2008년 2월

석사학위논문

Comparison of Conventional, Nested, Real-time PCR for Rapid and Accurate Diagnosis of Patients with *Vibrio vulnificus*

조 선 대 학 교 대 학 원

바이오신약개발학과

김 형 선

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이 논문을 이학석사학위 청구논문으로 제출함

2007年 10月

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2007年 11月

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비브리오 패혈증 환자들의 빠르고 정확한 진단을 위한 실시간 중합효소 연쇄반응의 임상적 유용성

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배 경: 비브리오 불니피쿠스 패혈증은 빠른 진행과 사망률이 50%에 이르는 치명적인 질환이다. 지금까지 비브리오 불니피쿠스균에만 특이성을 갖는 동일 유전자를 대상으로 실시간 중합효소연쇄반응 (Q-PCR), 이중중합효소연쇄반응 (N-PCR), 중합효소연쇄반응 (C-PCR)를 비교한 연구가 거의 시행된바 없다. 저자는 실시간 중합효소연쇄반응이 진단기술로써 유용한지 알아보기 위해 두 개의 병원에 피부와 연조직 감염으로 내원한 환자들의 샘플을 가지고 실험을 하였다. 이 논문에서 세 종류의 중합효소연쇄반응을 실행하고 그 결과와 현재 표준 진단법으로 사용되는 미생물 배양 결과의 비교 연구를 수행하였다.

방 법: 시동체와 탐색자는 비브리오 불니피쿠스 내에 *ToxR* 유전자를 표적으로 디자인하였다. 표준균주 30개, 2006~2007년 봉와직염과 괴사성근막염이 의심되어 내원한 환자중 비브리오 불니피쿠스 감염증으로 확진된 환자 22명의 혈액, 비브리오 불니피쿠스에 감염된 환자로부터 얻은 임상 분리주 30개, 비브리오 불니피쿠스 이외의 다른 원인균이 확인된 환자 19명의 혈액을 이용하여 중합효소연쇄반응 (Q-PCR), 이중중합효소연쇄반응 (N-PCR), 중합효소연쇄반응 (C-PCR)을 실행하여 미생물 배양검사법과 비교 연구 하였다.

결 과: 표준균주에서 Q-PCR은 $5x10^0$ copies/㎕까지 검출할 수 있고 양성과 음성을 나누는 cp값은 균에서는 30 cp값 이상으로 하였을 때 비브리오 불니피쿠스만 양성으로 읽었다. 임상분리주에서는 중합효소연쇄반응과 실시간 중합효소연쇄반응에서 비브리오 불니피쿠스만 양성으로 검출되었다. 환자들의 혈액을 이용한 고전적 중합효소연쇄반응 검사상 민감도가 45% 특이도는 100%였고, 이중중합효소연쇄반응의 민감도는 86%이고 특이도는 73% 였다. 양성과 음성을 나누는 cp값을 38 이상으로 정의 하였을 때 실시간 중합효소연쇄반응에서는 100%의 민감도와 특이도를 보였다. 실시간 중합효소연쇄반응이 가장 민감성과 특이성 좋은 검사임을 확인할 수 있었다.

결 론: 비브리오 불니피쿠스를 정확하고 빠른 진단하기 위해 세 가지의 실험법 중실시간 중합효소연쇄반응은 다른 실험법에 비해 특이성과 민감성이 좋을 뿐 아니라 빠르게 검출할 수 있는 방법임을 확인하였으며 실시간 중합효소의 적절한 사용은 환자들의 사망률을 줄이는 데 크게 기여할 수 있으리라 사료된다.

Key words: *Vibrio vulnificus*, conventional PCR, nested PCR, Real-time PCR

Ⅰ. INTRODUCTION

Vibrio vulnificus can cause severe and life-threatening diseases in those who eat contaminated seafood or have an open wound that is exposed to seawater (1-4). The speed disease goes on is fast and the mortality of *V. vulnificus* is high. So these patients need a rapid diagnosis and a treatment. Microbiological culture methods for identification of causative organisms - a selective agar media; TCBS and mCPC, requires a several days. This procedure are time consuming and laborious but it has good specificity (5).

Currently, PCR (Polymerase Chain Reaction) assay to amplify DNA has proven to be useful for the early diagnosis. On 1991, *V. vulnificus* was detected through PCR for the first time (7). Conventional PCR (C-PCR) is specific but sensitivity is low (1,5-6,25). To detect *V. vulnificus* many PCR techniques are developed: Nested PCR (N-PCR) has high specificity and sensitivity but it is possible to erroneously obtain a positive result due to DNA contamination (8). Multiplex PCR has advantage that can detect several target genes at the same time but time consuming and laborious like C-PCR and N-PCR (9-10). Real-time quantitative PCR (Q-PCR) can be detected *V. vulnificus* within 2 hours (11-12) and there is no agarose gel loading step (13). Q-PCR assay is not laborious, and has high sensitivity and specificity (24).

Until now there is a lack of data on the evaluation of 3 methods comparison; C-PCR, N-PCR and Q-PCR, as a target of *V. vulnificus* specific genes. The author conducted a prospective study to target *toxR* gene of *V. vulnificus* in samples of skin and soft tissue infection patients who were admitted to two tertiary care hospitals to assess the clinical usefulness of performing Q-PCR as a diagnostic technique. We carried out C-PCR, N-PCR and Q-PCR and compared results

of three kinds of PCR with the "gold standard" microbiologic conventional culture results.

Ⅱ. Materials and Methods

Bacterial strains and media

The type strains used in this study were listed in table 1. These strains were got from the American Type Culture Collection (ATCC), the Korea culture center of microorganisms (KCCM) and the Korean culture Type collection (KCTC).

