Molecular Analysis and Identification of Virulence Gene on \( p_{RST98} \) from Multi-Drug Resistant \( S. typhi \)

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\( p_{RST98} \) is a large and conjugative resistant plasmid (R plasmid) of 98.6 mega-dalton from multi-drug resistant \( S. typhi \) (\( S. typhi \)), which was classified to incompatibility group C (Inc C). It has been found that \( p_{RST98} \) made its host bacteria not only antibiotic resistant but also more virulent. In this study we explored the possibility of plasmid \( p_{RST98} \) in \( S. typhi \) carrying the \( Salmonella \) plasmid virulence gene - \( spv \). The plasmid \( p_{RST98} \) was isolated, purified and then digested by nine restriction endonucleases to make the plasmid enzyme profile. \( Spv \)-specific PCR and Southern blot were applied to identify the virulence gene on \( p_{RST98} \). The amplified \( spv \) fragments \( spvR \) and \( spvB \) were cloned into pGEM-T EASY and then the DNA sequences were analysed. The fragments of \( p_{RST98} \) digested by endonucleases Bgl II, Pst I and Sac II were identified, which may be useful for molecular analysis and further epidemiological surveillance of \( p_{RST98} \). The results of PCR and Southern blot showed that \( spv \) homologous genetic sequence which had been found in all pathogenesis \( Salmonella \) spp. except \( S. typhi \) was also presented on \( p_{RST98} \). The ORF of \( spvR \) and \( spvB \) of \( p_{RST98} \) were 894 bp and 1,776 bp, respectively. They have more than 99% homology with that of \( spvR \) and \( spvB \) on virulence plasmid in \( S. typhimurium \). The genotype research on \( p_{RST98} \) revealed that there is a plasmid carrying genes responsible for drug resistance and virulence in \( S. typhi \). This is the first report for such kind chimerical plasmid in \( S. typhi \). Cellular & Molecular Immunology. 2005;2(2):136-140.

Key Words: \( S. typhi \), R plasmid, virulence gene

Introduction

Typhoid fever is still an important cause of global morbidity and mortality, especially in developing countries (1-3). In a series of survey of antimicrobial susceptibility, 591 strains of \( Salmonella typhi \) (\( S. typhi \)) isolated in Suzhou during 1987-1992 were studied. It was found that more than 80% of isolates were multi-drug resistant and the resistance caused by a large and conjugative resistant plasmid (R plasmid) of 98.6 mega-dalton (159 kb), which was classified to incompatibility group C (Inc C). This 98.6 Md R plasmid of \( S. typhi \) was designated as \( p_{RST98} \). Our previous study showed that \( p_{RST98} \) could be transferred among \( E. coli \), \( S. typhimurium \), \( S. flexneri \) and \( S. typhi \) (4). Therefore, it was very difficult to block and prevent the spreading of antimicrobial resistance in bacterium. It has been also found that \( p_{RST98} \) caused its host bacteria not only antibiotic resistance but also virulence increasing (5). Previous researches in other labs have identified a highly conserved region of 8 kb, designated as \( spv \) for \( Salmonella \) plasmid virulence, presenting on the plasmids of all other pathogenesis \( Salmonella \) spp. except \( S. typhi \) and responsible for the virulence phenotype (6-12). Until now, in \( S. typhi \) neither virulence plasmid nor \( spv \) have been reported. Therefore, a hypothesis was proposed that the \( p_{RST98} \) might present a mosaic-like structure. In this study, nine restriction endonucleases were selected to make the \( p_{RST98} \) fingerprint and then the virulence gene on \( p_{RST98} \) was identified by \( spv \)-specific PCR, Southern blot and DNA sequence analysis. The results showed that \( spv \) homologous gene which had been found in other \( Salmonella \) spp. except \( S. typhi \) was also presented on \( p_{RST98} \). This is the first report revealing a chimerical plasmid carrying genes of drug resistance and virulence in \( S. typhi \).

