

Sequencing a 903 nucleotide *carA* gene of *Salmonella typhi* obtained through PCR employing Ca-3 and Ca-6 primers

P-7

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Abstrak

Salmonella typhi adalah bakteri patogen penyebab demam tifoid pada manusia dan informasi mengenai gen yang bertanggung jawab pada patogenitas belum diketahui. Penelitian ini merupakan bagian dari penelitian yang bertujuan untuk isolasi dan karakterisasi gen-gen *S. typhi* yang spesifik atau diekspresi hanya *in vivo* untuk pemahaman mekanisme molekuler penyakit demam tifoid, terutama pada tingkat DNA. Gen *ivi* (*in vivo* induced) *Salmonella typhimurium* yang ditemukan dengan cara IVET (*in vivo* expression technology) yang berperan pada patogenesis penyakit tifoid pada mencit, digunakan untuk identifikasi gen *S. typhi* yang dimaksud. Makalah ini mendiskusikan sekuens nukleotida *S. typhi* sebesar 0,9 kb, fragmen hasil amplifikasi PCR menggunakan primer CA-3 dan CA-6. Primer dirancang berdasarkan sekuens nukleotida gen struktural *carA* dari *E. coli* K12, dan dipergunakan untuk amplifikasi ekstensi bagian fragmen DNA sebesar 618 bp yang sebelumnya pernah dilaporkan. Fragmen 0,9 kb dianalisa menggunakan ensim restriksi *HinfI* dan *Sau3I* dan diklon ke dalam *MosBlue E. coli* dengan menggunakan vektor *pMosBlue T*. Sisipan plasmid rekombinan diisolasi menggunakan ensim restriksi *BamHI* dan *NdeI* dan disekuens dengan cara Sanger menggunakan primer universal T7, CA-3, CA-4 dan CA-6. Hasil sekuensing menunjukkan fragmen DNA 0,9 kb terdiri dari 903 bp. Analisa sekuens nukleotida dari fragmen DNA 903 bp ini dan menggabungkannya dengan sekuens dari fragmen 618 bp yang telah dilaporkan, menunjukkan bahwa gen *carA* yang lengkap dan sebagian kecil gen *carB* dari *S. typhi* telah diuraikan.

Abstract

Salmonella typhi is a pathogenic bacteria that causes typhoid fever in man and information regarding genes responsible for pathogenicity had not been known. This research had been part of a larger design with the objective to isolate and characterize *S. typhi* genes that are specific or expressed only *in vivo*. It is principally an effort to understand the molecular mechanism, especially at the DNA level, of the typhoid disease. The *ivi* (*in vivo* induced) genes of *Salmonella typhimurium*, discovered through IVET (*In Vivo* Expression Technology), that are thought to be intimately related to the pathogenesis mechanism of typhoid like diseases in mice were used in the identification of the intended *S. typhi* genes. This paper will discuss the nucleotide sequence of a 0.9 kb *S. typhi* PCR fragment amplified using CA-3 and CA-6 primers. These primers were designed based on the nucleotide sequence of the structural *carA* gene of *E. coli* K12 and can be used to amplify part of the extension of the 618 bp DNA fragment reported before. The 0.9 kb fragment was analyzed using *HinfI* and *Sau3I* restriction enzymes, then cloned into *E. coli* *MosBlue* using the *pMosBlue T* vector. The insert of the recombinant plasmids was isolated using *BamHI* and *NdeI* restriction enzymes and sequenced by the dideoxy Sanger method using universal, T7, CA-3, CA-4 and CA-6 primers. The results of all these sequencing experiments had shown without any doubt, that the insert - the 0.9 kb *S. typhi* DNA fragment - consisted of 903 bp. Analysing the nucleotide sequence of the 903 bp fragment and combining this with the nucleotide sequence of the previously reported 618 bp DNA fragment, revealed that the complete *carA* gene and a small part of the *carB* gene of *S. typhi* had been elucidated.

INTRODUCTION

Typhoid fever is globally very wide spread and is an endemic disease, mostly prevalent in developing countries. The disease is caused by a pathogenic bacteria, *Salmonella typhi* that enters the human body through food and drink. In Indonesia, the mortality

rate due to typhoid disease tends to increase, so that it had become a health problem, which need close and serious attention.