Clinical strains of patients with *V. vulnificus* were got from Chosun university hospital and Chonnam university hospital between 2006 and 2007. Clinical strains were obtained from various samples of patients such as blood, bulla aspiration, and other skin and soft tissue of the patients with skin and soft tissue infection. Clinical strains were identified with a VITECK II automated system. All strains were cultured in LB (Luria-Bertani) or BHI (Brain heart infusion) broth (Difco, USA) and agar (Difco, USA) but *Vibrio* species were cultured in broth and agar containing 2% NaCl. These strains were stored at -70℃.

ToxR cloning

The *toxR* gene of *V. vulnificus* was cloned according to the method described by Hajime Takahashi et.al (14). Briefly, *V. vulnificus* (KCCM 41665) was cultured in the TSB (Tryptic Soy Broth, Bacto, USA) containing 2% NaCl. Template genomic DNA for PCR was extracted using the QIAamp DNA mini kit (Qiagen, Germany).

The primers were designed to target sequence of *toxR* gene (GeneBank accession No. AF170883) from *V. vulnificus* in table 2 (573 bp). PCR was conducted in a 20 ㎕ mixture totally; 1 ㎕ of template DNA, 0.2 ㎕ of 2.5 U of TaKaRa Taq DNA polymerase (Takara Bio, japan), each 1 ㎕ of 10 μM of forward primer and reverse primer, 2 ㎕ of dNTPs, 2 ㎕ of 10X PCR buffer and 12.8 ㎕ of water. PCR was performed with predenaturation at 94℃ for 5 min followed by 39 cycles of 3 steps:

denaturation at 94℃ for 30 sec, annealing at 62℃ for 30 sec and extension at 72℃ for 1 min. The elongation step was prolonged to 7 min in the last cycle using Applied Biosystems Veriti™ 96-well Thermal cycler. The PCR product was electrophoresed on 1.2% agarose gel (Seakem® LE agarose) with Etbr (Ethidium bromide) at 100 V (0.5X TBE buffer). Amplified DNA was got from gel elution using QIAquick® gel extraction kit (Qiagen, Germany). Target DNA was ligated in pGEM-T easy vector and transformed into E.coli. After we confirmed it through sequencing, the *toxR* gene inserted in pGEM-T easy vector within E.coli was cultured in LB broth containing ampicillin (50 mg/ml). The plasmid DNA was extracted with Gene ALL™ Plasmid Quick (general bio system).

DNA extraction

DNA was extracted according to the manual of a QIAamp DNA mini kit (Qiagen, germany). Briefly, first 200 ㎕ of a sample such as whole blood, serum, buffy coat, or strain in PBS was added 20 ㎕ proteinase K (20 mg/ml) and 200 ㎕ AL buffer. After the samples were incubated at 56℃ for 10 min, you added to 200 ㎕ Ethanol (100%), centrifugation. Then mixture in a tube was transferred to a column and centrifugation. The column was added Washing buffer 1,2 (500 ㎕) and centrifuge. The last, 50 ㎕~80 ㎕ of AE buffer was added in a column and centrifuge at 8000 rpm for 1~3 min. After genomic DNA was extracted, we always devided DNA to protect contamination and stored -20℃.

Primer and Probe

Primers and a probe used in this study were listed in table 2. Primers, tox-130 and tox-200, and probe, tox-152, are used as described previosly by Hajime Takahashi et.al (14). The tox-130 and tox-200 were used in C-PCR, nested 2nd PCR and Q-PCR. Primers, tox-100 and tox-303, were designed using the Basic Local Alignment Search Tool (BLAST) database search program in the National Center for

Biotechnologh Information (NCBI) and primer 3. It is used in nested 1st PCR. 5 end and 3 end of probe toxR-152 were labeled by FAM (6-carboxyfluorescein) and BHQ-1 (Black Hole Quencher-1), respectively. The position of primers and a probe sequence we designed was showed in figure 1.

PCR

ⅰ) C-PCR

Primers, toxR-130 and toxR-200, were used in C-PCR (Table 2). Template DNA, each 1 ㎕ of 10 pmole/㎕ of forward primer and reverse primer, 10 ul of 2X EXCEL-Taq™ PreMix (Taq polymerase 2U, 400 μM dNTP, 2.0 mM $MgCl_2$ KCl, Tris-Cl) and the volume was adjusted to 20 ㎕ with distilled water. PCR condition is followed; preincubation at 94℃ for 5 min, 3 steps of denaturation at 94℃ for 1 min, annealing at 60℃ for 1 min and elongation at 72℃ for 1 min (35 cycles). A final extension step was done for 7 min using Applied Biosystems Veriti™ 96-well Thermal cycler. PCR product was electrophoresed on a 2% agarose gel (Seakem® LE agarose) with Etbr (Ethidium bromide) for 30 min at 100 V (0.5X TBE).

ⅱ) N-PCR

The internal primers, toxR-130 and toxR-200, were listed in Table 2. The external primer was designed by program of Primer 3 site (http://frodo.wi.mit.edu/)(Table 2). First PCR was performed with tox-100 and tox-303 primers (10 pmoles/㎕), 2X EXCEL-Taq™ PreMix (Taq polymerase 2U, 400 μM dNTPs, 2.0 mM $MgCl_2$ KCl and Tris-Cl), template DNA and distilled water in a total volume of 50 ㎕. First PCR condition is the same C-PCR condition. The second PCR mixture was the same mixture of first PCR except primer; tox-130 and tox-200 primer (10 pmoles/ul). The template DNA of the second PCR is the first PCR product (2 ㎕/ 50 ㎕ total volume). PCR products were electrophoresed

on a 2% agarose gel at 100 V.

iii) Q-PCR

The principal of Q-PCR is to detect fluorescent dye emitted during PCR. Plasmid DNA analyzed quantity using a spectrophotometer (DU® 530 Life science UV/vis spectrophotometer, BECKMAN COULTER). We can change units from ng/㎕ to copies/plasmid molecules According to formula of Promega protocol.

