PRIMERS FOR *SALMONELLA* SEROVAR DETECTION BY POLYMERASE CHAIN REACTION

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Abstract. *Salmonella* serovar detection was studied by polymerase chain reaction (PCR). The primers were designed from *Salmonella* specific clone, A18:2 which was previously constructed and studied for genus specificity through colony hybridization. The primers were subsequently tested for specificity and sensitivity and showed that they amplified DNA fragment of all Salmonellae tested but did not amplify all isolates of non-Salmonellae tested. The amplified fragment was confirmed and increased sensitivity by nested PCR. *Salmonella* isolates amplified by the primers in the first round PCR were all positive in the second round. The sensitivity in the first and second round were 7 pg and 80 fg, respectively. The result indicated that the primers can be used as molecular tool for future field survey of *Salmonella* both in food and in clinical specimens.

INTRODUCTION

Salmonellosis is one of a potential public health problem in food-borne disease. Recently a remarkable increase both in incidence and severity of human salmonellosis has been reported. The incidence in some European countries shows currently a 20-fold increase during the last 10-15 years (WHO, 1997). The majority of cases has been caused by serotypes of *S. enteritidis* and *S. typhimurium*. Since the begining of the 1990s, strains of *S. typhimurium* which are multi-drug resistant have emerged and are threatening to become a serious public health problem.

The changing trend of salmonellosis also occurred in Southeast Asia including Thailand (Wong *et al*, 1994; Jayanetra, 1990). The main contributing factor to the problem is the consumption of various contaminated foods, the main sources being eggs, egg products, poultry and other meat products (Cowden *et al*, 1988; Hopper, 1988). In Thailand there have been periodic shifts in the prevalence of various serovars encountered in food contamination. Certain serovars resulted in health problems such as *S. krefeld* which was epidemic in Thailand during 1976 to 1978 (Jayanetra *et al*, 1990). In early 1990, increasing incidence of *S. enteritidis* was observed in Thailand and was reported elsewhere, in Europe and North America (Bangtrakulnon *et al*, 1997; Rodrigue, 1990; Hickman-Brenner, 1991). The situation suggested global spread of this serovar of *Salmonella*. The incidence of *S. enteritidis* in Thailand remains high up to the present time. In early 1996, the emergence of *S. paratyphi* A was encountered in Thailand. Although an attempt has been made to control the problem, the incidence of cases indicated that this was unsatisfactory. It is obvious that salmonellosis is currently a global public health concern. It causes not only medical problems but also contributes significantly to economic loss for food exporter countries. The need for rapid detection of *Salmonella* in food and in clinical specimens has become a demand. We previously reported the construction of DNA probe A 18 : 2 from genomic DNA of *S. typhimurium* LT2 ATCC 23566. However, the probe also gave a weak signal to *Shigella* and *Citrobacter*. In the present communication, we developed primers from the nucleotide sequence of such clones and studied the sensitivity and specificity of the primers in detecting various *Salmonella* serovars.

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MATERIALS AND METHODS

Bacterial strains

The *Salmonella* serovars and other bacterial species involved in this study were summarized in Table 1. *Salmonella* serovars were provided or confirmed by the National Salmonella and Shigella Center, Thailand. Non-*Salmonella* strains were obtained from the Clinical Microbiology Division, Department of Pathology, Ramathibodi Hospital, Thailand.

Sequencing of clone A 18:2

Clone A 18:2 was constructed by random recombination cloning of genomic DNA of *S. typhimurium* LT 2 ATCC 23566 (Pilantanapak *et al*, 1997). The clone was sequenced by the dideoxynucleotide chain termination method carried out according to the recommendation of the manufacturer using a commercially available kit of silver sequence DNA sequencing system (Promega). Modification was made in the last step of making the hard copy by using photographic black and white bromide paper instead of the film recommended by the company. The results were confirmed from both the hard copy and the dried gel. The sequence of the clone was submitted to the GenBank database and the assigned accession number is AF025748.

PCR of clone A18:2 inserted fragment

The primers obtained from the nucleotide sequence of clone A 18:2 was analysed by McVector version 4.1.1. Their sequences were 5'GAC-

Table 1a

Specificity of primers* to Salmonellae.

Microorganisms	No. of isolates tested	No. of positive isolated in PCR (590bp)
S. enteritidis	10	10
S. derby	1	1
S. muenchen	1	1
S. cerro	1	1
S. london	1	1
S. anatum	1	1
S. weltvreden	1	1
S. panama	2	2
S. stanley	1	1
S. parathypi A	3	3
S. worthington	1	1
S. emek	1	1
S. bovismorbificans	1	1
S. agona	1	1
S. senftenberg	1	1
S. montevideo	1	1
S. blockley	1	1
S. rissen	1	1
S. hardar	1	1
S. typhimurium	4	4

* Primers: 5' GACTTTTGCGGTATTGA 3'/ 5' GCTCATCAGGGCTTTTT 3'.

