgAgTATATTAgCATCCgCA-3'), P-1254 (5'-CCg CAgCC-AA-3'), and OPB-17 (5'-AgggAACgAg-3'), respectively. REP-PCR generates DNA fragments, which are the genomic elements separating the primer sites. PCR mixture and reaction programs were utilized with some modifications after previous optimization tests, and other related conditions were predicated on previous accounts (Calson *et al.*, 1999; Garaizar *et al.*, 2000). Fingerprinting profiles generated by both PFGE and PCR were analyzed with GelCompar II software (version II, Applied Math, Belgium). The profiles were scored for the presence and absence of DNA bands, and strains differing by one DNA band were assigned different pulsed-field profiles (PFP). In the absence of the Gold Standard (e.g., complete genome sequencing) as a basis for genetic comparison, the extent of variability was determined using the Dice coefficient. Clustering was predicated on the unweighted pair group average method (UPGMA) (Liebana *et al.*, 2001; Woo, 2005). Finally, a comparison and evaluation of DA (discriminative ability) among the seven individual typing methods was conducted on the basis of the Simpson's index of diversity (SID) method (Hunter and Gaston, 1988).

Multiplex-PCR (M6-PCR)

In order to detect the genes encoded the specific antimicrobial drug resistance, multiplex-PCR (M6-PCR) simultaneously using six type primers (*spv*B, *clm/tet*R, *Tem*, *inv*A, *Sip*B/C and *PSE*1) was conducted. M6-PCR was performed with an automated thermocycler (Applied Biosystems, USA). PCR mixtures and reactions were conducted by Carlson's protocol (Carlson *et al.,* 1999).

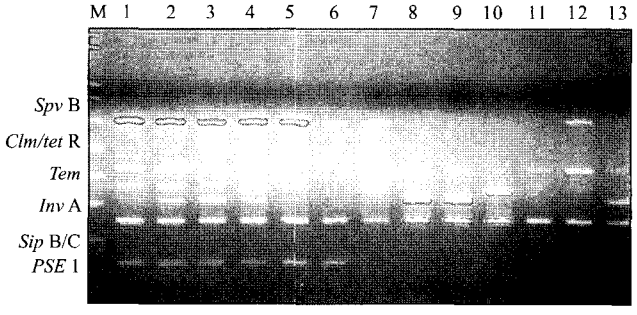

Fig. 1. Multiplex PCR amplification products for rapid and specific detection of R-type (ACSSuT) *S. typhimurium* DT104 (MRSTDT 104) strains. DNA bands were indicated *spv* B (650 bp), *clm/tet* R (280 bp), *Tem* (300 bp), *inv*A (284 bp), *SipB/C* (250 bp) and *PSE*1 (150 bp), in order. M shows the 1 kb Plus DNA ladder (Invitrogen, USA), lanes 1 to 4; the MRSTDT 104 reference strains, lanes 5 to 13; R-type (ACSSuT) *S. typhimurium* field isolates from swine, chicken and humans in Korea.

Table 1. Comparison of genotyping methods based on the discriminative ability on 118 *Salmonella typhimurium* isolates including the MRSTDT 104 from animals and humans in Korea

Typing method	No. of genetic type	Prevalence of dominant type (%)	Genetic similarity (%)	SID[a]
PFGE (*Xba*I)	11	5 (13.2)	31.47	0.924
REP-PCR	18	11 (9.3)	0.0	0.957
ERIC-PCR	21	11 (9.3)	21.83	0.948
SERE-PCR	25	12 (10.2)	30.27	0.959
BOX-PCR	17	16 (13.6)	30.27	0.936
RAPD (OPB-17)	25	14 (11.9)	0.0	0.946
RAPD (P-1254)	18	11 (9.3)	12.67	0.941
Combined method[b]	31	8 (6.8)	6.18	0.969

a. SID was assessed according to the study of Hunter and Gaston.
b. This method shows the combined analysis results of both six kinds of PCR and PFGE method.