Plasmid DNA was diluted 10 fold serially after the concentration of the standard plasmid DNA set 1 x 10^8 copies /㎕,

Q-PCR was conducted in 20 ㎕ reaction ; 5 ㎕ of genomic DNA, each 1 ㎕ of 5 pmole/㎕ forward primer and reverse primer (toxR-130 and toxR-200), 1 ㎕ of 2 pmole/㎕ probe, 4 ㎕ of master mix (reaction buffer, FastStart Taq DNA polymerase, $MgCl_2$ and dNTP(with dUTP instead of dTTP)), and water. The amplification condition consisted of preincubation at 95℃ for 10 min, 2 step (45 cycles) at 95℃ for 10 sec and 60℃ for 30 sec, followed by cooling at 40℃ for 30 sec using LightCycler 2.0. We can analysis the result using the LightCycler software 4.0 on the computer.

Patients selection

We enrolled adult patients (aged ≥ 18 years) suffering from skin and soft tissue infections such as cellulitis or necrotizing fasciitis. An informed consent was obtained from the patients or their guardians. Each patient was admitted from 2006 to 2007 to Chosun University Hospital or Chonnam University Hospital. Whole blood samples for PCR assay of patients were collected for this study. Clinical strains were isolated from the blood, bulla and other skin and soft tissue. Identification of the clinical isolates was initially performed with a VITEK II automated system (bioMe´rieux, Marcy l'Etoile, France).

The lab personal who carrying out PCR assay did not know any of the clinical information and definite diagnosis and the physician who treat the patients did not know the PCR result.

Ⅲ. RESULTS

Ⅰ. Bacteria standard strains

ⅰ) Detection Sensitivity

To determine the detection sensitivity of serially diluted plasmid DNA from $5X10^8$ copies/㎕ to $5X10^0$ copies/㎕ and detection limit of primers and a probe to target *toxR* gene of *V. vulnificus,* three PCR; Q-PCR, N-PCR and C-PCR were performed.

Figure 2 was described the detection sensitivity of C-PCR and N-PCR serially diluted plasmid DNA from $5X10^8$ to $5X10^0$ copies/㎕ using ABi Veriti™ 96-well Thermal cycler machine. C-PCP (70 bp) using tox-130 and tox-200 primers could detect to $5X10^3$ copies/㎕. When N-PCR was done, first PCR (204 bp) using external primer, tox-100 and tox-303, can detect to $5X10^3$ copies/㎕ and second PCR (70 bp) using internal primer, tox-130 and tox-200, can detect to $5X10^2$ copies/㎕.

Figure 3 showed the detection sensitivity of serially diluted plasmid DNA using Q-PCR, Roche. Q-PCR using tox-130 and tox-200 primers and tox-152 probe could detect to $5X10^0$ copies/㎕.

ⅱ) Detection Specificity

To confirm the specificity of this primers (Tox-130 and Tox-200) and a probe (Tox-152) to target *toxR* gene, we conducted Q-PCR, N-PCR and C-PCR with the type strains.

The bands of C-PCR and N-PCR of type strains were not detected except *V. vulnificus* (data not shown). As shown in Figure 4, Only *V. vulnificus* among *Vibrio* species was detected but others not. Q-PCR also had high cp value (> 30 cp) except *V. vulnificus* (10.2 cp) (Table 3). We decided that cp value of type strain had more than 30 was negative (15-18).

II. Clinical isolates

C-PCR, N-PCR and Q-PCR were performed with the clinical isolates identified as *V. vulnificus* by VITECK II system.

29 out of 30 clinical isolates identified as *V. vulnificus* by VITECK II system showed positive results on the C-PCR. 30 of 30 clinical isolates showed positive results on N-PCR (Table 4). If we use negative cut off value as > 30 cp of Q-PCR (15-18), 29 out of 30 clinical isolates showed positive results except the one isolate (CHU-A-47; cp value of a strain is > 36 cp), which was identified initially as *V. vulnficus* by VITECK II system, but identified finally as *Pseudomonas aeruginosa* by 16S rRNA gene sequence analysis (data not shown). Actually 29 of 29 positive clinical isolates of *V. vulnificus* showed positive results on the C-PCR and Q-PCR. It showed 100% specificity. Positive results on N-PCR of CHU-A-47 clinical isolates finally identified as *Pseudomonas aeruginosa* turned out to be false positive.

III. Clinical usefulness of Q-PCR as a diagnostic method for *Vibrio vulnificus*

Total 86 patients with skin soft tissue infection were enrolled in our study. Of these patients, pathogens was isolated in 41 patients' sterile fluids by microbiologic methods. Three kinds of PCR assays were performed using blood of these 41 patients. We could detect 10 patients of 22 *V. vulnificus* patients through C-PCR, 19 patients of 22 positive patients through N-PCR (Table 5). Comparison of the results of the C-PCR assay of the blood with the results of conventional microbiological methods, demonstrated a sensitivity of 45% and a specificity of 100%. The results of the N-PCR assay of the blood showed a sensitivity of 86% and a specificity of 73%. If we adopt cut off value > 38 cp as positive results (15,19-20), the results of Q-PCR

using blood samples showed 100% sensitivity and 100% specificity (Table 6).

IV. Discussion

V. vulnificus sepsis is a rapidly progressing and lethal disease entity whose mortality is reported to ≥ 50% (1-4). It is very important to administer adequate antibiotics immediately after early diagnosis. It takes several days to isolate causative infectious organisms from patients samples such as blood, bulla, CSF, and skin tissue. For the PCR assays, It takes at least 6 hours to detect *V. vulnificus*, but sensitivity is not good in C-PCR. Although N-PCR requires longer than C-PCR (9 hours), sensitivity is known to be better than C-PCR. Q-PCR assay can give results within 2 hours, and it is very useful for establishing an early diagnosis and providing the potential for automation for high throughput and quantitative information for the assessment of prognosis or responses to treatment. Until now there have been no data on the evaluation of 3 PCR methods comparison; C-PCR, N-PCR and Q-PCR, against the same target gene specific for *V. vulnificus.* We selected tox R gene, which is known to be a house-keeping regulatory gene regarded as an effective taxonomic marker for identification of *Vibrio species* (14).