Table 1b

Cross-reaction of primers with other enteric organisms.

Microorganisms	No. of tested isolates	No. of positive isolated in PCR (590bp)
Shigella flexneri	4	0
Shigella dysenteriae	10	0
Escherichia coli	8	0
Staphylococcus spp	1	0
Aeromonas spp	2	0
Acinetobacter spp	1	0
Klebsiella pneumoniae	4	0
Klebsiella spp	3	0
Haemophilus influenzae	1	0
Pseudomonas aeroginosa	1	0
Citrobacter spp	5	0
Proteus spp	1	0
Vibrio parahaemolyticus	1	0
Vibrio cholerae	1	0
Vibrio cholerae ogawa	1	0
Neisseria spp	1	0
Proteus mirabilis	1	0
Enterobacter cloacae	5	0
Providencia spp	2	0
Providencia rettgeri	3	0
Staphylococcus coag+	1	0
Acinetobacter calcoaceticus	4	0
Edwardsiella spp	1	0
Enterobacter spp	2	0
Serratia spp	1	0
Yersinia spp	2	740

TTTTGCGGTATTGA3' and 5'GCTCATCAGGG-TTTTT3' corresponding to nucleotides 44-60 and 617-633 of *Bam*HI-*Pst*I fragments of clone A18:2. The amplified product was therefore expected to be 590 bp for *Salmonella* serovars. To increase the sensitivity and confirm specificity for detection, the inner primers (nested PCR) were designed. Their sequences were 5'GCTACCAAATATCC-CCA3' and 5'CGGCGTTAGAACACTAAAGA3' located in positions 91-110 and 592-611, respectively. The target product from the second round PCR should be 521 bp. Amplifications were performed in 50 µl reaction mixtures containing 10 µM Tris-HCl(pH8.3), 50mM KCl, 2.0mM $MgCl_2$, 1M each primer, 100 µM each dNTP, 1.25 units of Taq DNA polymerase. The first round PCR was amplified for 38 cycles as the following : 95°C 40 seconds, 57°C 50 seconds and 72°C 60 seconds. In the last cycle the extending step was expanded to 5 minutes. The second round PCR mixture contained 10 µl of the first PCR product as template. The reaction was similarly amplified in the same manner as the first round excepted the annealing step was 60°C instead of 57°C. PCR products were visualized by gel electrophoresis on 1% agarose stained with ethidium bromide.

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GGATCCCCGC  CATCACGCCG  CCTAACGCCT  GCGATCCGCC   40
AAAGACTTTC  GCGGTATTGA  CGCCAACCAG  AATGTTCATG   80
ATAGCAACAC  CCGCGCTACC  AAATATCCCC  ATCAGGCCCA  120
GCAGGTTAGG  ATAATGGACG  GCAACATCGC  CGACGATATC  160
CGGACGTTTA  AGAATGTTGA  TAATGCCGGT  AATCAAGCCG  200
GATGCGATAA  AGGCGGGGAT  AAGCGGTATA  AAAACATTCG  240
CCAGCTTACG  CAACGCATCG  CTCATCGGCG  CTTTATACTT  280
CGCTTTCGCC  TCGGATTTGG  TACGCGCCAT  AGCGTCATCC  320
GGTTTTACAC  CACCTGCGGC  AATCTGCACG  CGCATCGCAT  360
CAACCACCTG  CGCCAGCTTC  CCCGGCCCAA  CGATCAGCTG  400
ATGTTGCTCC  CCTTGCTTTA  CGTATCCACT  GACGCCAGGC  440
AACGCTTTTA  AGGCCGGAAT  ATCCAGTTGG  CTGTCGTCCT  480
GTACCTCCAC  CCTGACTCGC  GTCATACAGT  TTTCCAGGCG  520
CAGAATATTT  TGCTCTCCGC  CGATACCACG  TAAAATGTCG  560
CTGGCGAGCG  CTGCCGTCTT  ATCCATACAC  ACCTCTTTAG  600
TGTTCTAACG  CCGCCCTTAA  AAAGCCCTGA  TGAGCGGCAA  640
GTTTTTCCCT  GGCGGCTGCA  G
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Fig 1–Nucleotide sequence of 661 bp inserted fragment of clone A18:2, *Salmonella* specific clone. The first PCR primers are in positions 44-60 and 617-633 and nested primers are in positions 91-110 and 592-611.

Sensitivity of PCR

To examine the least amount of DNA of *Salmonella* which can be detected by PCR, serial dilution in the range of 100ng - 1 fg of extracted chromosomal DNA of *Salmonella* was used as template in the PCR reaction in the first round. The 10 µl of PCR product which was not visualized in the electrophoresis gel staining was used for amplification in the second round to detect the sensitivity of nested PCR.