Information regarding molecular pathogenesis and response of the bacterial host cell is very essential for efforts to combat the disease through diagnosis, therapy and immunization. Some light had been shed by the development of a technique called IVET (*In Vivo* Expression Technology) by Mekalanos et. al., who had discovered the *ivi* (*in vivo* induced) genes of *Salmonella typhimurium* which are induced and expressed only in the host cell in mice. These genes are thought to be intimately related to the pathogenesis

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mechanism of typhoid-like diseases in mice, and were used in the identification of the intended *S. typhi* genes.

This research had been part of a larger design with the objective to isolate and characterize *S. typhi* genes that are specific or expressed only in vivo. It is principally an effort to understand the molecular mechanism, especially at the DNA level, of typhoid disease through the recombinant DNA and PCR technologies approach.

0.6 kb PCR amplified fragments of *S. typhi*, *S. typhimurium*, and *E. coli* K12 had been obtained using CA-1/CA-2 primers; these fragments were then sequenced. Analysis of the nucleotide sequences of the *S. typhi* fragment showed a similar sequence to the upstream region of the *carA* gene of *S. typhimurium* LT2. Some nucleotide differences in the sequences were noticed^{1,2}.

Attempts had been successfully made to isolate the complete *carA* genes of *S. typhi* and *S. typhimurium* in order to obtain information related to the degree of homology between the two *carA* genes and between these two genes and the *carA* gene of *E. coli* K12. For this purpose, CA3/CA-6 primers were constructed and used for PCR amplification of those *carA* genes, resulting in 0.9 kb DNA fragments. The 0.9 kb DNA fragment of *S. typhi* was proven to be an extension of the 0.6 kb fragment obtained earlier from PCR with CA-1/CA-2 primers.

METHODOLOGY

This research consists of the following five steps, namely: designing the primers that will be used in a PCR process; amplifications of the 0.9 kb DNA fragment of *S. typhi*; restriction analyses of the DNA fragment using *Sau3AI* and *HinfI* restriction enzymes; cloning the DNA fragment using the pMosBlue T vector with the *E. coli* MosBlue host. The plasmids of several white recombinant clones were then subjected to restriction analyses using *BamHI* and *NdeI* enzymes and sequencing of the insert of recombinant plasmids of *S. typhi* were also carried out.

Designing the primers

The CA-3/CA-6 primers that were used for the PCR process, were designed based on the nucleotide sequence of the conserved area of the *carA* and part of the *carB* structural genes, which form part of the *carAB* operon of *E. coli* K12. The construction of

primers were carried out using the Genmon, the proteolize, and the primer detective computer softwares.

Amplification of the 0.9 kb DNA fragment of *S. typhi*

The chromosomal DNA template that was used in the PCR process was obtained by lysis of a single colony of the culture of *S. typhi* using a lysis buffer³.

The reaction of the PCR process had a total volume of 50 µl and consisted of 1x PCR buffer (Pharmacia), 200 µM of dNTP mixture, 10 µl of DNA template, and 20 pmol each of the primers. The amplification reaction was carried out in 30 cycles of denaturation at 94°C for 60 seconds, annealing at the various temperatures (50°C, 52°C, 53°C, 54°C, and 55°C) for 60 seconds, and extension at 72°C for 60 seconds. For the last cycle, the extension time was four minutes. The PCR product was separated on 2 % agarose gel and stained by ethidium bromide. As DNA marker, DNA fragments of pUC19 cut with *HinfI* (1,419 bp; 517 bp; 397 bp, 214 bp; and 75 bp) were used.

Restriction analyses of the PCR products

Restriction analyses were carried out on the 0.9 kb DNA fragment of *S. typhi* using the 0.9 kb DNA fragment of *E. coli* K12 as positive control. Restriction analyses were done with *Sau3AI* and *HinfI* enzymes (Amersham). The restriction fragments were separated on 2 % agarose gel and stained by ethidium bromide. As DNA marker, DNA fragments of pUC19 cut with *HinfI* were used.