For type strains, the specificity of three kind of PCR for various microorganisms was very good. No positive results in C-PCR and N-PCR was detected except *V. vulnificus*. Q-PCR also had high cp value (> 30 cp) for various microorganisms other than *V. vulnificus.* Only *V. vulnificus* had low cp value (10.2 cp).

Takahashi reported that Q-PCR targeted to the toxR for detection of *V. vulnificus* was very sensitive detecting as few as 10 microbes per milliliter of seawater and oyster homogenate (14). In our study, the detection sensitivity in three kind of PCR assays showed some differences. The lower detection limit of C-PCR was $5x10^3$ copies/$\mu\ell$, and the lower detection limit of N-PCR was $5x10^2$ copies/$\mu\ell$. However the

lower detection limit of Q-PCR was $5x10^0$ copies/㎕, and had the best detection sensitivity.

Even though various cut off cp value have beeb used for various microorganisms. Cp value for cultured microorganisms is lower than that of clinical specimens such as blood, tissue fluids, biopsy samples and so on. Cp value for cultured microorganisms were commonly used > 30 cp (15-18), > 28 cp (21) as negative results. The cp for clinical specimens from stool and biopsy were reported to get agreement > 40 and > 38, respectively (22). The cp value of blood has higher than 38 (15,19-20) was reported to be negative. In our study, in case of cp value more than 38, PCR products turned out to be dimers formation, not *V. vulnificus* genes through gene sequencing of the PCR products. It was also turn out to be *V. vulnificus* tox R genes in case of cp value lower than 38. Therefore, we adopted cp value of Q-PCR > 38 using blood as negative results. We adopted cut off cp value of Q-PCR >30, when we performed Q-PCR with cultured bacterial isolates.
For clinical isolates, all clinical *V. vulnificus* isolates could be detected as positive using C-PCR except one clinical isolate of CUH-A-47.If we use cut off value of Q-PCR more than 30 cp as negative, all clinical isolates were positive except clinical isolate of CUH-A-47 (Table 3), which was identified originally as *V. vulnificus* using VITECK II automatic system. The cp value in Q-PCR performed with clinical isolate of CUH-A-47 was more 30 (36.2 cp). We got the same results in repeated experiments. So we performed conventional microbiology and 16S rRNA gene sequence analysis for the CHU-A-47 isolate, and identified finally as *Pseudomonas aeruginosa.*

To assess the clinical usefulness of performing Q-PCR in practice as a diagnostic technique, we compared blindly the Q-PCR results using blood samples of the patients with skin and soft tissue infection enrolled between 2006 and 2007 with the other PCR assays and the microbiologic culture results. If we use cut off value > 38 cp as

positive results, the results of Q-PCR using blood sample showed 100% sensitivity and specificity. Therefore Q-PCR was not only the most sensitive and specific techniques to detect *V. vulnificus* , but also the most rapid diagnostic method.

In conclusion, the results of our study suggest that Q-PCR assay of the blood is a rapid and reliable method for the diagnosis of *V. vulnificus* sepsis.

V. ABSTRACT

Comparison of Conventional, Nested, Real-time PCR for Rapid and Accurate Diagnosis of Patients with *Vibrio vulnificus*

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Introduction: *V. vulnificus* sepsis is a rapidly progressing and lethal disease entity whose mortality is reported to ≥ 50%. Until now there is a lack of data on the evaluation of comparison 3 kinds of PCR methods ; conventional PCR (C-PCR), nested PCR (N-PCR) and real-time quantitative TaqMan PCR (Q-PCR), against the same target gene specific for *V. vulnificus*. The author conducted a prospective study to target *toxR* gene of *V. vulnificus* in samples of skin and soft tissue infection patients who were admitted to two tertiary care hospitals to assess the clinical usefulness of performing Q-PCR as a diagnostic technique. We carried out C-PCR, N-PCR and Q-PCR and compared results of three kinds of PCR with the "gold standard" microbiologic conventional culture results.

Materials and Methods: We performed C-PCR, N-PCR and Q-PCR to detect *V. vulnificus*. Primers and a probe were designed from *toxR* gene of *V. vulnificus*. We got the samples; 30 type strains, 30 clinical strains and 41 bloods of patients. We compared between Sensitivity and

specificity of primers and a probe from among C-PCR, N-PCR and Q-PCR

Results: In type strains, The lower detection limit of Q-PCR was $5x10^0$ copies/$\mu\ell$. Among 30 type strains, only *V. vulnificus* was detected by Q-PCR. Q-PCR assay can give results within 2 hours. In clinical strains, C-PCR and Q-PCR could detect only *V. vulnificus*. The sensitivity of C-PCR and N-PCR was 45% and 86% in bloods of patients, respectively. C-PCR and N-PCR assay showed a specificity of 100% and 73% in bloods of patients, respectively. If we adopt cut off value > 38 cp as positive results, Q-PCR assay showed 100% sensitivity and specificity

Conclusion: Q-PCR was not only the most sensitive and specific techniques to detect *V. vulnificus*, but also the most rapid diagnostic method. Therefore our study suggest that Q-PCR assay using the blood is a rapid and reliable method for the diagnosis of *V. vulnificus* sepsis.