RESULTS

Specificity test

Inserted DNA, the *Bam*HI-*Pst*I fragment of clone A18:2 was sequenced. The fragment length was 661 bp; the nucleotide sequence is shown in Fig 1. DNA homologous alignment was performed with the DNA database of GenBank, EMBL, DDBJ and PDB. No 100% homologous sequence was presented. But in the smaller region of the minus strand, between positions 570-478 showed 60% homology with 3 clones of *E. coli* genomic DNA (Accession no. dbj/D90873, dbj/D90872 and gb/AE000330). Based on the sequence outside the homologous region, PCR primers were designed and evaluated for their specificity for *Salmonella*. With extracted chromosomal DNA from 35 isolates of 20 serovars of *Salmonella* tested, all were amplified by the primers, the predicted length was 590 bp, shown in Fig 2. Non-*Salmonella* isolates tested gave no amplified product except *Providencia* spp. However the location of the amplified product detected by gel electrophoresis was different from the *Salmonella* product, indicating a different sequence from *Salmonella*. To confirm that *Salmonella* amplified fragments were the target sequences, the nested primers were used with the products of all positive isolates from the first round PCR. All resulted in a 521 bp product which is shown in Fig 3. The results indicated that primers developed from clone A18:2 had no cross reaction with non-*Salmonella* serovars tested, especially *Citrobacter* and *Shigella* which gave weak signals in colony hybridization using inserted fragments of clone A18:2 as probes (Pilantanapak *et al*, 1997).

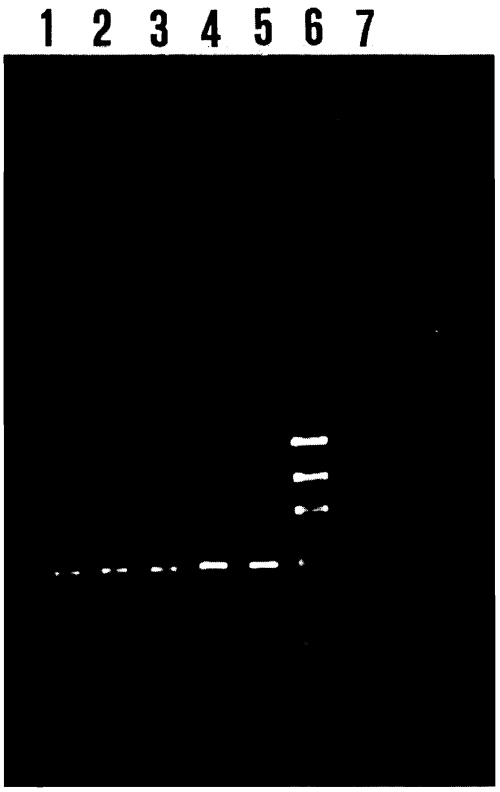

Fig 2–Demonstration of 590 bp. PCR product of *S. typhimurium, S. enteritidis, S. derby, S. anatum* and A18:2 (lane 1-5, respectively) with primer 5'GACTTTTGCGGTATTGA3' and 5'GCTCATCAGGGCTTTTT3'. Lane 6 and 7 present PHiX174/HaeIII and negative control.

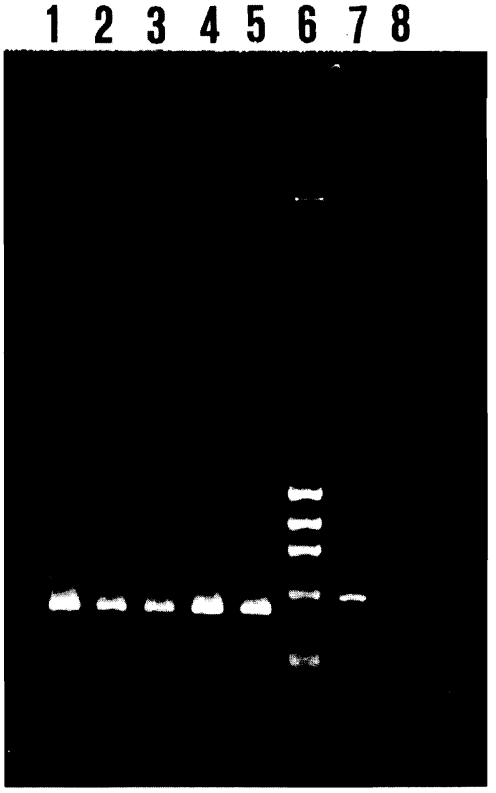

Fig 3–Demonstration of 521 bp PCR prducts of *S. thyphimurium, S. enteritidis, S. derby, S. anatum* and A18:2 (lane 1-5, respectively) with nested primer 5'GCTACCAAATATCCCC3' and 5'CGGCGTTAGAACATAAAGA3'. Lane 6, 7 and 8 present PhiX174/HaeIII, first PCR product and negative control.