Materials and Methods

**Bacterial strains**
1) *S. typhi* strain STpRST98 was obtained from the blood of a patient during the outbreak of typhoid fever in Jiangsu, China in 1987. It was used as a representing strain which naturally harbored pRST98 and resistant to chloramphenicol, streptomycin, trimethoprin and sulphonamide, gentamicin, neomycin, kanamycin, cephaplorin, ampicillin, carbencillin and tetracycline. 2) The antibiotic sensitive plasmid free *S. typhi* (pRST98), used as the negative control. 3) *E.coli* K12 W1485 Rif' F' Lac' that had a rifampicin resistant gene on chromosome was chosen as a recipient. Trans-conjugant pRST98/E.coli K12 W1485 was used to insure that the *spv* gene was located on the plasmid rather than the chromosome. 4) *S. typhimurium* strain carrying plasmid pGTR061, which harbored a 7.1 kb *spv*-cloning fragment obtained from a virulence plasmid of *Salmonella Dublin*. 5) The wild type *S. typhimurium* strain UK-1 and SR-11 carrying a 90 kb and a 100 kb virulence plasmid respectively, were used as the positive control (4 and 5 were kind gift of Prof. R Curtiss III Washington University in St. Louis USA). 6) *E.coli* V517 (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8 and 1.4 mD) and *S. flexneri* 2A57 (140, 105, 26.2 and 2.0 mD) harbored standard Mr plasmid. LB-broth and LB-agar plates were served as growth media except those with special explanation.

### Plasmid DNA extraction

The plasmid DNA extraction and electrophoresis analysis of isolates for making the pRST98 profile was used the method recommended by Takahashi et al. (13). For restriction enzyme analysis, choosing one colony on L-agar plates, transferred it to 2 ml LB broth containing antimicrobial agents and incubated with gentle agitation at 37°C for 8 h (300 rpm), then transferred the 2 ml bacteria broth to 100 ml LB broth containing gentamicin, cephalosporin, ampicillin, carbencillin and tetracycline. Then samples were the same as the amplification primers. The restriction endonuclease analysis of pRST98 Nine restriction endonucleases (BamH I, Bgl II, EcoR I, EcoRV V, Hind III, Pst I, Sac I, Sac II and Sph I) were selected to make the enzyme profile by digestion of pRST98 DNA. The restriction enzymes were products of Promega (USA) and Biolabs (New England). BSA was added to benefit the reaction and plasmid DNA were digested overnight at 37°C, other conditions were set down according to the manufacturer’s instructions. The digested DNA and marker λDNA/Hind III (23.2, 9.4, 6.6, 4.4, 2.3 and 2.0 kb, product of Promega) were separated on 0.5% agarose gels at 3 V/cm for 6 h. After stained with EB, the fragments of digested pRST98 DNA were visualized under UV light.

### PCR amplification

To insure that *spv* was located on plasmid pRST98, trans-conjugant pRST98/E.coli K12 W1485 was used as the templet. The primers sequences were designed according to the reported *spvR* and *spvB* sequences. The *spvR* primers were (sense) 5’-ATG GAT TTC ATT AAT AAA AAA TTA-3’ and (anti-sense) 5’-TCA GAA GGT GGA CTG TTT CAG TTT-3’. The *spvB* primers were (sense) 5’-ATG TTG ATA CTA AAT GGT TTT CTA-3’ and (anti-sense) 5’-CTA TGA GTT GAG TAC CCT CAT GTT-3’. The condition for amplification with all primer sets was 95°C for 10 min, followed by 32 cycles at 95°C for 20 s, 55°C for 20 s, and 72°C for 1 min, then followed by a single extension cycle at 72°C for 5 min. The amplified products were electrophoresed on a 1.2% agarose gel and stained with EB and UV light was used to visualize. The antibiotic sensitive *E.coli* K12 W1485 and *S. typhimurium* SR-11 were used as *spv* negative and positive control, respectively.

### Spv probe preparation and Southern blot

pGTR061 was digested by Sal I and Xho I according to the manufacturer’s instructions. 30 μl of the digested fragments and 1 kb DNA ladder (Gibco BRL) were electrophoresed in 0.7% agarose gel extraction kits. Then samples were transferred to Gene Screen Plus nylon membrane (New England Nuclear, Boston, Mass., USA). The hybridization procedures were according to the manufacturer’s instructions. The result was confirmed by autoradiography.

### Nucleotide sequencing

PCR products were purified prior to DNA sequence analysis with the PCR purification kit (QIAGEN GmbH, Germany), then direct sequencing of the amplified fragments was performed using an automatic DNA sequencer (ABI 3700, Applera Co., Foster City, CA, USA). Sequencing primers were the same as the amplification primers.

