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A Sensitive Polymerase Chain Reaction-based Detection of *Yersinia Pseudotuberculosis* and Pathogenic *Yersinia Enterocolitica* and Its Application to Mountain Water

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= 국문초록 =

중합효소 연쇄반응법에 의한 *Yersinia pseudotuberculosis*와 병원성 *Yersinia enterocolitica*의 검출법과 산악 자연수 검사에서의 적용

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배 경 : *Yersinia pseudotuberculosis(Y. pseudotuberculosis)*와 병원성 *Yersinia enterocolitica(Y. enterocolitica)*의 인체감염은 주로 이 균들에 오염된 물과 음식물등에 의해 발생되어지며 특히 *Y. pseudotuberculosis*균 감염인 경우 한국에서는 옹달샘 물 음용이 중요한 원인 중에 하나이다. 환자 진단은 환자 대변에서 적절한 선택배지를 사용하여 세균을 배양, 진단할 수 있으나 옹달샘 물에서의 균배양은 여과및 알카리처리법을 사용함에도 불구하고 성공율이 매우 낮으므로 중합효소연쇄반응(PCR)법을 사용하여 물에서 *Yersinia*균 진단을 시도 하였다.

방 법 : *Y. pseudotuberculosis*균 진단을 위해서는 *inv* gene primer, *Y. enterocolitica* 균 진단을 위해서는 *ail* gene primer를 사용하였으며 plasmid 존재유무를 관찰하기 위하여 *vir* F gene primer를 사용하여 PCR법을 실시하였고 PCR법의 측정 한계(민감도)에 대한 실험도 실시하였다.

결 과 : *inv, ail* 및 *vir* F gene들의 primer들의 조합을 사용하면 *Y. pseudotuberculosis* 및 *Y. enterocolitica*균을 효과적으로 감별 진단할 수 있으며 PCR법의 민감도는 단회(30 cycle) PCR인 경우는 재래식 배양법보다 결과가 민감치 못하였으나 중복(60 cycle) PCR인 경우는 *Y. pseudotuberculosis*균인 경우 10 CFU/ml까지, Y. enterocolitica균인 경우 100 CFU/ml까지 검출할 수 있으므로 재래식 방법보다 매우 민감하며 이 방법을 이용하여 산악 자연수에 존재하는 소수의 세균을 검출할 수 있었다.

결 론 : 중복 PCR 방법은 재래식 배양법에 비하여 매우 민감하며 이 방법은 재래식 배양법으로 검출할 수 없는 자연수에 존재하는 소수의 *Yersinia*균 진단에 적절하다. 또한 인체 감염 진단시에도 재래식 배양법으로 배양되지 않는 소수의 균 진단에 사용될

본 논문은 1994년도 교육부 대학교수 국비 해외 파견(공동연구)에 의한 결과임

수 있으리라 사료되며 향후 환경검체에 대한 역학조사에도 효과적으로 사용될 수 있으리라 사료된다.

INTRODUCTION

Y. pseudotuberculosis causes gastroenteritis, mesenteric lymphadenitis and terminal ileitis, particularly in children[1], and could bring up Kawasaki disease, nephropathy, erythema nodosum, and arthritis among many others as complications[2]. Another species of *Yersinia*, pathogenic *Y. enterocolitica* causes mesenteric lymphadenitis and terminal ileitis often mistakenly diagnosed as appendicitis.

The clinical manifestation of these two *Yersinia* species are similar and they gain access to susceptible host by ingestion of tainted food or drink[1]. We[3] isolated *Y. pseudotuberculosis* from mountain spring waters and human stools and suggested that drinking of mountain spring water might be one of the main sources of *Y. pseudotuberculosis* infections in Korea. Traditionally, the epidemiological researches depend on the culture of *Yersinia* species. The method takes relatively long time and appeared to be insensitive because only 10-30% of the suspected spring water samples yielded positive results in our experience.

Recently polymerase chain reaction(PCR) was introduced for the detection of *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* successfully. It involved the amplification of three loci, *inv* gene for *Y. pseudotuberculosis*, *ail* gene for *Y. enterocolitica*, and plasmid-coded *vir* F gene for both *Yersinia* species[4-7]. We evaluated the PCR method for sensitivity and examined water samples from Korea and Japan.

