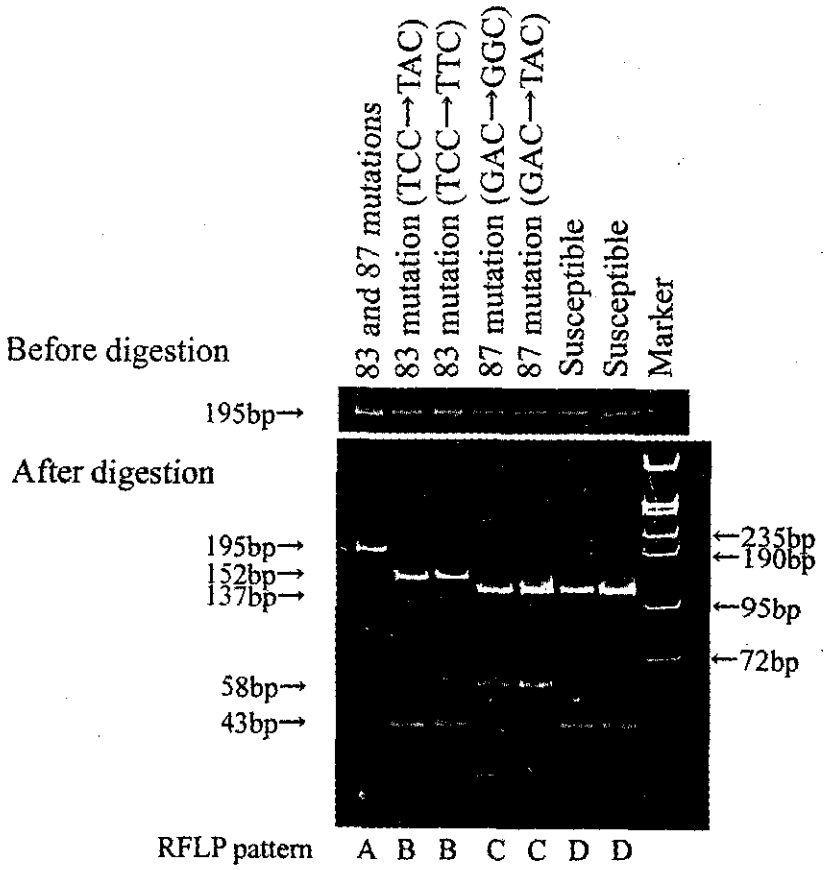

Fig. 3 PCR-RFLP patterns of the representative strains after *Hinf*I digestion. The digested PCR products were separated by 15% polyacrylamide gel electrophoresis.

Table 4. Primers used for PCR-RFLP.

Primers	Sequence		primer position [1]
gyrA-F	5'- TGT CCG AGA TGG CCT GAA GC	-3'	108-127
gyrA-HinfI-as	5'- ATG TAA CGC AGC GAG AAT GGC TGC GCC ATA CGA ACG CTG GA*[2]G	-3'	302-261

1) The primer positions were indicated by the number of nucleotide sequences from the start codon of *gyrA* gene.
2) The mismatch sequence to produce a *Hinf*I site into the amplified fragment is indicated by asterisc on the primer of gyrA-HinfI-as

susceptibility to fluoroquinolones. The purpose of this study is to develop more rapid screening method for the detection of fluoroquinolone resistant strains and reduced susceptibility strains than the ordinary culture method.

We designed the PCR-RFLP to detect common mutations related to fluoroquinolone resistance at codon 83 and 87 of GyrA. The PCR was performed with the primers, gyrA-F and gyrA-HinfI-as, which are expected to produce a 195-bp amplified fragment with a *Hinf*I restriction site at the codon corresponding to

Ser-83 of GyrA (Table 4). The reverse primer gyrA-HinfI-as, whose sequence is different by one base from the original gene sequence, introduced an artificial *Hinf*I cleavage site at Asp-87 codon of GyrA according to the primer-specified restriction site modification method. Restriction enzyme digestions were performed in a total of 20 μl of the mixture containing 10 μl of PCR product, 2 μl of 10x digestion buffer and 5U of *Hinf*I for 60 min. incubation at 37°C. The digested PCR products were separated in 15% polyacrylamide gel electrophoresis.