VI. 감사의 말씀

먼저 하나님께 감사와 영광을 돌립니다.
석사 입학했을 때가 엊그제 같은데 벌써 2년이란 세월이 흘러 학위 논문을 내니 감회가 남다릅니다. 저를 받아주시고 가르쳐 주시고 믿어주시고 공부도 실험도 열심히 할 수 있게 기회를 주시고 항상 밝고 온화한 미소를 지니며 칭찬을 아끼지 아니하신 김동민 교수님께 진심으로 무한 감사드립니다. 부족한 저를 지도해 주시고 바쁘신 중에도 늘 따뜻하게 관심 가져주시는 박해령 박사님, 임용 교수님, 장숙진 교수님, 최철희 교수님, 저의 첫 실험실 선배님으로 많은 가르침과 도움을 주신 권안성 선생님, 이주영 선생님, 바쁜 실험 중에도 저의 실험을 많이 도와주고 늘 즐거움을 주는 유미씨와 수미씨, Ganesh, 학민씨, 효정씨, 논문 작성시 많은 조언과 도움을 주신 지윤언니, 주미언니, 이태범 선생님 모두들 감사드립니다.

특히 가장 많은 감사를 드리고 싶은 분들이 있습니다. 늘 기도로 밀어주시고 크신 사랑을 듬뿍 주시며 믿어주시고 힘이 되어주시는 아버지(김용연)와 어머니(오순덕)께 진심으로 무한무한 감사드립니다. 정말로 감사합니다. 부족한 언니를 늘 이해해 주고 가장 크게 응원해주고 위로와 격려를 아끼지 않은 착하고 이쁜 동생 미선이, 자기가 하는 일에 열정을 가지고 믿음으로 도전하는 멋진 남동생 석중이, 항상 기도로 응원해 주시는 박요섭 목사님, 첨단중앙교회 집사님들, BEDTS, 학교 친구들 진심으로 감사합니다.

첫 발을 떼었습니다.

Work as though your strength were limitless. <S.Bernhardt>
They can do all because they think they can. <Virgil>
Living without an aim is like sailing without a compass. <J.Ruskin>
할 수 있다는 긍정적인 사고방식을 가지고 목표를 향해 열심히 달리겠습니다.

2007.11. 김형선

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Table 1. Bacterial strains used in this study

Type strains
Aeromonas hydrophilia subsp. hydrophila KCTC 2358
Vibrio alginolyticus KCCM 40513
Vibrio cholerae KCCM 41626
Vibrio fluvialis KCCM 40827
Vibrio furnissii KCCM 41679
Vibrio mimicus KCCM 42257
Vibrio proteolyticus KCCM 11992
Vibrio vulnificus KCCM 41665
Streptococcus agalactiae KCCM 40417
Streptococcus mitis KCTC 3556
Streptococcus mutans KCTC 3065
Streptococcus pyogenes KCTC 3208
Streptococcus salivarius KCTC 3960
Streptococcus sobrinus KCTC 3288
Staphylococcus aureus subsp. Aureus (MRSA) KCCM 40510
Staphylococcus aureus(MRSA) KCTC 29213
Staphylococcus epidermidis KCTC 1917
Staphylococcus saprophyticus subsp. saprophyticus KCTC 3345
Salmonella pneumoniae KCTC 1925
Klebsiella pneumoniae KCTC 2242
Shigella sonnei KCTC 2518
Pseudomonas aeruginosa KCTC 27853
Aeromonas caviae KCTC 1653
aeromonas salmonicida subsp. salmonicida KCTC 12266
Aeromonas hydrophila subsp. Anaerogenes KCTC 12487
Vibrio hollisae KCCM 41680
Vibrio parahaemolyticus KCCM 11965
Streptococcus pneumoniae KCTC 3932
Streptococcus sanguinis KCTC 3299
Clostridium perfringens KCTC 3269
Clostridium difficile KCTC 5009

Table 2. Oligonucleotide primers and a probe used in this study

Primers and a probe Sequence	Location (Length)	Amplicon Size (bp)	Tm (℃)	Ref	PCR
ToxAll 1 : 5V-GAG CAG GGG TTT GAG GTG GAT GAT-3V	1-24 (24 mer)	573	63.1	1	Cloning Primer
ToxAll 2 : 5V-GTT TTG GCC CCC CGT CGC GAT CAC-3V	550-573 (24 mer)		72.7	1	
Tox-130 : 5' -TGTTCGGTTGAGCGCATTAA- 3'	130-149 (20 mer)	70	56.4	1	Q-PCR Primer C-PCR Primer N-PCR Internal Primer
Tox-200 : 5' -GCTTCAGAGCTGCGTCATTC- 3'	180-200 (21 mer)		56.3	1	
Tox-152 : 5' -FAM-CGCTCCTGTCAGATTCAACCAACAACG-BHQ1- 3'	152-188 (27 mer)		63.8	1	Q-PCR Probe
Tox-100 : 5' -ACGGTTCCAAAACGTGGTTA- 3'	100-119 (20 mer)	204	60		N-PCR External Primer
Tox-303 : 5' -TGTTGACGTGCCAGCATTAT- 3'	284-303 (20 mer)		60		

Table 3. The results of C-PCR, N-PCR and Q-PCR for detection of *V. vulnificus* in type strains