MATERIALS AND METHODS

1. Bacterial strains

Y. pseudotuberculosis(serotype 4b, P+ (with plasmid)) was isolated from human stool and from spring water in Korea. *Y. enterocolitica* (serotype O:8, P+) and *Y. enterocolitica*(serotype O:8, P-) were from wild animal stool in Japan.

2. Water samples

Fifteen stream water samples were collected in Aomori prefecture of Japan and spring water samples at 12 places in Korea.

3. PCR

DNA samples were amplified using Takara PCR Amplification Kit(Takara Biochemicals, Japan). The reaction mixture contained 200 uM dNTP, 20 uM primers, 1 u Taq polymerase, 5 ul 10x PCR buffer and 5 ul DNA sample in a total volume of 50 ul and overlaid with a drop of paraffin. The reaction was carried out in the Thermal cycler(PC700,Japan) with 94℃ 1 min, 55 ℃ 2 min, 72℃ 1 min for 30 cycles. For two-stage PCR, another round of 30 cycle PCR was performed with additional dose of Taq polymerase. Ten microliter of the amplified DNA was examined on a 0.8% agarose gel after ethidium bromide-staining.

The 3 sets of primers used were 5'-TAA-GGG-TAC-TAT-CGC-GGC-GGA-3' and 5'-CGT-GAA-ATT-AAC-CGT-CAC-ACT-3' for *inv*, 5'-ACT-CGA-TGA-TAA-CTG-GGG-AG-3' and 5'-CCC-CCA-GTA-ATC-CAT-AAA-GG-3' for *ail* and 5'-TCA-TGG-CAG-AAC-AGC-AGT-CAG-3' and 5'-ACT-CAT-CTT-ACC-ATT-AAG-AAG-3' for *vir* F. The amplified DNA fragments would be 295 bp, 170 bp, and 591 bp long for

inv, ail, and *vir F,* respectively[61].

4. Colony counting and preparation of DNA for the detection limit

Yersinia spp. were grown on trypticase soy broth overnight at 25℃. A series of ten-fold dilutions were made with normal saline or with stream water and 0.1 ml was plated for cell counting. A 100 ul aliquot of each dilution was mixed with equal volume of Tween 20 and boiled for 5 min. Supernatants were obtained by centrifugation at 15,000 rpm for 3 min and used for

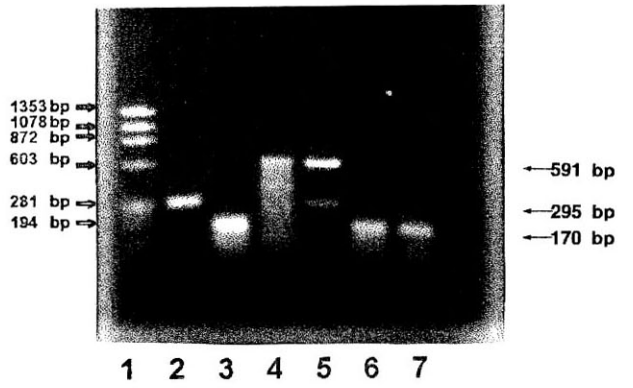

Fig. 1. Differentiation among yersinia bacilli by PCR with *inv, ail* and *vir* F primers, Lanes : 1. DNA base pairs marker, 2. 295 bp product from pathogenic *Y. pseudotuberculosis*(4b, P+) with *inv* primers, 3. 170 bp product from pathogenic *Y. enterocolitica*(0 : 8, P+) with ail primers, 4. 591 bp product from pathogenic *Y. enterocolitica*(0 : 8, P+) with *vir* F primers, 5. 295 & 591 bp product from pathogenic *Y. pseudotuberculosis*(4b, P+) with mixture of *inv* & *vir* F primers, 6 & 7 : 170 bp product from non-pathogenic *Y. enterocolitica*(0 : 8, P-) with ail primers.