The cloning processes of the 0.9 kb DNA fragments of *S. typhi*

The 0.9 kb DNA fragments of *S. typhi* was cloned using pMosBlue T vector with *E. coli* MosBlue host. The cloning process was carried out according to the procedure that was in the pMosBlue T kit (Amersham). Several white recombinant clones were taken, followed by the isolation of the plasmids from those clones. The plasmid isolation was done using the minipreparation lysis by alkali method. Those recombinant plasmids were then restriction analysed using *BamHI* and *NdeI* enzymes (Amersham).

Sequencing of the insert of the recombinant plasmids of *S. typhi*

The sequencing processes were done using the T7 sequencing kit (Pharmacia) and Thermo Sequenase Radiolabeled Terminator Cycle Sequencing kit (Amer-

sham). As the primers of the sequencing processes, the universal, T7 promoter, CA-3, CA-4, and CA-6 primers were used.

RESULTS AND DISCUSSION

The computer analysis resulted in a set of a sense primer, termed CA-3 and an antisense primer, termed CA-6. The CA-3 primer has the following DNA sequence: 5' GGCAAAGAAGT GACCACCGC 3' and the CA-6 primer has the following DNA sequence 5' CAGTGAATCGGCT GATGTAGG 3'.

Using the genomic DNA of *S. typhi* as template, the PCR with the above primers resulted in the amplification of a 0.9 kb DNA fragment, and the genomic DNA of *E. coli* K12 was used as a positive control for the PCR process. This 0.9 kb DNA fragment of *S. typhi* was proven to be an extension of the 0.6 kb fragment obtained earlier from PCR with CA-1/CA-2 primers. The analyses of the PCR products are shown in Figure 1.

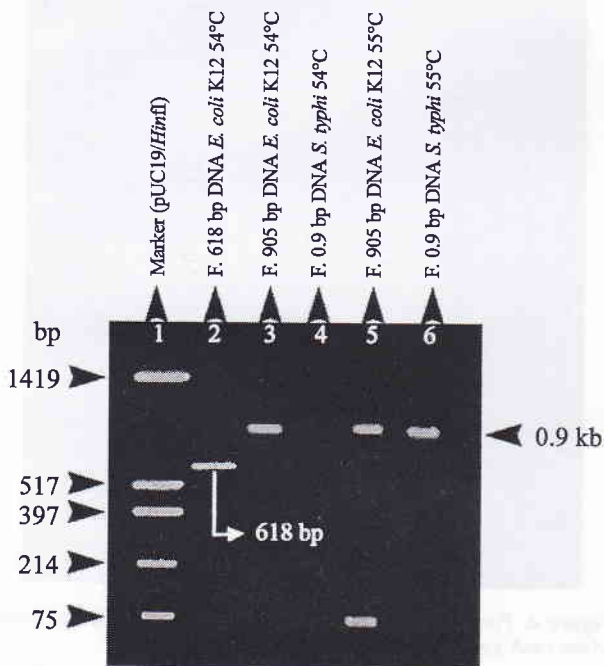


Figure 1. 0.9 kb PCR Amplified Fragments of *S. typhi* and *E. coli* K12.

Line 1: DNA marker, the DNA fragments of pUC19 cut with *HinfI* (1419 bp, 517 bp, 396 bp, 214 bp and 75 bp); line 2: 0.6 kb PCR amplified fragment of *E. coli* K12 using CA-1/CA-2 primers; line 3: 0.9 kb PCR amplified fragment of *E. coli* K12 using CA-3/CA-6 primers, annealing temperature at 54°C; line 4: 0.9 kb PCR amplified fragment of *S. typhi* using CA-3/CA-6 primers, annealing temperature at 54°C; line 5: 0.9 kb PCR amplified fragment of *E. coli* K12 using CA-3/CA-6 primers, annealing temperature at 55°C; line 6: 0.9 kb PCR amplified fragment of *S. typhi* using CA-3/CA-6 primers, annealing temperature at 55°C.

According to the literature, the 905 bp DNA fragment of *E. coli* K12 was known to contain one recognition site of *Sau3AI* and three recognition site of *HinfI*. Restriction of this DNA fragment with *Sau3AI* enzyme would then result in two fragments of 444 bp and 461 bp length. And restriction of this DNA fragment with *HinfI* would then result in four fragments of 220 bp, 506 bp, 171 bp and 18 bp length.