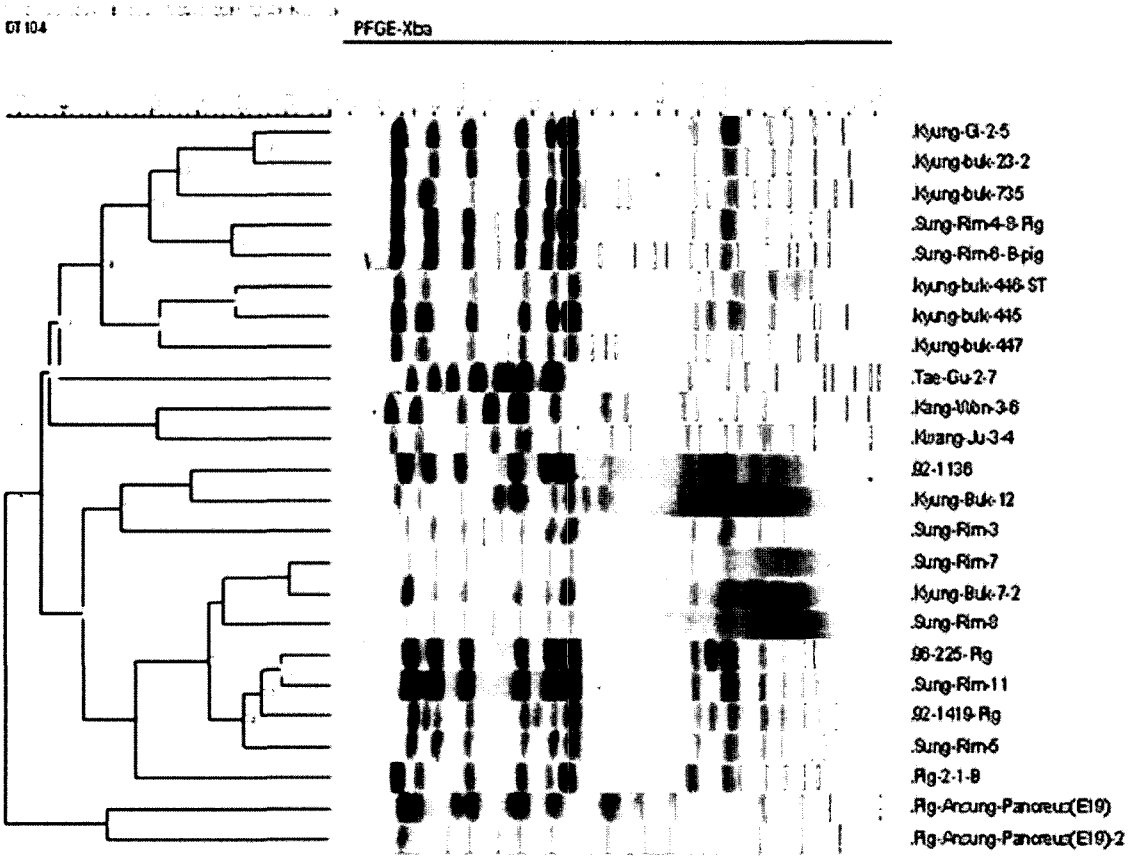

Fig. 2. Dendrogram (Left panel) and DNA fingerprints (Right panel) generated by the GelCompar II software showing the relationship of representing PFGE fingerprints for 24 *Salmonella typhimurium* isolates including the MRSTDT 104 strains from animals and humans in Korea. DNA was digested with *Xba*I. Pattern clustering on a matrix of dice coefficient was based on the unweighted pair group method with arithmetic averages (UPGMA).