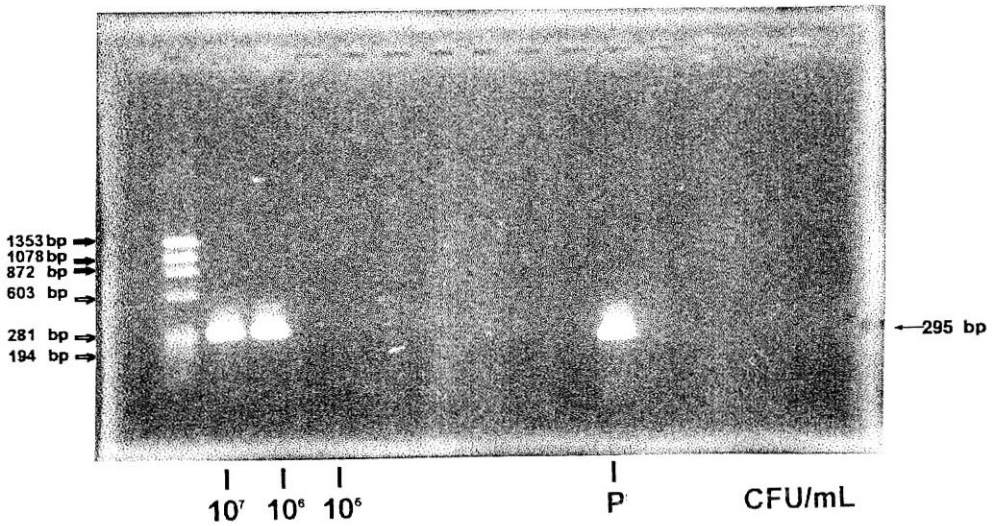

Fig. 2. Detection limit of single PCR to *Y. pseudotuberculosis* with *inv* primers. : positive control(a *Y. pseudotuberculosis* colony).

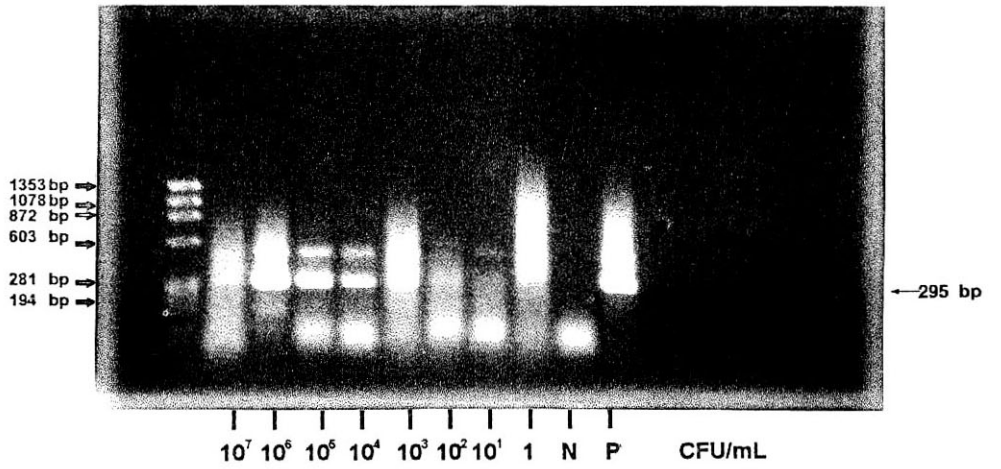

Fig. 3. Detection limit of duplicate PCR to *Y. pseudotuberculosis* with *inv* primer. N: negative control(distilled water), P : positive control(a Y. *pseudotuberculosis* colony).

PCR.

5. PCR of mountain water

The mountain water samples were centrifuged at 8,000 rpm for 20 min. Pellets were resuspended in 0.5 ml PBS and 0.1 ml was mixed with Tween 20 and treated as described above.

6. Traditional culture of *Yersinia spp.* from mountain water

Two liters of water samples were filtered with suction and the filter paper(pore size, 0.45 μm) [Millipore Co. USA] was rinsed with 10 ml PBS. The suspension was centrifuged at 3,500 rpm for 30 min. On the next day. The pellet was resuspended with 0.5 ml PBS and treated with equal volume of 0.5% KOH for 15 sec. 0.1ml of treated suspension was plated on Irgasan Novobiocin(IN) agar or modified MacConkey agar and incubated at 25℃ for two days.

RESULTS

PCR amplification of three genetic loci, *inv, ail,* and *vir F* can be used to diagnose both *Yersinia spp.*(Fig. 1) and *vir* F showed the presence of the plasmid.

A single run of PCR for *inv* detected up to 10^6 CFU/ml of *Y. pseudotuberculosis* or perhaps even lower concentrations(Fig. 2) but the two-stage PCR for *inv* detected up to 10 CFU/ml (Fig. 3) and the two-stage PCR for *ail* detected up to 100 CFU/ml of *Y. enterocolitica*(Fig. 4).

