Article

Molecular Analysis and Identification of Virulence Gene on pR_{ST98} from Multi-Drug Resistant *Salmonella typhi*

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pR_{ST98} is a large and conjugative resistant plasmid (R plasmid) of 98.6 mega-dalton from multi-drug resistant *Salmonella typhi (S. typhi)*, which was classified to incompatibility group C (Inc C). It has been found that pR_{ST98} made its host bacteria not only antibiotic resistant but also more virulent. In this study we explored the possibility of plasmid pR_{ST98} in *S. typhi* carrying the *Salmonella* plasmid virulence gene - *spv*. The plasmid pR_{ST98} 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 pR_{ST98}. The amplified *spv* fragments *spv*R and *spv*B were cloned into pGEM-T EASY and then the DNA sequences were analysed. The fragments of pR_{ST98} digested by endonucleases Bgl II, Pst I and Sac II were identified, which may be useful for molecular analysis and further epidemiological surveillance of pR_{ST98}. 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 pR_{ST98}. The ORF of *spv*R and *spv*B of pR_{ST98} were 894 bp and 1,776 bp, respectively. They have more than 99% homology with that of *spv*R and *spv*B on virulence plasmid in *S. typhmurium*. The genotype research on pR_{ST98} 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 pR_{ST98}. Our previous study showed that pR_{ST98} 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 anti-microbial resistance in bacterium. It has been also found that pR_{ST98} 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 for *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 pR_{ST98} might present a mosaic-like structure. In this study, nine restriction endonucleases were selected to make the pR_{ST98} fingerprint and then the virulence gene on pR_{ST98} was identified by s*pv*-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 pR_{ST98}. This is the first report revealing a chimerical plasmid carrying genes of drug resistance and virulence in *S. typhi*.

Materials and Methods

Bacterial strains

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1) *S. typhi* strain STpR_{ST98} 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 pR_{ST98} and resistant to chloramphenicol, streptomycin, trimethoprin and sulphonamide, gentamicin, neomycin, kanamycin, cephalosporin, ampicillin, carbencillin and tetracycline. 2) The antibiotic sensitive plasmid free *S. typhi* (pR_{ST98}^-), used as the negative control. 3) *E.coli* $K_{12}W_{1485}$ Rif^r F^-Lac^+ that had a rifampicin resistant gene on chromosome was chosen as a recipient. Trans-conjugant pR_{ST98}/*E.coli* $K_{12}W_{1485}$ 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* V_{517} (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8 and 1.4 mD) and *S. flexneri*$_{24570}$ (140, 105, 2.6 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 pR_{ST98} 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 antimicrobial agents and incubated for 16 h as described above. Log-phase cultured bacteria were separated by centrifugation at 6,000 rpm for 15 min. QIAGEN Pasmid Maxi Kits were used to isolate and purify plasmid pR_{ST98}, then analyzed by electrophoresis on 0.7% agarose gels, stained with ethidium bromide (EB, 1 μg/ml) for 30 min and determined DNA quantity by UV-reflection spectro-photometry.

The restriction endonuclease analysis of pR_{ST98}

Nine restriction endonucleases (BamH I, Bgl II, EcoR I, EcoR V, Hind III, Pst I, Sac I, Sac II and Sph I) were selected to make the enzyme profile by digestion of pR_{ST98} 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 pR_{ST98} DNA were visualized under UV light.

PCR amplification

To insure that *spv* was located on plasmid pR_{ST98}, trans-conjugant pR_{ST98}/*E.coli* $K_{12}W_{1485}$ was used as the templet. The primers sequences were designed according to the reported *spv*R and *spv*B sequences. The *spv*R 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 *spv*B primers were (sense) 5'-ATG TTG ATA CTA AAT GGT TTT TCA-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* $K_{12}W_{1485}$ and *S. typhimurium* SR-11 were used as *spv* negative and positive control, respectively.

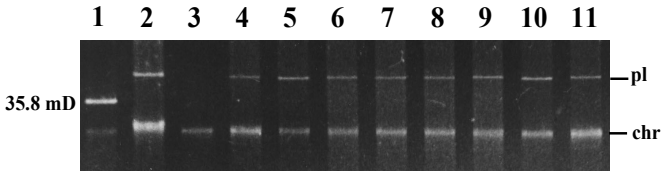

Figure 1. Electrophoresis pasmid profile of pR_{ST98}. Lane 1, *E.coli* V_{517}, plasmid size marker; Lane 2, *S. flexneri*$_{24570}$, plasmid size marker; Lane 3, antibiotic sensitive *S. typhi*, which were plasmid free, and used as the negative control; Lanes 4-11, multi-drug resistant *S. typhi* were used as the representing strains which naturally harbored pR_{ST98} and resistant to chloramphenicol, streptomycin, trimethoprin and sulphonamide, gentamicin, neomycin, kanamycin, cephalosporin, ampicillin, carbencillin and tetracycline.

