

Discriminative PCR of *Bordetella pertussis* from closely related *Bordetella* species using 16S rDNA Gene

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16S rDNA 유전자를 이용한 *Bordetella* 근연종으로부터 *Bordetella pertussis*의 감별 PCR

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Background : Polymerase-chain reaction (PCR) detection is useful to diagnosis of pertussis at initial stage because the growth rate of *Bordetella pertussis* (*B. pertussis*) is relatively slow. Currently, the primer set for the insertion sequence IS481 (BP primer) is used widely for PCR detection of *B. pertussis*. However, the cross-reactivity of BP primer set with *Bordetella holmesii* (*B. holmesii*) was reported recently. Therefore, discrimination of *B. pertussis* and *B. holmesii* is needed in PCR step. For this reason, we developed new primer sets based on 16S rDNA sequence for diagnostic use and estimated the efficiency of these new primer sets.

Materials and Methods : The specific PCR primers were designed from the aligned sequence matrix of 16S rDNA genes of various *Bordetella* species. The specificity of designed primers were estimated using clinically important 4 *Bordetella* species, *B. pertussis*, *B. holmesii*, *Bordetella parapertussis* (*B. parapertussis*) and *Bordetella bronchiseptica* (*B. bronchiseptica*). The sensitivity to *B. pertussis* of designed primers was also estimated and compared with BP primer set.

Results : As the results, the developed new primer set successfully distinguished *B. pertussis* and other *Bordetella* species containing *B. holmesii*. In the sensitivity assay, the detectable limits of 16S-F2/16S-R1 primer set for *B. pertussis* were revealed as 5 pg of genomic DNA and 10^5 cells/mL of cell suspension. In addition to these, identical results between BP with primer and new primer were obtained in clinical samples.

Conclusion : In this study, the specific primer set for *B. pertussis* was developed based on 16S rDNA sequence and this primer set did not show cross-reactivity to *B. holmesii*. In addition to these, the applicability of this primer set to the clinical specimens was also confirmed.

Key Words : *Bordetella pertussis*, *Bordetella holmesii*, 16S rDNA, species-specific PCR primer

INTRODUCTION

Pertussis is one of the respiratory diseases caused by bacterial infection and specifically severe in the infants. The high global incidences of pertussis were reported until 1980's (1,982,384 cases/year)(1) but they were greatly reduced by vaccination campaign, which started

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from 1940. However, local outbreaks of pertussis were reported between 1990–2000 in well-vaccinated countries like USA, Netherlands, Japan, and Australia(2–4). Although there were many efforts to find out the reason of these outbreaks, they were not yet found. Therefore, to prevent the disease from spreading in early stage, the diagnostic process plays an important role.

Usually, the diagnostic gold standard is to confirm the pathogen by culture. However, the growth rate of *Bordetella pertussis*, the pathogen of pertussis, is quite slow. For this reason, the PCR detection is a critical step to determine the result in early phase. Until now, various kinds of diagnostic primer sets were developed. From these primer sets, BP1/2 for IS481 element(5, 6), PTp1/p2 for pertussis toxin promoter region(5, 7, 8) and P1/2/3 for porin gene upstream region(9) are common primer sets used in clinical application. Especially, because of its high sensitivity and specificity, BP primer set is most widely used primer. However, despite of high sensitivity of BP primer, the cross-reactivity of BP primer set to *B. holmesii* was recently reported(10–13).

B. holmesii is one of the species of *Bordetella* Genus and shows highest similarity to *B. pertussis* in 16S rDNA sequence analysis(14). There were few reports about the pathogenicity and infectivity of *B. holmesii* for human, this strain was isolated from the nasopharyngeal specimens of patients with pertussis-like symptoms(14, 15). Moreover, the specific primer for *B. holmesii* was not reported yet and BP primers are currently applied to *B. holmesii* detection(10, 12, 13). Therefore, in diagnostic PCR diagnosis step, discriminative analysis between *B. pertussis* and *B. holmesii* is required.

For this reason, we designed new diagnostic primer sets for 4 major species of *Bordetella* Genus based on 16S rDNA sequence and the efficiency of designed primer sets was estimated.

MATERIALS AND METHODS

1. Bacterial Strains

The reference bacterial strains used in this study were *Bordetella pertussis* (ATCC 9797), *Bordetella holmesii* (ATCC 51541), *Bordetella parapertussis* (ATCC

15237) and *Bordetella bronchiseptica* (ATCC 31124). In addition to these strains, the nasopharyngeal specimens collected from pertussis patients were also used as confirmation test.

2. Preparation of PCR templates

The genomic DNAs of 4 *Bordetella* species were purified from cultured cells and used as PCR templates. For this, all of strains were incubated on Regan–Lowe medium without cephalexin for 5–7 days at 37°C. After incubation, the bacterial cells were collected by scrapping and genomic DNA was isolated using commercial kit according to the manufacturer's instruction (QIAamp DNA Mini Kit, QIAGEN).

For clinical specimens, collected nasopharyngeal aspirates (NPA) were directly used as templates for PCR reaction. Some portion (0.5–1 mL) of the specimens was heated for 5 min in boiling water. Then after centrifugation, 1–2 µL of supernatant was used as PCR template.