Based on the analysis results using gel agarose electrophoresis, restriction analyses of the 0.9 kb PCR amplified DNA fragment of *E. coli* K12 using *Sau3AI* and *HinfI* enzymes yielded the same theoretically intended fragments. Whereas, restriction analysis of the 0.9 kb PCR amplified DNA fragment of *S. typhi* using the *Sau3AI* enzyme gave three fragments of 0.45 kb, 0.25 kb and 0.2 kb length. Using the *HinfI* enzyme, the 0.9 kb *S. typhi* DNA fragment gave three fragments of 0.4 kb, 0.2 kb, and 0.1 kb length. The restriction analyses results were shown in Figure 2.

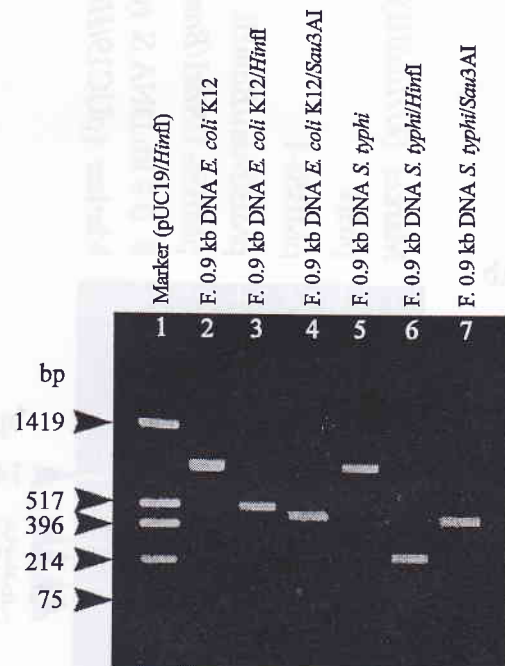


Figure 2. Restriction analyses of 0.9 kb PCR amplified DNA fragments of *S. typhi* and *E. coli* K12 using *Sau3AI* and *HinfI* enzymes.

Line 1: DNA marker, the DNA fragments of pUC19 cut with *HinfI* (1419 bp, 517 bp, 396 bp, 214 bp and 75 bp); line 2: 0.9 kb PCR amplified DNA fragment of *E. coli* K12; line 3: 0.9 kb PCR amplified DNA fragment of *E. coli* K12 cut with *HinfI*; line 4: 0.9 kb PCR amplified DNA fragment of *E. coli* K12 cut with *Sau3AI*; line 5: 0.9 kb PCR amplified DNA fragment of *S. typhi*; line 6: 0.9 kb PCR amplified DNA fragment of *S. typhi* cut with *HinfI*; line 7: 0.9 kb PCR amplified DNA fragment of *S. typhi* cut with *Sau3AI*.

These restriction fragments of *S. typhi* were found to be different from those of *E. coli* K12. This is probably due to the difference in position of the recognition site of these enzymes on each of the DNA fragments. This will later on be proven in the nucleotide sequence of the 0.9 kb DNA fragment of *S. typhi*.

These DNA fragments were then cloned using the pMosBlueT vector and *E. coli* MosBlue host. Several white recombinant clones were obtained. The plasmids of several clones were then subjected to restriction analyses using *Bam*HI and *Nde*I. This characterisation was carried out to prove that the insert in the recombinant plasmid is the 0.9 kb DNA fragment of *S. typhi*. These restriction analyses had shown that the the insert of the recombinant plasmids were the intended 0.9 kb DNA fragment of *S. typhi*. The restriction analyses results are shown in Figure 3.