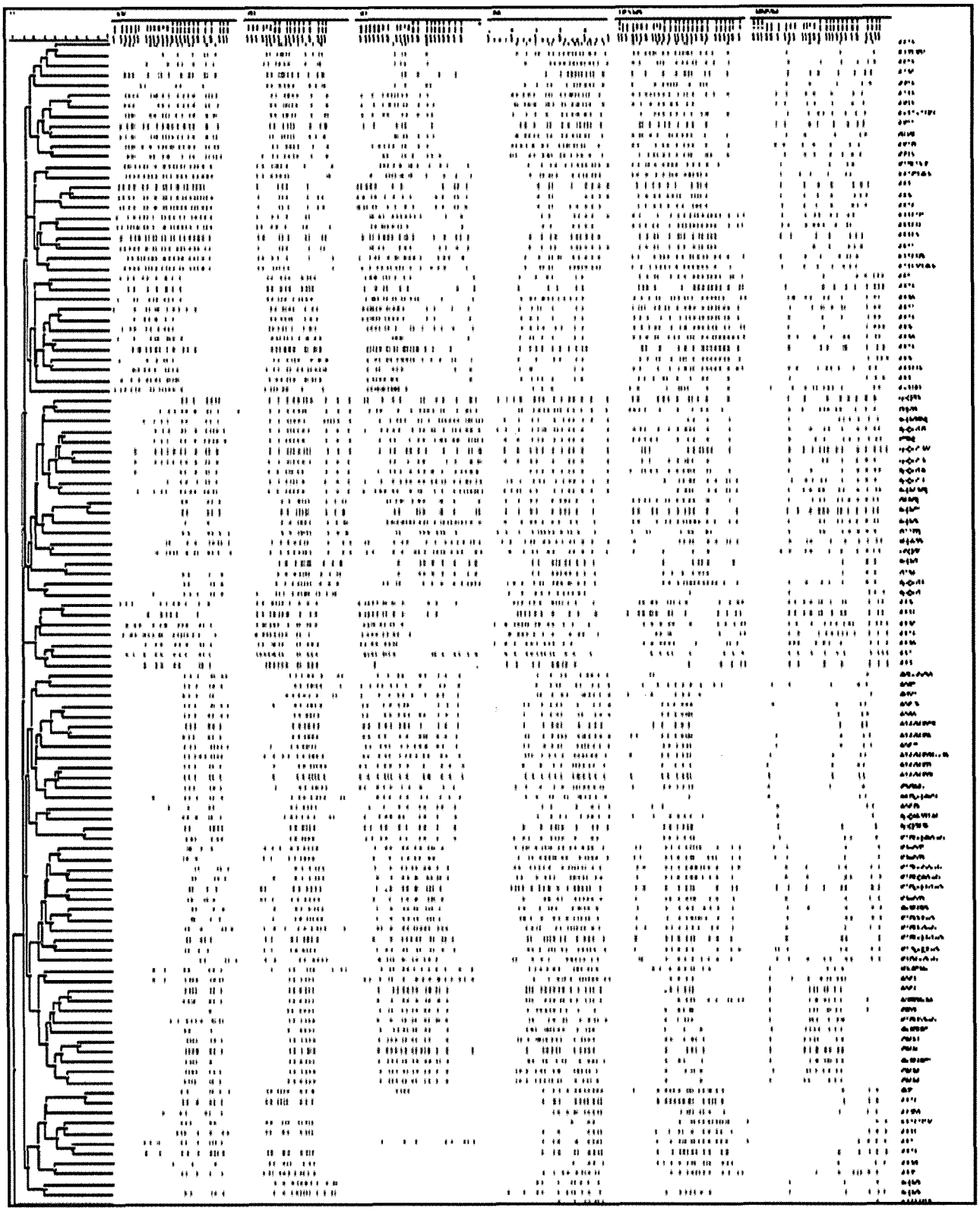

Fig. 3. Clustering of 115 *Salmonella typhimurium* isolates by REP-PCR, ERIC-PCR, BOX-PCR, SERE-PCR, RAPD and PFGE combined fingerprints. Pattern clustering on a matrix of dice coefficient was based on the unweighted pair group method with arithmetic averages (UPGMA) and dendrogram was constructed with the GelCompar II analysis software.