The alterations of the *gyrA* gene have a major role in the fluoroquinolone resistance of gram negative bacteria among the mechanisms of quinolone resistance. Our data in previous study showed that only the strains with reduced susceptibility to fluoroquinolones were found in the clinical isolates, and that the typical resistant strains (minimum inhibitory concentration (MIC) of ciprofloxacin; ≥ 4 μg/ml) were never found in the clinical isolates (8, 10). We determined the *gyrA* mutations of more than 100 strains of *S. enterica* serovar Typhi and serovar Paratyphi A with reduced susceptibility to fluoroquinolones. *S. enterica* serovar Typhi and serovar Paratyphi A clinical isolates with reduced susceptibility to fluoroquinolones had only a single mutation in the *gyrA* gene at the position of either 83 or 87 of GyrA, and other *gyrA* mutations were never found in the clinical isolates of *S. enterica* serovar Typhi and serovar Paratyphi A. The alterations in the QRDR of the *gyrB* and *parE* genes were not found in all of the strain tested (8). Our findings clearly showed that only *gyrA* mutations were involved in the fluoroquinolone resistance of *S. enterica* serovar Typhi and serovar Paratyphi A, and also support that *gyrA* mutations are principally important for the *S. enterica* serovar Typhi and serovar Paratyphi A fluoroquinolone resistance. Therefore, the screening of *gyrA* mutations is considered to be a necessary and sufficient for the screening of the strains with reduced susceptibility to fluoroquinolones in *S. enterica* serovar Typhi and serovar Paratyphi A.

A fragment including *gyrA* QRDR was amplified by PCR with the primers gyrA-F and gyrA-HinfI-as (Table 4). The size of the amplified fragment was 195 bp, which is the same in all *S. enterica* serovar Typhi and serovar Paratyphi A strains tested. After *Hinf*I digestion, the cleaved fragments of the susceptible strains of *S. enterica* serovar Typhi and serovar Paratyphi A consisted of 3 bands (137, 43 and 15 bp; RFLP pattern D in Fig.3), which was produced by the *Hinf*I sites at the nucleotide sequences corresponding to Ser-83 and Asp-87 of GyrA . The 15 bp-smallest fragment, which was produced by the cleavage between Ser-83 and Asp-87 of GyrA, was invisible in Fig.3, however, it is easy to be differentiated from the other types. The digested fragment of the strains which have a mutation in codon Ser-83 of GyrA consists of 2 bands (152 and 43 bp; RFLP pattern B in Fig.3), which lost the *Hinf*I site corresponding to the Ser-83 of GyrA. The PCR fragment of the strains which have a mutation in codon Asp-87 of GyrA consists of 2 bands (137 and 58 bp; RFLP pattern C in Fig. 3). The fragment of the strains which have mutations in both Ser-83 and Asp-87 were not digested by *Hinf*I restriction enzyme due to the point mutations at the both sites, and the fragment size did not change after the digestion with *Hinf*I (RFLP pattern A in Fig.3). We examined with this method several strains of *S. enterica* serovar Typhi and serovar Paratyphi A, which include fluoroquinolone resistant strains, the strains with

reduced susceptibility to fluoroquinolone, and fluoroquinolone susceptible strains, and we successfully screened the strains with reduced susceptibility to fluoroquinolones by this method (Table 3). Establishment of surveillance system for the detection of *gyrA* mutations will be the most important to find out the fluoroquinolone resistance of *S. enterica* serovar Typhi and serovar Paratyphi A. PCR-RFLP method described here may be one of methods for the rapid detection of such mutations.

Conclusions

The surveillance for antimicrobial resistance of *S. enterica* serovar Typhi and serovar Paratyphi A should be continued. Particularly monitoring the emergence of strains with double mutations in the *gyrA* genes, that are fully resistant to fluoroquinolones, is important for the antimicrobial resistance surveillance of clinically important *S. enterica* serovar Typhi and serovar Paratyphi A.

In conclusion, PCR diagnosis method for typhoid fever and paratyphoid fever and PCR-RFLP for screening for *gyrA* mutations, that are described here, may make it possible to get earlier diagnosis and earlier screening for fluoroquinolone resistance.

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