Type strains	C-PCR	N-PCR	Q-PCR	
			CP values	Interpretation
Aeromonas hydrophilia subsp. hydrophila KCTC 2358	-	-	38.35	-
Vibrio alginolyticus KCCM 40513	-	-	35.67	-
Vibrio cholerae KCCM 41626	-	-	37.37	-
Vibrio fluvialis KCCM 40827	-	-	35.8	-
Vibrio furnissii KCCM 41679	-	-	37.2	-
Vibrio mimicus KCCM 42257	-	-	34.37	-
Vibrio proteolyticus KCCM 11992	-	-	37.52	-
Vibrio vulnificus KCCM 41665	+	+	10.2	+
Streptococcus agalactiae KCCM 40417	-	-	>35	-
Streptococcus mitis KCTC 3556	-	-	35.15	-
Streptococcus mutans KCTC 3065	-	-	>40	-
Streptococcus pyogenes KCTC 3208	-	-	>40	-
Streptococcus salivarius KCTC 3960	-	-	35.25	-
Streptococcus sobrinus KCTC 3288	-	-	>35	-
Staphylococcus aureus subsp. Aureus (MRSA) KCCM 40510	-	-	36.25	-
Staphylococcus aureus(MRSA) KCTC 29213	-	-	>35	-
Staphylococcus epidermidis KCTC 1917	-	-	>35	-
Staphylococcus saprophyticus subsp. saprophyticus KCTC 3345	-	-	>35	-
Salmonella pneumoniae KCTC 1925	-	-	37.08	-
Klebsiella pneumoniae KCTC 2242	-	-	>40	-
Shigella sonnei KCTC 2518	-	-	33.5	-
Pseudomonas aeruginosa KCTC 27853	-	-	39.64	-
Aeromonas caviae KCTC 1653	-	-	33.78	-

aeromonas salmonicida subsp. salmonicida KCTC 12266	-	-	38.27	-
Aeromonas hydrophila subsp. Anaerogenes KCTC 12487	-	-	>40	-
Vibrio hollisae KCCM 41680	-	-	36.46	-
Vibrio parahaemolyticus KCCM 11965	-	-	36.17	-
Streptococcus pneumoniae KCTC 3932	-	-	33.81	-
Streptococcus sanguinis KCTC 3299	-	-	36.95	-
Clostridium perfringens KCTC 3269	-	-	30.96	-
Clostridium difficile KCTC 5009	-	-	36.67	-

Table 4. The results of C-PCR, N-PCR and Q-PCR for detection of *V. vulnificus* from 30 clinical isolates

Name	Clinical isolates	Q-PCR		C-PCR	N-PCR
		CP values	Interpretation		
CUH-0-11	*Vibrio vulnificus*	12.21	+	+	+
CUH-0-21	*Vibrio vulnificus*	12.52	+	+	+
CUH-0-31	*Vibrio vulnificus*	11.91	+	+	+
CUH-0-41	*Vibrio vulnificus*	12.19	+	+	+
CUH-0-51	*Vibrio vulnificus*	12.99	+	+	+
CUH-0-61	*Vibrio vulnificus*	18.26	+	+	+
CUH-0-71	*Vibrio vulnificus*	11.92	+	+	+
CUH-0-81	*Vibrio vulnificus*	11.9	+	+	+
CUH-A-79	*Vibrio vulnificus*	11.67	+	+	+
CUH-0-91	*Vibrio vulnificus*	11.45	+	+	+
CUH-0-13	*Vibrio vulnificus*	12.86	+	+	+
CUH-08-95	*Vibrio vulnificus*	11.82	+	+	+
CUH-A-47	*Pseudomonas aeruginosa*	36.2	-	-	+
CUH-0-23	*Vibrio vulnificus*	11.3	+	+	+
CUH-0-33	*Vibrio vulnificus*	11.13	+	+	+
CUH-41-63	*Vibrio vulnificus*	12.8	+	+	+
CUH-0-43	*Vibrio vulnificus*	13.5	+	+	+
CUH-42-31	*Vibrio vulnificus*	12.8	+	+	+
CUH-42-32	*Vibrio vulnificus*	12.9	+	+	+
CUH-44-65	*Vibrio vulnificus*	12	+	+	+
CUH-42-14	*Vibrio vulnificus*	11.9	+	+	+
CUH-43-52	*Vibrio vulnificus*	10.5	+	+	+
CUH-43-53	*Vibrio vulnificus*	10.6	+	+	+
CUH-43-54	*Vibrio vulnificus*	10	+	+	+
CUH-43-55	*Vibrio vulnificus*	11.1	+	+	+
CUH-43-56	*Vibrio vulnificus*	10.5	+	+	+
CUH-43-57	*Vibrio vulnificus*	8.96	+	+	+
CUH-43-58	*Vibrio vulnificus*	9.77	+	+	+
CUH-43-59	*Vibrio vulnificus*	10.2	+	+	+
CUH-43-61	*Vibrio vulnificus*	11.7	+	+	+

Table 5. The results of C-PCR, N-PCR and Q-PCR for Detection of 41 blood samples of patients with *V. vulnificus* infection and others

Patients	Pathogens	Q-PCR		C-PCR	N-PCR
		CP values	Interpretation		
Patient 1	*Vibrio vulnificus*	30.25	+	+	+
Patient 2	*Vibrio vulnificus*	36.1	+	-	+
Patient 3	*Vibrio vulnificus*	35.08	+	-	-
Patient 4	*Vibrio vulnificus*	31.7	+	+	+
Patient 5	*Vibrio vulnificus*	31.61	+	+	+
Patient 6	*Vibrio vulnificus*	34.87	+	-	+
Patient 7	*Vibrio vulnificus*	25.85	+	+	+
Patient 8	*Vibrio vulnificus*	31.85	+	+	+
Patient 9	*Vibrio vulnificus*	34.3	+	-	+
Patient 10	*Vibrio vulnificus*	36.91	+	-	+
Patient 11	*Vibrio vulnificus*	32.09	+	-	+
Patient 12	*Vibrio vulnificus*	34.96	+	-	-
Patient 13	*Vibrio vulnificus*	33.75	+	-	-
Patient 14	*Vibrio vulnificus*	29.16	+	+	+
Patient 15	*Vibrio vulnificus*	27.16	+	+	+
Patient 16	*Vibrio vulnificus*	34.14	+	-	+
Patient 17	*Vibrio vulnificus*	30.77	+	+	+
Patient 18	*Vibrio vulnificus*	33.16	+	-	+
Patient 19	*Vibrio vulnificus*	29.89	+	+	+
Patient 20	*Vibrio vulnificus*	33.68	+	-	+
Patient 21	*Vibrio vulnificus*	18.02	+	-	+
Patient 22	*Vibrio vulnificus*	28.92	+	+	+
Patient 23	*Shewanella putrefaciens*	>40	-	-	-
Patient 24	*Staphylococcus aureus*	>40	-	-	-
Patient 25	*S. pyogens*	>40	-	-	-
Patient 26	*Cellulomonas spp.*	>35	-	-	+
Patient 27	*S. viridans*	>35	-	-	+
Patient 28	*S. equi subsp.*	>35	-	-	+
Patient 29	*S. hominis*	>35	-	-	+
Patient 30	*Streptococcus dysgalactiae*	>40	-	-	-
Patient 31	*Aeromonas hydrophila*	>40	-	-	-
Patient 32	*Pseudomonas aeruginosa*	>40	-	-	-
Patient 33	*Peptostreptococicus prevot*	>40	-	-	-
Patient 34	*Staphylococcus aureus*	>40	-	-	+
Patient 35	*Aeromonas hydrophila*	>40	-	-	-
Patient 36	*Aeromonas spp.*	>40	-	-	-
Patient 37	*Fusobacterium necrophorum*	>40	-	-	-
Patient 38	*S. pyogens*	>40	-	-	-