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**Figure 1. Electrophoresis plasmid profile of pRST98.** Lane 1, *E.coli* V517, plasmid size marker; Lane 2, *S. flexneri* 2A57, plasmid size marker; Lane 3, antibiotic sensitive *S. typhi*, which were plasmid free, and used as the negative control; Lanes 4-11, multi-drug resistant *S. typhi* were used as the representing strains which naturally harbored pRST98 and resistant to chloramphenicol, streptomycin, trimethoprin and sulphonamide, gentamicin, neomycin, kanamycin, cephaplorin, ampicillin, carbencillin and tetracycline.
Results

The plasmid profile and the restriction endonuclease pattern of pRST98
The plasmid profile of pRST98 was shown in Figure 1. The restriction endonuclease pattern of pRST98 was made by digestion of pRST98 DNA using nine restriction endonucleases. Lanes 1-8, the restriction fragments of pRST98 DNA were digested by EcoR I, BamH I, Hind III, Pst I, Bgl II, EcoR V, Sac I, Sac II, respectively; Lane 9, Molecular marker of λDNA/Hind III.

The PCR amplification of spv
There were five ORF in spv genes - spvRABCD. In this study, the primers designed according to the sequences of spvR and spvB could amplify the spvR and spvB homologous genetic sequences on pRST98. The size of the product was the same as the reported spvR and spvB (Figure 3).

The result of Southern blot
The probe was obtained from the Sac I-Xho I fragment of spv cloned into a pGTR061 plasmid (Figure 4). As shown in Figure 5, the agarose gel electrophoresis of plasmid pRST98, the restriction fragments of pRST98 digested by Bgl II, Pst I and Sac I were well separated and identified. But the fragments digested by EcoR I, BamH I and EcoR V were not very clear. The poorest fragments were digested by Sac I, Hind III and Sph I.

DNA sequences analysis
The DNA sequences of spvR and spvB from pRST98 were compared with the reported sequences in Microsoft NCBI. The ORF of spvR and spvB of pRST98 were 894 bp and 1,776 bp, respectively. They had more than 99% homologous with that of spvR and spvB on virulence plasmid in S. typhimurium.

Discussion
The epidemic of multi-drug resistant S. typhi affected as many as 13 provinces and cities in China in the mid and late 1980s. According to the reports of plasmid profile analysis from many other labs in China, it was demonstrated that the drug-resistant strains with a plasmid about 100 mD were the main causative agent for the epidemic (14). The patients infected with the pRST98 containing S. typhi were more severe and protracted with a higher rate of complications and mortality than usually seen in typhoid fever patients. But since 1991, S. typhi strains recovered their drug sensitivity and pRST98 could no longer be detected from S. typhi strains. However, in recent years, the epidemic of drug-resistant S. typhi in other countries of the world took place again owing to the repossessing R plasmid of the bacteria (15).

These phenomena initiated our thinking: where was the origin of pRST98? How this plasmid was transferred in different species of enteric bacilli? Was pRST98 a mosaic plasmid, carrying genes responsible for drug resistance and virulence increasing in S. typhi? To investigate the virulence of pRST98, several experiments have been carried out. Our previous research showed that 1) pRST98 could be transferred easily in laboratory from S. typhi to E.coli and further transferred easily to sensitive and resistant pathogenic bacilli, such as S. flexneri and S. typhimurium; 2) pRST98 was

Figure 2. Restriction endonuclease digestion of plasmid pRST98. The restriction endonuclease pattern of pRST98 was made by digestion of pRST98 DNA using nine restriction endonucleases. Lanes 1-8, the restriction fragments of pRST98 DNA were digested by EcoR I, BamH I, Hind III, Pst I, Bgl II, EcoR V, Sac I, Sac II, respectively; Lane 9, Molecular marker of λDNA/Hind III.

Figure 3. PCR fragments of spvR and spvB. Lane 1, DNA ladder; Lane 2, S. typhi pRST98; Lane 3, S. typhimurium SR-11; Lane 4, S. typhi pRST98; Lane 5, S. typhimurium SR-11. There were five ORF in spv genes - spvRABCD. The primers designed according to spvR and spvB sequences could amplify the spvR and spvB homologous genetic sequences on pRST98. The size of the product was same as the reported spvR and spvB.

Figure 4. spv probe from pGTR061. Lane 1, spv probe fragment; Lane 2, DNA ladder.
drugs and resistance and virulence in *S. typhi*. This is the first report revealing a chimerical plasmid carrying genes of drug resistance and virulence in *S. typhi*.

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**References**