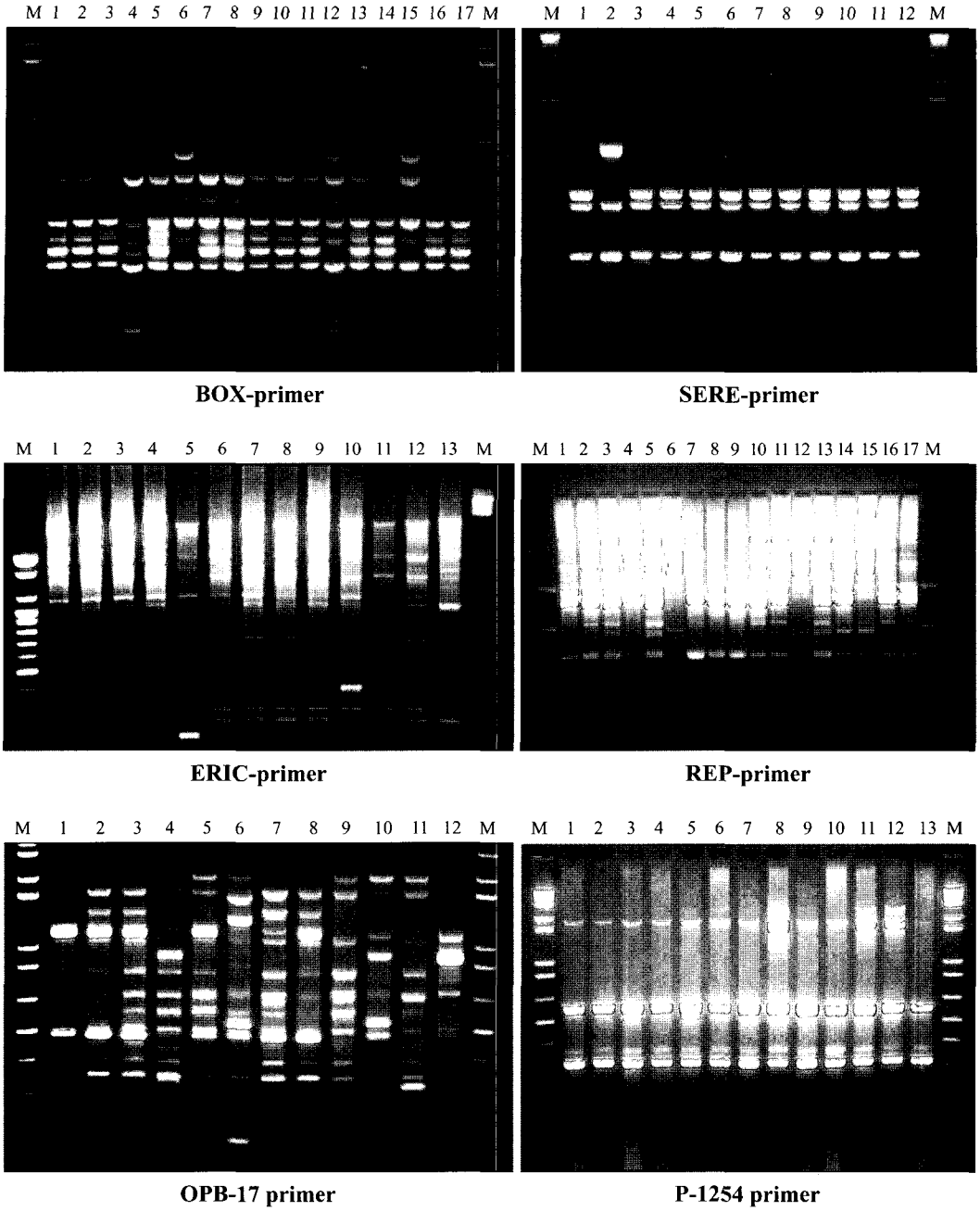

Fig. 4. Photographs of electrophoretic gels from reliability testing for REP-PCR, using BOX, SERE, ERIC and REP primers and RRAP (P-1254 and OPB-17 primer). Numbers identify the individual *Salmonella typhimurium* isolates and "M" identifies the marker lanes (100 bp ladder, Bioneer, Korea).