Sensitivity test

Sensitivity was determined with the serial dilution of extracted chromosomal DNA of *S. enteritidis*. The visualized DNA band in an ethidium bromide-stained gel indicated that the minimal detectable DNA amount was 7 pg in the first round and 80 fg in the second round (Table 2). Besides specificity confirmation, nested PCR also gives advantage in sensitivity enhancement.

DISCUSSION

Salmonella is having increasing impact on human health and on economic endeavor, particularly on food export. These facts demand the development of an effective method for rapid detection of the agent in food, in humans, animals and the environment. To achieve this purpose, we used polymerase chain reaction (PCR) which proved to provide a more sensitive and rapid tool for detection of microorganisms than the conventional cultural method. In a traditional bacteriology laboratory where the method of pre-enrichment, growth on selective media and serology tests were employed, *Salmonella* identification required at least one week. This time consuming process adds considerable expense to food industries. Since it takes only one day to detect *Salmonella* from the pre-enriched media, PCR is therefore considered to be more advantageous. The method has also been

Table 2

Sensitivity of *Salmonella* detection by 2 steps PCR.

Salmonella DNA (μg)	PCR product visualized in	
	1st round PCR	2nd round PCR
100	+	+
10	+	+
1	+	ND
10^{-1}	+	ND
10^{-2}	+	ND
10^{-3}	+	ND
10^{-4}	+	ND
10^{-5}	+	ND
9×10^{-6}	+	ND
8×10^{-6}	+	ND
7×10^{-6}	+	ND
6×10^{-6}	-	+
5×10^{-6}	-	+
10^{-7}	-	+
9×10^{-8}	-	+
8×10^{-8}	-	+
7×10^{-8}	-	-

ND = not done

reported to be very useful in detection of various agents in clinical specimens such as *Mycobacterium tuberculosis* (Shawar *et al*, 1993), enteropathogenic *E. coli* (Franke *et al*, 1994), *Streptomyces archromogenes* (Hornemann *et al*, 1989).

In this study we started initially from the sequence of clone A18, which was established by random recombination cloning of *S. typhimurium* LT2 ATCC23566. The clone was intensively screened for specificity by colony hybridization with 247 *Salmonella* isolates and 59 non-*Salmonella* isolates. The results were positive for all *Salmonella* and negative for all non-*Salmonella* except *Citrobacter* and *Shigella* sp (Pilantanapak *et al*, 1997). Its sequence was studied by homologous alignment test with DNA databases of GenBank, EMBL, DDBJ and PDB. This search indicated no homologous sequence with other organisms thus far submitted to these databases except *E. coli* at position 570-478 on the minus strand. The primers were designed and examined for specificity with *Salmonella* and non-*Salmonella*. The primer amplified the DNA fragment of all *Salmonella* tested but did not amplify any non-*Salmonella* including *Citrobacter* and *Shigella*. The amplified DNA was confirmed by nested PCR and most showed the correct target. Sensitivity test of the first round PCR was 7 pg and was 80 fg in the second round.

In other studies the utilization of PCR for *Salmonella* detection can be summarized as follows : firstly, for the diagnosis of virulence and pathogenic properties of *Salmonella* including toxicity, virulence, invasion of intestinal epithelium, flagellin gene, etc (Song *et al*, 1993; Rahn *et al*, 1992; Stone *et al*, 1994). Secondly, for the utilization of non-pathogenic genes such as *oric*, IS 200, 16 sRNA (Widjojoatmodjo *et al*, 1991; Cano *et al*, 1993; Lin *et al*, 1996) for identification and for other purposes *ie* detection of *Salmonella* serovars in feces and food specimens; however, the results of such investigations remain a controversy. Thirdly, the random search for specific fragments of DNA from library clones as investigated by Aoba *et al* (1995), Cohen *et al* (1994) and Ngvyen and Khan, (1994). The results of these investigations were very promising for identification and detection of *Salmonella*. Our study gave similar results to those previously described, but our primers are very sensitive, they can detect a nucleotide sequence of *Salmonella* as small as 80 fg. The use of the PCR method based on these primers looks promising for mass screening in food industries and of clinical specimens. Futhermore, the fact that we found 60% similarity of a nucleotide sequence of *E. coli* with the nucleotide sequence of *Salmonella* on the minus strand at position 570-478 provides a challenging idea for possible design of primers for simultaneous detection of both *Salmonella* and *E. coli* in any specimens.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Ola Skold and Gote Swedberg for their assistance in initial DNA sequencing and Mrs Aroon Bangtrakulnonth, WHO Salmonella and Shigella Center, Thailand for providing *Salmonella* serovars. This work was successed with the part of grant supported from NSTDA to Dr Panida Jayanetra.

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