Patient 39	*S. anginosus, S. epidermidis*	>40	-	-	-
Patient 40	*S. pyogens*	>40	-	-	-
Patient 41	*Streptococcus dysgalactiae*	>40	-	-	-

Table 6. Results of C-PCR, N-PCR and Q-PCR using blood as a diagnostic technique in comparison to microbiologic methods for the patients with skin and soft tissue infections

Test	Screening tests			
	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)
C-PCR	0.45 (0.25-0.67)	1 (0.79-1)	1 (0.65-1)	0.61 (0.42-0.78)
N-PCR	0.86 (0.64-0.96)	0.74 (0.49-0.90)	0.79 (0.57-0.92)	0.82 (0.56-0.95)
Q-PCR	1 (0.82-1)	1 (0.79-1)	1 (0.82-1)	1 (0.79-1)

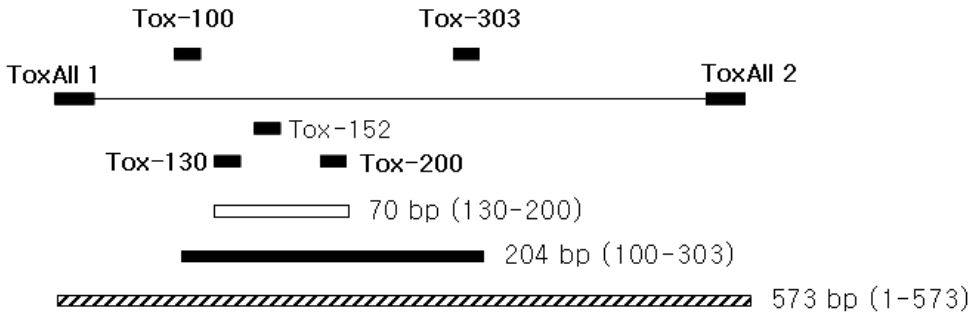


Figure 1. The diagram of primers' position of *V. vulnificus* transmembrane regulatory protein *toxR* gene (AF170883). white bar: The amplicon size 573 bp primer for cloning (ToxAll1 and ToxAll2); Black bar: The amplicon size 204 bp external primer for 1st PCR of nested PCR (Tox-100 and Tox-303); The bar with slanting lines pattern: The amplicon size 70 bp primer for real time, conventional PCR and 2nd PCR of nested PCR (Tox-130 and Tox-200) and a probe (Tox-152) for real time PCR.

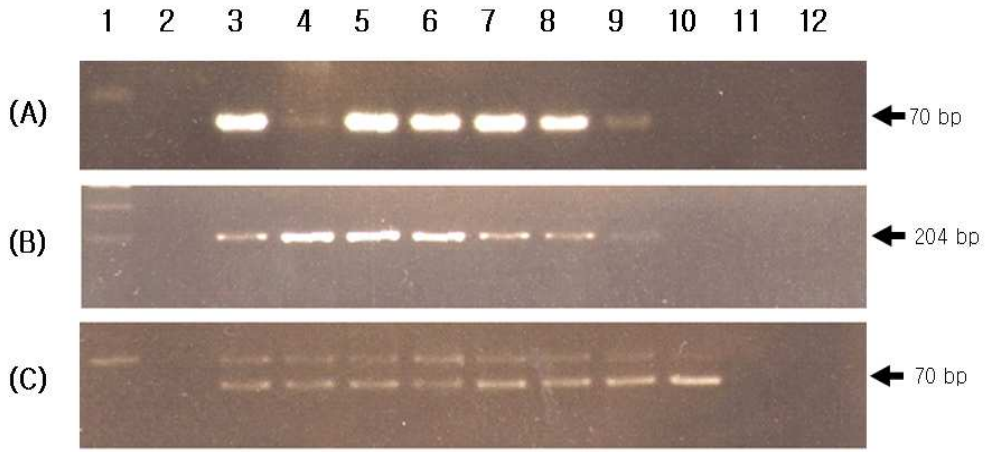
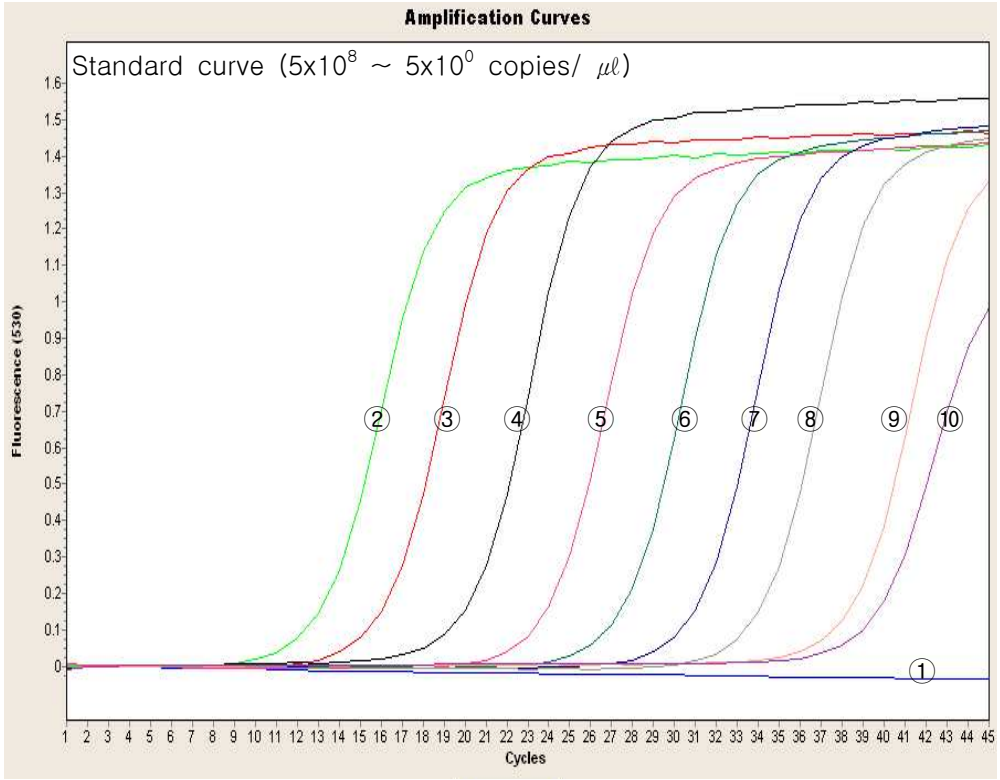