Results

A total of 118 isolates of *S. typhimurium* (ST) were initially screened for the existence of six types of antimicrobial resistance genes via the M6-PCR technique, due to the epidemiologic importance of the MRSTDT 104 strain as an epidemic and zoonotic pathogen. Fortunately, we were unable to detect the MRSTDT 104 strain in this study, whereas the R-type (ACSSuT) multi-resistant ST isolates were detected only in the ST isolates from domestic animals based on the antibiogram profiles (Fig. 1). Due to the ineffectiveness of the phage typing system for *S. typhimurium*, the exact phage types of *S. typhimurium* isolates submitted in the

present study could not be determined. DA of PFGE, REP-PCR (ERIC-primer, SERE-primer, BOX-primer, and REP-primer) and RAPD (both P-1254 and OPB-17 primer) methods, as well as a combined analysis method of the above 7 typing methods, were compared in an attempt to select the most efficient and reliable analysis method (Table 1).

In PFGE (Fig. 2 & 3), 38 chromosomal DNAs of *S. typhimurium* isolates, including the MRSTDT 104 strains, were digested with *Xba*I, and analyzed on the basis of their pulsed-field profiles (PFP). Although other enzymes (*Bln*I & *Spe*I) were also compared on the same ST isolates, in an effort to select the most suitable enzyme in the previous study, the *Xba*I enzyme was finally determined to be the most suitable and cost-effective enzyme, considering our diverse laboratory conditions (data not shown). This enzyme was discriminated into 13 to 21 fragments, ranging from 903.5 kb to 35.0 kb, and was also able to be differentiated into 11 different pulsotypes (PFP). In the present study, PFGE could discriminated with a 61.5% genetic relationship between the ST isolates from swine and chickens. This analysis method evidenced the lowest DA (SID, 0.924) among the tested methods, but the reliability of PFGE method was found to be sufficient (Table 1). As an overabundance of genetic clones was detected in this study, we were ultimately unable to determine any specific sources, and also to find out the definitive animal reservoir of *S. typhimurium* isolates.

In the present PCR methods (Fig. 4), both the ERIC and REP-primers allowed for discrimination into 21 and 18 genotypes on the basis of their DNA profiles, which were far more complex than those of any of the other REP-PCR primers (BOX and SERE). Whereas the SERE primer generated the most genotypes (n=25), along with the OPB-17 primer (RAPD), the BOX primer generated only 17 genotypes (Fig. 3 & 4). According to our results of the genetic properties of *S. typhimurium* isolates from domestic animals and humans, the SERE-primer, which generated the smallest number of DNA bands and also the simplest DNA profiles, showed the most powerful DA (SID; 0.959), followed by REP (SID; 0.957), ERIC (SID; 0.948), and BOX (SID; 0.936), in order (Fig. 3 & 4). In general, the number of genotypes generated by individual PCR primers was found to be directly proportional to the DA of the PCR technique. In RAPD analysis, although the P-1254 primer generated a more monotonous DNA profile than that associated with the OPB-17 primer, this primer allowed for discrimination into only 18 genotypes, and evidenced a far lower DA (SID; 0.941) than did the OPB-17 primer (SID; 0.946). When the seven genotyping methods were combined and their results were analyzed, the combined method showed the highest DA (0.969), with the most genotypes (n=31) (Table 1).

Discussion

S. typhimurium constituted the major pathogen responsible for salmonellosis in both animals and humans, until the unexpected appearance of *S. enteritidis* in domestic poultry in the mid-1990's (Woo *et al.*, 2000 (a) & (b); 2005). Possibly due to the inappropriate use and abuse of antibiotics for the treatment of salmonellosis, R-type (ACSSuT) and other multi-resistant (more than five drugs) *S. typhimurium* isolates have already been seen in domestic livestock farms, as evidenced by antimicrobial drugs susceptibility tests (Woo *et al.*, 2000 (a); 2001 (b)). On M6-PCR, reference MRSTDT 104 strains simultaneously amplified a six positive DNA bands of the expected size, but both the R-type (ACSSuT) and other multi-resistant *S. typhimurium* isolates were amplified with less than five DNA bands (Fig. 1). From this perspective, it was revealed that the phage typing data of the R-type strains represents the most definitive of the epidemiological data. However, phage types could not be determined in our study, due to the limitations of the phage typing system and standard typing phages for *S. typhimurium*, but an effective *S. enteritidis* phage typing system had already been established in our laboratory (Woo, 2005).