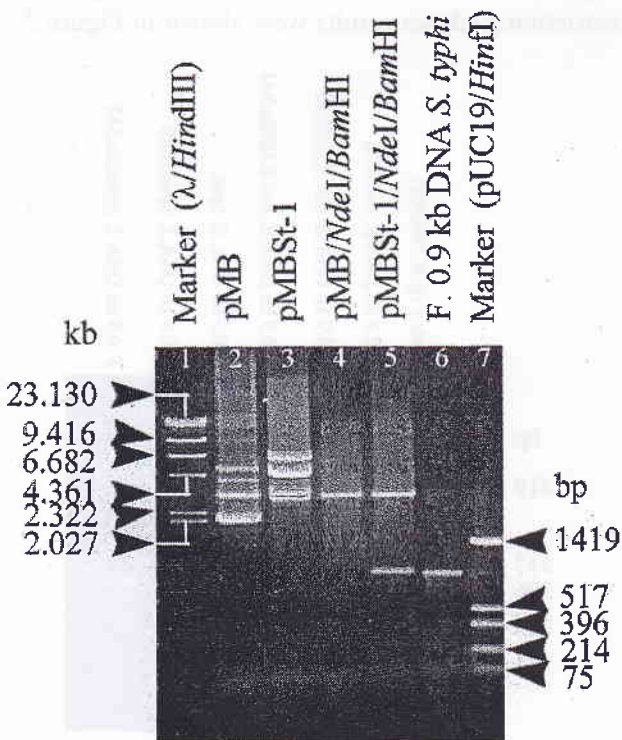


Figure 3. Analysis of *S. typhi* Recombinant DNA
 Line 1: DNA marker, the DNA fragments of the DNA cut with *Hind*III (23.130 kb, 9.416 kb, 6.682 kb, 4.361 kb, 2.322 kb and 2.027 kb); line 2: *E. coli* MosBlue recombinant plasmid uncut; line 3: *S. typhi* recombinant plasmid uncut; line 4: *E. coli* MosBlue recombinant plasmid cut with *Bam*HI and *Nde*I; line 5: *S. typhi* recombinant plasmid cut with *Bam*HI and *Nde*I; line 6: 0.9 kb PCR amplified DNA fragment of *S. typhi*; line 7: DNA marker, the DNA fragments of pUC19 cut with *Hin*FI (1419 bp, 517 bp, 396 bp, 214 bp and 75 bp).

Sequencing of the insert of the *S. typhi* recombinant plasmid (pMB-St1) using universal, T7, CA-3, CA-4, and CA-5 primers had shown, that the inserts were 903 bp DNA fragments. Combining this with the nucleotide sequence of the 618 bp DNA fragment of *S. typhi* that was obtained earlier, the result was the proof of existence of a 1499 bp DNA fragment in *S. typhi*.

This 1499 bp DNA fragment consists of the the *car*AB operon promotor, the *car*A structural gene, the *car*A-*car*B intermediate region and the upstream part of the *car*B structural gene. The nucleotide sequence of the *car*AB operon promotor and of the *car*A gene of *S. typhi* had been submitted to and accepted by the GenBank Database with the access number AF012246, 3-July-1997. The part of the autoradiogram that showed the nucleotide sequence of the specific region in the *car*A gene of *S. typhi* is shown in Figure 4 as an example.

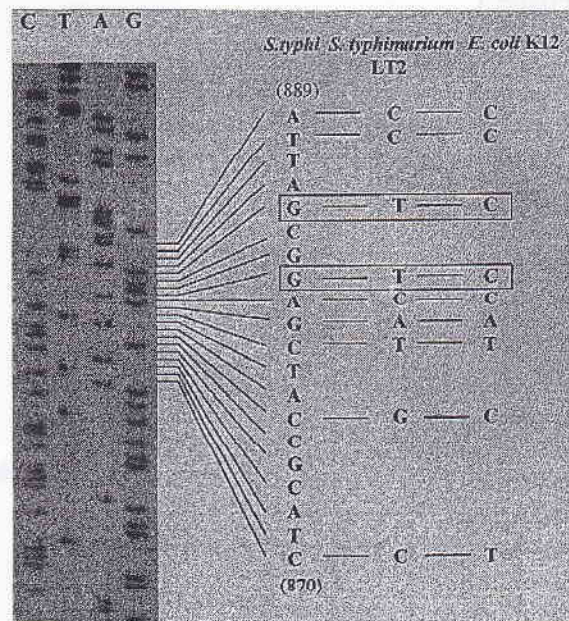


Figure 4. Part of the autoradiogram of the nucleotide sequence of the *car*A gene of *S. typhi*.

Nucleotide sequences of corresponding parts of *S. typhi*, *S. typhimurium* LT2 (from GenBank) and *E. coli* K12 (from GenBank) were shown.