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 at 3 V/cm for 5 h. Then they were stained and visualized under UV light. The 7.1 kb fragment was cut and extracted by QIAEX II agarose gel extraction kits. Then the *spv* gene probe was labeled with digoxin (Roche Molecular Bio-chemicals DIG High Prime DNA Labeling and Detection Starter Kit II). pR_{ST98} was digested with Bgl II, Pst I and Sac I (Promega), which were electro- phoresed together with plasmids carrying *spv* virulence gene in *S. typhimuruim* UK-1 and *S. typhimuruim* SR-11. Then samples on the gel 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.

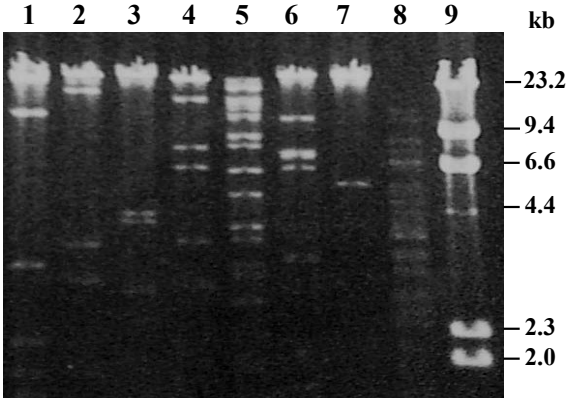

Figure 2. Restriction endonuclease digestion of plasmid pR_{ST98}. The restriction endonuclease pattern of pR_{ST98} was made by digestion of pR_{ST98} DNA using nine restriction endonucleases. Lanes 1-8, the restriction fragments of pR_{ST98} DNA were digested by EcoR I, BamH I, Hind III, Pst I, Bgl II, EcoR V, Sac I, Sac II, respectively; Lane 9, Molecular marker of λDNA/Hind III.

Results

The plasmid profile and the restriction endonuclease pattern of pR_{ST98}

The plasmid profile of pR_{ST98} was shown in Figure 1. The restriction endonuclease pattern of pR_{ST98} was made by digestion of pR_{ST98} DNA using nine restriction endonucleases (Figure 2). The result showed that the fragments of pR_{ST98} digested by endonucleases Bgl II, Pst I and Sac II 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.

The PCR amplification of spv

There were five ORF in *spv* genes - *spv*RABCD. In this study, the primers designed according to the sequences of *spv*R and *spv*B could amplify the *spv*R and *spv*B homologous genetic sequences on pR_{ST98}. The size of the product was the same as *spv*R and *spv*B (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 pR_{ST98}, the restriction fragments of pR_{ST98} digested by Bgl II, Pst I and Sac I, and *spv* positive control from *S. typhimurium* UK-1, SR-11. The Southern blot results of pR_{ST98} with *spv* probe were shown in Figure 6.

DNA sequences analysis

The DNA sequences of *spv*R and *spv*B from pR_{ST98} were compared with the reported sequences in Microsoft NCBI. The ORF of *spv*R and *spv*B of pR_{ST98} were 894 bp and 1,776 bp, respectively. They had more than 99% homologus with that of *spv*R and *spv*B on virulence plasmid in *S. typhmurium*.

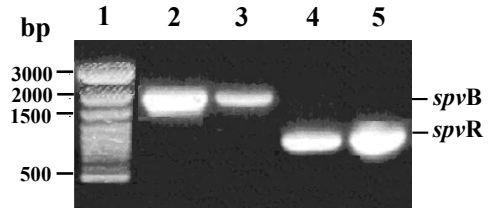

Figure 3. PCR fragments of *spvR* and *spv*B. Lane 1, DNA ladder; Lane 2, *S. typhi* pR_{ST98}; Lane 3, *S. typhimurium* SR-11; Lane 4, *S. typhi* pR_{ST98}; Lane 5, *S. typhimurium* SR-11. There were five ORF in *spv* genes - *spv*RABCD. The primers designed according to *spv*R and *spv*B sequences could amplify the *spv*R and *spv*B homologous genetic sequences on pR_{ST98}. The size of the product was same as the reported *spv*R and *spvB*.