The typing of bacterial strains originating from diverse sources is a prerequisite tool for the prevention and control of specific infection, and also for risk assessment studies of the sources of zoonotic diseases, including salmonellosis. In general, the validation of typing methods necessarily includes the evaluation of their performance. Several performance criteria are important in this regard, including type-ability, reproducibility, stability, discriminatory power, and typing system concordance (Liebana *et al.*, 2001; Thong *et al.*, 2002). The standardization of protocols for analysis could enhance reproducibility between laboratories, as well as the flow of epidemiological information. This ability can be improved via the use of software for image acquisition and analysis (Lim *et al.* 2002; Garaizar *et al.*, 2000).

In the comparison of the DA's of the methods conducted in this study, the combined method (total seven methods) was ultimately determined to be the most discriminative (SID, 0.969) followed by (in order) the SERE-primer, REP-primer, ERIC-primer, OPB-17 primer, P-1254 primer, BOX-primer, and PFGE (0.924) methods. In total, seven genotyping methods resulted in high level type-ability (over 0.900; standard SID value) on 118 *S. typhimurium* isolates (Fig. 3). According to the known reports, the primers for REP-PCR are selected on short repetitive sequences (REP, ERIC, SERE, and

BOX), which have also been applied to the identification of other members of the *Enterobacteriaceae* family, as well as some gram-positive bacteria and certain fungi (Burr *et al.*, 1998; Garaizar *et al.*, 2000; Lim *et al.*, 2002).

In conclusion, our results implicate REP-PCR as the genotyping method of choice, even in comparison to PFGE, although the fundamental principles underlying the two methods are quite different.

In general, PFGE allows for the discrimination of the entirety of the chromosomal DNA, and has been applied to a wide range of organisms. Other than the complete whole gene sequencing method, PFGE has become the gold standard in laboratories worldwide, and has also shown itself to be an invaluable epidemiological tool for the genotyping of *Salmonella* serotypes, due to its high reproducibility and discriminative power (Liebana *et al.*, 2001; Garaizar *et al.*, 2000; Woo, 2005). In specific cases in which such a high degree of precision is necessary, e.g., the trace-back of an individual isolate to a specific contamination source, PFGE is absolutely considered to be the best method (Kaufmann and Pitt, 1994). Typically, PFGE targets the intact and large chromosomal DNA embedded in an agarose plug (Fig. 2). This is why PFGE is capable of producing a more highly reproducible and reliable result than other methods (Liebana *et al.*, 2001; Thong *et al.*, 2002).

The PCR method could not differentiate efficiently among MRSTDT 104 strains, but could discriminate within the same genotype, whereas PFGE could discriminate efficiently between MRSTDT 104 (n=7) and the other multi-resistant *S. typhimurium* isolates. According to these results, we determined that the PFPs of the *S. typhimurium* isolates from domestic swine were quite different from those of chickens, and also appeared to show a generally low genetic relationship (< 40%) between the ST isolates of both animals. This suggests that the sources of the *S. typhimurium* isolates from animals and humans were different from one another (Fig. 2 & 3). Interestingly, only one swine strain (Sung-Rim-6-B) exhibited a relatively pronounced genetic relationship (> 60%) with the chicken strains. Therefore, evaluations based on SID have been considered very easy, efficient, and objective evaluation methods for the comparison of objective results between the very qualitatively different PCR and PFGE methods (Hunter and Gaston, 1988).

All laboratory conditions, including experimental costs, rapidity, and labor-intensiveness were considered prior to the selection of the most efficient *Salmonella* genotyping method. Although the PFGE method had already been established as the general gold standard for the genotyping of diverse bacteria, we suggest that the REP-PCR method may represent an efficient and time-saving analysis tool for the genotyping of *Salmonella* serotypes, including *S. typhimurium,* considering of our laboratory conditions.

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