Analysis of the 1499 bp nucleotide sequence resulted in a two tandem promotor that was termed promotor-1 (P1) and promotor-2 (P2). P1 starts at base no. 24 and ends at base no. 91 while P2 starts at base no. 92 and ends at base no. 123. The start codon (TTG) of the *car*A gene is at base no. 124 - 126, and its stop codon (TAA) is at base no. 1270 - 1272. The start co-

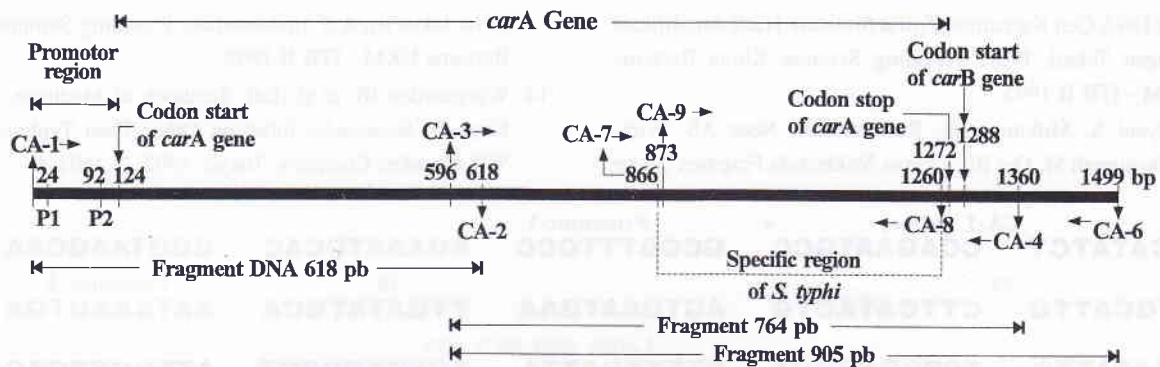


Figure 5. Analysis diagram of the nucleotide sequence of the 1499 bp PCR DNA fragment of *S. typhi*.

don of the *carB* gene (ATG) is at base no. 1288-1290. Diagram analysis of the nucleotide sequence of the 1499 bp PCR DNA fragment of *S. typhi* is shown in Figure 5.

CONCLUSIONS

- 0.6 kb and 0.9 kb DNA fragments of *S. typhi* had been successfully amplified using PCR with CA-1 / CA-2 and CA-3 / CA-4 primers. The PCR amplified 0.6 kb and 0.9 kb DNA fragments of *S. typhi* had been successfully cloned with the pMosblue T vector and *E. coli* MosBlue host.
- The recombinant plasmids of the recombinant clones were subsequently subjected to restriction analyses using *Hind*III and *Eco*RI for the 0.6 kb clone and *Bam*HI and *Nde*I for the 0.9 kb clone. The analyses had shown that the inserts of the recombinant plasmids were the intended 0.6 kb and 0.9 kb DNA fragments.
- Sequencing of the inserts of the recombinant plasmids were carried out by the dideoxy Sanger method, and showed that the inserts were a 618 bp and 903 bp DNA fragment respectively.
- Analysis of the nucleotide sequences of these two DNA fragments and combination thereof resulted in a 1499 bp. DNA fragment consisting of a 123 bp *carAB* operon promotor, a 1149 bp *carA* structural gene, a 15 bp *carA-carB* intermediate region, and a 212 bp upstream part of the *carB* structural gene.
- Further analysis of the 1499 bp DNA fragment in relation to the restriction analyses previously carried out on the 618 bp and 903 bp DNA fragments using *Eco*RV, *Cla*I, *Sau*3AI, and *Hin*fI restriction enzymes, is in agreement with the known restriction sites starting at : bp no. 459 for *Eco*RV, bp no. 461 for *Cla*I, bp no. 256, 370, 754, and 1041 for *Sau*3AI, and bp no. 330, 363, 816, 1078, 1320, and 1491 for *Hin*fI.