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 pR_{ST98} 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 pR_{ST98} 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 pR_{ST98}? How this plasmid was transferred in different species of enteric bacilli? Was pR_{ST98} a mosaic plasmid, carrying genes responsible for drug resistance and virulence increasing in *S. typhi*? To investigate the virulence of pR_{ST98}, several experiments have been carried out. Our previous research showed that 1) pR_{ST98} 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. flexnier* and *S. typhimurium*; 2) pR_{ST98} was

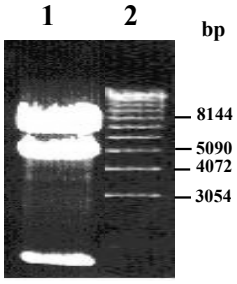

Figure 4. *spv* probe from pGTR061. Lane 1, *spv* probe fragment; Lane 2, DNA ladder.

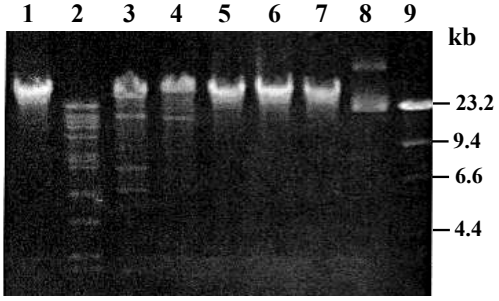

Figure 5. Agarose gel electrophoresis of plasmid and restriction fragment of pR_{ST98}, plasmids from *S. typhimurium* UK-1, SR-11 and pR_{ST98}/*E.coli* $K_{12}W_{1485}$. Lane 1, plasmid pR_{ST98}; Lanes 2-4, the restriction fragments of pR_{ST98} digested by Bgl II, Pst I and Sac I, respectively; Lane 5, pR_{ST98}/*E.coli* $K_{12}W_{1485}$; Lanes 6 and 7, the *spv* positive control from *S. typhimurium* UK-1 and SR-11, respectively; Lane 8, pGTR061; Lane 9, molecular marker λDNA/ Hind III.

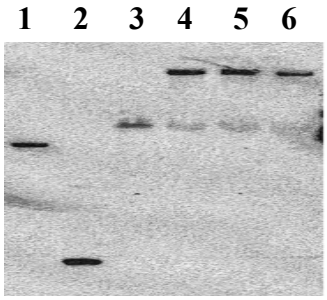

Figure 6. Southern blot of pR_{ST98} with *spv* probe. Lane 1, pR_{ST98}/Bgl II; Lane 2, pR_{ST98}/Pst I; Lane 3, pR_{ST98}/Sac I; Lane 4, pR_{ST98}/*E.coli* $K_{12}W_{1485}$; Lane 5, plasmid of *S. typhimurium* UK-1; Lane 6, plasmid of *S. typhimurium* SR-11.

associated with the virulence increasing in mice, such as lethality, infection of spleen, liver and mesenteric lymph nodes and serum resistance; 3) pR_{ST98} could also cause its host bacteria resistance to the phagocytes and enhance the intracellular growth of *Salmonella* strains in phagocytes (5). Therefore, it hinted that pR_{ST98} had not only genes of drug resistance but also genes of virulence, and novel virulence genes or related sequences which are important in the pathogenesis of *Salmonella* infection.

To characterize pR_{ST98}, nine restriction endonucleases were selected to make the enzyme profile by digestion pR_{ST98} DNA. The results showed that when using the QIAGEN Kits, enough and purified plasmid DNA could be gotten, which was very important in getting an identical restriction pattern. Endonucleases Bgl II, Pst I and Sac II were especially suitable for the molecular analysis of pR_{ST98} and were very useful tools for further research. The restriction endonuclease pattern of pR_{ST98} is of much help in character molecular marks, epidemiological surveillance and the location of the functional genes of the plasmid.

Previous researches in other labs in the world 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 for *S. typhi* and responsible for the virulence phenotype. Until now, in *S. typhi* neither virulence plasmid nor *spv* have been reported. The founding of PCR and Southern blot illustrated that *spv* homologous genetic sequence was also presented on pR_{ST98}. The ORF of *spv*R and *spv*B of pR_{ST98} were 894 bp and 1,776 bp, respectively. They had more than 99% homologus with that of *spv*R and *spv*B on virulence plasmid in *S.typhmurium*. Two mechanisms could be considered to explain the appearance. One is homologous recombination of the R plasmid and the virulence plasmid, and the other is the transposon genes coding for virulence proteins insertion into the drug resistance plasmid (16). The genotype research of pR_{ST98} revealed that a plasmid carrying genes responsible for drug 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*.

Acknowledgements

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