Out of 15 stream water samples from Japan, 6 were positive for *ail* by PCR. *Y. enterocolitica* was isolated from 2 of the 6 PCR-positive samples by the traditional culture method(Fig. 5). Out of 12 spring water samples collected in Korea, 11 were positive by PCR for *inv*. However, *Y. pseudotuberculosis* was not detected in any samples by the traditional culture method (Fig. 6).

DISCUSSION

We amplified three chromosomal locations using PCR to identify *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* The three sets of primers were derived from the sequences of *inv, ail,* and *vir F* genes. The *inv* encodes invasin which appeared to be required for the invasion of epithelial cells by *Y. pseudotuberculosis*[8]. The ail from *Y. enterocolitica* promoted attachment and invasion of *E. coli* cells to HEp-2 cells. The ail is for attachment-invasion-locus and has no ho-

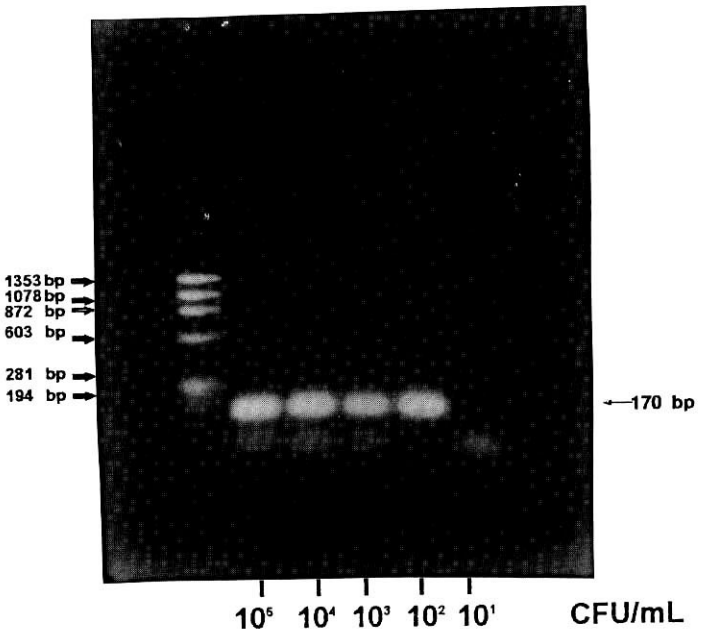

Fig. 4. Detection limit of duplicate PCR to pathogenic *Y. enterocolitica* with *ail* primers.

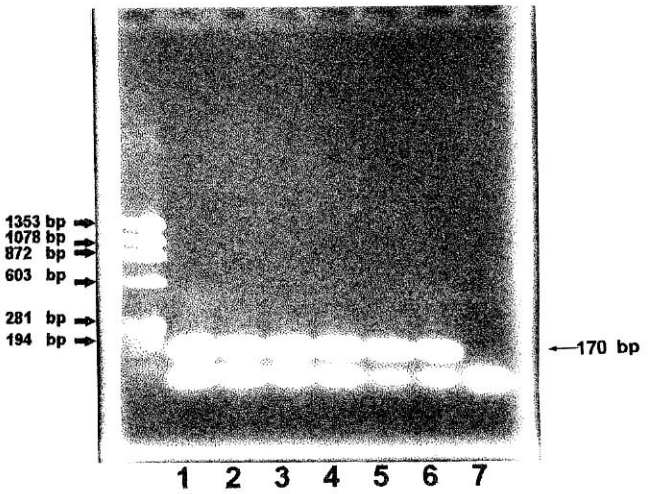

Fig. 5. Application of PCR to mountain stream waters of Aomori prefecture in Japan by ail primer to detect Y. enterocolitica. Lanes 1-6 : positive, Lane 7 : negative.

mology to *inv* of *Y. pseudotuberculosis*[9]. While these two genes were unique to the two *Yersinia spp.* respectively, The *vir* F was carried on a plasmid, pYV, found in both species. This 70 kb plasmid is responsible for the pathogenicity of *Yersinia spp.* as revealed in animal studies[10]. It also carries several indirect markers of virulence such as calcium dependence and autoagglutination. The *vir* F is related to the expression of yop gene which codes for a protein found on the outer membrane of *Yersinia spp.*[11].