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GCCCATATCT	CA-1 primer → CCAGAATGCC	Promotor-1 GCCGTTTGCC	AGAAATCCAC	GGGTAAGCAA
ATTTGCATTG	- 35 CTTCATACTG	ACTGAATGAA	- 10 TTGATATGCA	Promotor-2 AATAAAGTGA
GTGAATATTC	TCTGGAGGGT	(Codon start <i>carA</i> gene) GTTTTGATTA	AGTCAGCGCT	ATTGGTTCTG
GAAGAGGGAA	CCCAGTTTCA	CGGTCGGGCC	ATAGGGGCAA	CAGGTTCCGGC
GTTTGGGGAA	GTCTTTTTCA	ATACTTCAAT	GACCGGTTAT	CAAGAAATCC
<i>Sau3AI</i> TCACTGATCT	TCCTATTCCG	CAAATCGTC	ACTCTAGCTA	TCCCATATTG
GTAATGTCGG	CACCAATGAA	GCCGATGAAG	<i>HinfI</i> AATCTTTCTC	AGGTTTCATGC
GCAAGGTTTA	<i>HinfI</i> GTGATTTCGG	<i>Sau3AI</i> ATCTGCCGTT	GATTGCCAGC	AACTTCCGTA
ACACCGAAGA	CCTCTCTTCC	TTGAGCCTGA	AACGCCATAA	CATCGTGGCG
	<i>Clal</i>			
<i>EcoRV</i> ATTGCCGATA	TCGATTTCGG	CAAGCTGACC	CGACTGCTGC	AACTTAAAGG
CGCACAGAAC	GGCTGCATCA	TTGCAGGCGA	TAGCCCGGAT	GCGAACGTGG
CGCTGGAAAA	AGCGAAAGCA	TTTCTGAGAC	TGAACGGTAT	GGATCTGGCA
CA-3 primer →	← cCA-2 primer			
AAAGAAGTGA	CCACCGCGGA	AACTTACCGC	TGGACGCAGG	GTAGCTGGAC
GCTGAAAGAC	GGTTTACCGG	AAGCGAAATC	TGAGGATGAC	CTGCCGTTCC
ACGTGGTGGC	CTACGATTTT	GGCGCCAAGC	GCAACATTCT	ACGTATGCTG
<i>Sau3AI</i> GTGGATCGCG	GCTGCCGCCT	GACGGTGGTG	CCAGCGCAAA	CCTCTGCTGA
AGAGGTGCTG	<i>HinfI</i> AAAATGAATC	CGGACGGCAT	CTTCCTGTCTG	AACGGCCCTG
GCGACCCGGC	GCCGTGCGAC	TACGCCATCG	AGGCGATTAA	GAAATTCCTC
CAGACCGACA	TTCCTGTGTT	CGGCATCTGC	CTCGGCCATC	AACTGCTGGC
GCTGGCGAGC	GGTGCGAAGA	CCGTTAAGAT	GAAGTTCCGGC	CATCACGGCG
GCAACCATCC	GGTAAAAGAT	ATGGACAGAA	ACGTGGTGAT	<i>Sau3AI</i> GATCACCGCG
CAGAACCACG	GATTTGCGGT	GGATGAAGAC	<i>HinfI</i> TCGCTGCCCG	CCAACCTGCG
CGTGACCCAC	AAGTCGCTGT	TCGACGGGCA	CCCTGCAGGG	CATTCACCGT
ACCGACAAAC	CGGGCGTTCA	GCTTCCAGGG	ACACCCGGGC	GGACGCCGGG
CCCGACGATG	CGGCGCCGCT	GTTCCGACCAC	TTTATCGAAT	TGATTAAGCA
CTACCGTTCT	TCAGCAAAAT	(Codon stop <i>carA</i> gene) AATCAGGAGC	(Codon start <i>carB</i> gene) CAACAAAATC	CCAAAACGTA
CAGATATAAA	AAGCATCCTG	<i>HinfI</i> ATTCTGGCCG	← cCA-4 primer GGGCCCGATT	GTTATCGGTC
← AGCGTGTGG	ATTTGACTAC	TCCGGCGCTC	AGGCGTGTA	ACCGGTGCGC
GAAGAACGGT	ACCCGGGTTA	TTCTGGTGAA	CTCTAACCCG	GCCACCATCA
			← cCA-4 primer	
TGACCGACCC	GGAAATGGCC	GGTGCCACCT	ACATCGAGCC	<i>HinfI</i> GATTCACCTC

Figure 6. The nucleotide sequence of 1499 bp DNA fragment of *S.typhi*.

Note : The nucleotide sequence of the *carA* gene of *S.typhi* had been submitted to and accepted by the GenBank Database with the accession number AF012246, 3-July-1997.