Figure 2. Sensitivity of C-PCR (A) or N-PCR with the external primer Tox100 - Tox303 (B) or internal primer Tox130 - Tox200 (C) to detect *V. vulnificus* from plasmid DNA.

Lane 1: 100bp ladder marker (bioneer), Lane 2: negative-Sterilized distill water, Lane 3: positive control *Vibrio vulnificus,* lane 4-12: Plasmid DNA serially diluted 10 fold from $5x10^{8}$ copies/㎕ to $5x10^{0}$ copies/㎕.



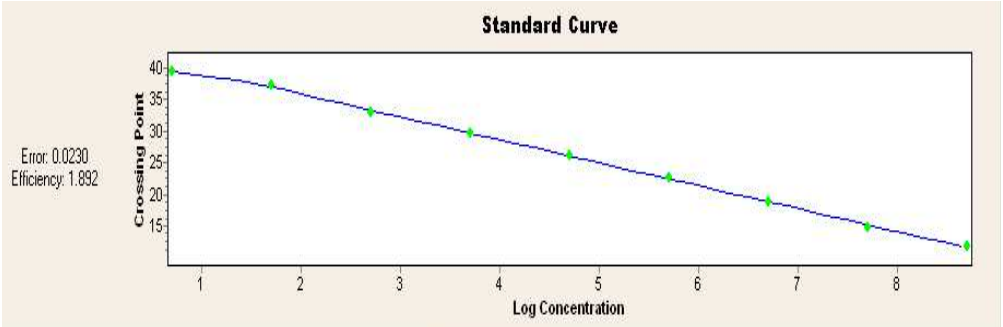


Figure 3. Standard curve ($5x10^8$ ~ $5x10^0$ copies/㎕) of Q-PCR. Plasmid DNA was used as template. ① curve: negative-Sterilized distill water, ②~⑩ curve: Plasmid DNA serially diluted 10 fold from $5x10^8$ copies/㎕ to $5x10^0$ copies/㎕.

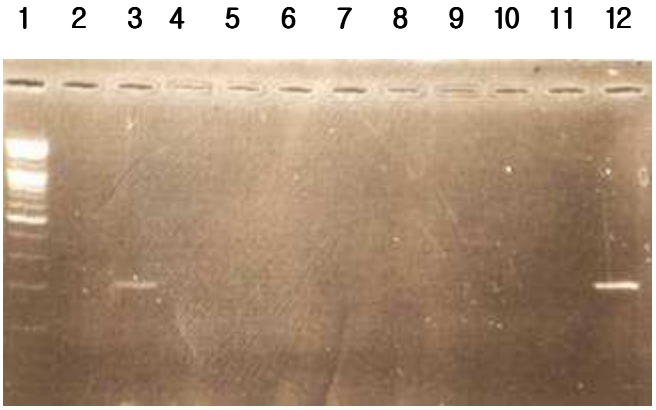


Figure 4. The specificity of external primer in the N-PCR to target *toxR* gene among *vibrio* species. Lane 1: 100bp ladder marker (bioneer), Lane 2: negative- Sterilized distill water. Lane 3: positive control *V. vulnificus* lane 4: *V. alginolyticus*. Lane 5: *V. cholerae*. Lane 6: *V. fluvialis*. Lane 7: *V. furnissii*. Lane 8: *V. hollisae*. Lane 9: *V. mimicus*. Lane 10: *V. parahaemolyticus*. Lane 11: *V. proteolyticus*. Lane 12: *V. vulnificus*.

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논문제목	한글 : 비브리오 패혈증 환자들의 빠르고 정확한 진단을 위한 실시간 중합효소 연쇄반응의 임상적 유용성 영어 : Comparison of Conventional, Nested, Real-time PCR for Rapid and Accurate Diagnosis of Patients with *Vibrio vulnificus*				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

동의여부 : 동의(o) 반대( )

2007 년 12 월 10 일

저작자: 김 형 선 (서명 또는 인)

조선대학교 총장 귀하