There have been attempts to identify *Y.*

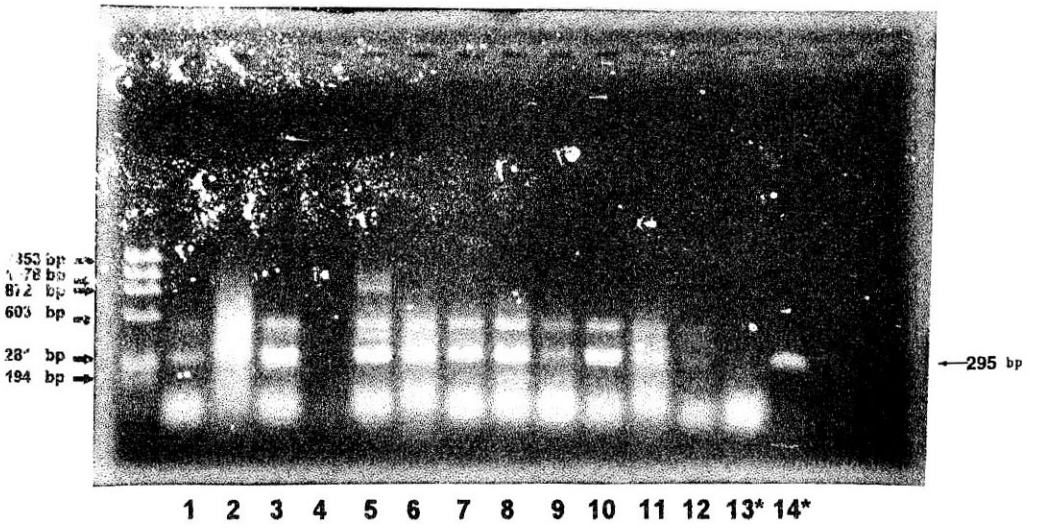

Fig. 6. Application of PCR to mountain spring waters of Seoul by *inv* primer to detect *Y. pseudotuberculosis*. Lanes 1-12 : positive, except lane 4, Lane 13 : negative control (distilled water) Lane 14 : positive control(a Y. *pseudotuberculosis* colony)

pseudotuberculosis and *Y. enterocolitica* by amplification of these gene segments by PCR[4-7]. Simultaneous amplification of the above three loci appeared to be a convenient and adequate procedure for routine detection of these pathogenic *Yersinia spp.*[6]. Furthermore, Nakajima et al. reported that the above three segments were not amplified in non-pathogenic *Yersinia spp.* nor in *Salmonella spp. Campylobacter spp.*, or in pathogenic *Escherichia spp.*[6].

We confirmed that *Y. pseudotuberculosis* and *Y. enterocolitica* could be identified by a similar PCR procedure.

The single-stage PCR involving 30 cycles for *inv* could detect up to 10^6 CFU/ml. However, a two-stage PCR involving additional 30 cycles with another dose of Taq polymerase raised the sensitivity to 10 CFU/ml. This is consistent with the observation made by Nakajima et al.[6]. This sensitivity was confirmed both with normal saline as well as with stream waters.

We examined natural water samples from Korea and Japan using this PCR method and compared the result with that obtained with the culture method. Out of 12 spring water samples from Korea, 11 were PCR-positive for *inv.* but all were negative by the traditional culture. Considering that the samples were collected during August when the water temperature reached the highest and that *Y. pseudotuberculosis* is a psychrophile, the result suggests that the PCR method could be much more sensitive than the traditional culture method in practice.

The detection of *yersinia spp.* by PCR would be useful for the epidemiological study of other environmental specimens.

SUMMARY

A PCR-based detection of *Y. pseudotuberculosis* and pathogenic *Y. enterocolitica* was evaluated.

Methods : Three loci, *inv* for *Y. pseudotuberculosis*, ail for *Y. enterocolitica* and plasmid-coded *vir* F for both, were amplified and the results were compared with that obtained by the traditional culture method.

Results : A single-stage PCR detected *Y. pseudotuberculosis* to 10^6 CFU/ml, which was not more sensitive than culture method. However, two-

stage PCR could detect 10 CFU/ml of *Y. pseudotuberculosis* for *inv* and 100 CFU/ml of *Y. enterocolitica* for ail. When the natural samples were examined, PCR method proved its superiority more than expected.

Conclusion : The PCR method should be useful in detecting *Y. pseudotuberculosis* and *Y. enterocolitica* in natural water samples and may also be applied for rapid and sensitive diagnosis of human yersiniosis.

감사의 글

본 논문의 영문 원고 교정을 도와주신 서울백병원 분자생물학 연구실의 정연보 교수님께 감사를 드립니다.

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