3. PCR primers

The 4 PCR primers were designed from 16S rDNA sequences of 4 *Bordetella* species (*B. holmesii*, *B. bronchiseptica*, *B. pertussis* and *B. parapertussis*). The DNA sequence information of 16S rDNA gene was retrieved from NCBI GenBank. These collected nucleotide sequences were aligned using multiple alignment method by MEGA program(16) and the sequence similarity of 16S rDNA gene was calculated by p-distance method at Family level and Genus level(17). From these aligned data matrix, the region containing different nucleotide was analyzed by Sequence Output program and the PCR primers were designed by these differences (Fig. 1). For reference, the BP primers sequence (BP1: 5'-GAT TCA ATA GGT TGT ATG CAT GGT T-3', BP2: 5'-TTC AGG CAC ACA AAC TTG ATG GGC G-3') was cited from the published paper(12).

4. PCR condition

The standard reaction mixture (20 µL) was composed of 2 µL of 10x buffer, 0.5 µL of dNTPs mix (2.5 mM

each), 1 μ L of F-primer (5 pmol/ μ L), 1 μ L of R-primer (5 pmol/L), 0.2 μ L of SP-Taq polymerase (2.5 Unit/ μ L), 1 μ L of template (purified genomic DNA or bacterial suspension) and distilled water was added to make total 20 μ L of reaction volume. For 16S primer sets, dimethylsulfoxide (DMSO) was added to make 2.5% of final concentration.

The temperature condition for new 16S primer sets was 35 cycles of denaturation at 98°C for 10 sec and annealing/extension at 64°C or 66°C for 15 sec according

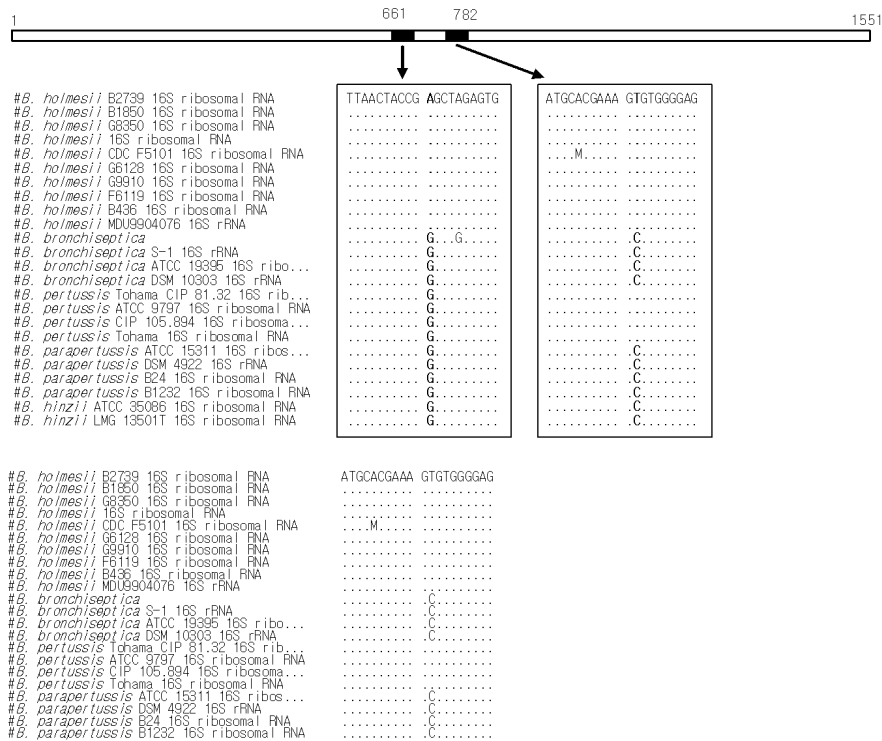
to primer sets. In the case of BP primer sets, 35 cycles of denaturation at 95°C for 5 sec and annealing/extension at 54°C for 10 sec were performed.

RESULTS

1. Cross reactivity of BP primer set with

B. holmesii

The cross reactivity of BP primer to *B. holmesii* and other 2 *Bordetella* species (*B. bronchiseptica* and *B.*



B

Designed PCR primers

16S-F1	5'-AACTGCATTTTAACTACCG-A-3'
16S-F2	5'-AACTGCATTTTAACTACCG-G-3'
16S-R1	5'-ATCCTGTTTGCTCCCCAC-A-3'
16S-R2	5'-ATCCTGTTTGCTCCCCAC-G-3'

PCR specificity

<i>B. holmesii</i>	16S-F1 x 16S-R1
<i>B. pertussis</i>	16S-F2 x 16S-R1
<i>B. bronchiseptica</i> , <i>B. parapertussis</i> , <i>B. hinzii</i>	16S-F2 x 16S-R2

Fig. 1. Design of PCR primers. (A) Multiple alignment of 16S rDNA sequences from *B. holmesii*, *B. bronchiseptica*, *B. pertussis*, *B. parapertussis* and *B. hinzii*. (B) Designed PCR primers sequences and specificity by combination of primers. The 16S rDNA sequences of indicated *Bordetella* strains were retrieved from NCBI GenBank and the collected sequences were aligned by multiple alignment method. From the generated data matrix (A), the variable sites were searched and 4 PCR primers were designed. The specificity for each target species was derived from the combinational sets of 4 primers, as shown in (B).

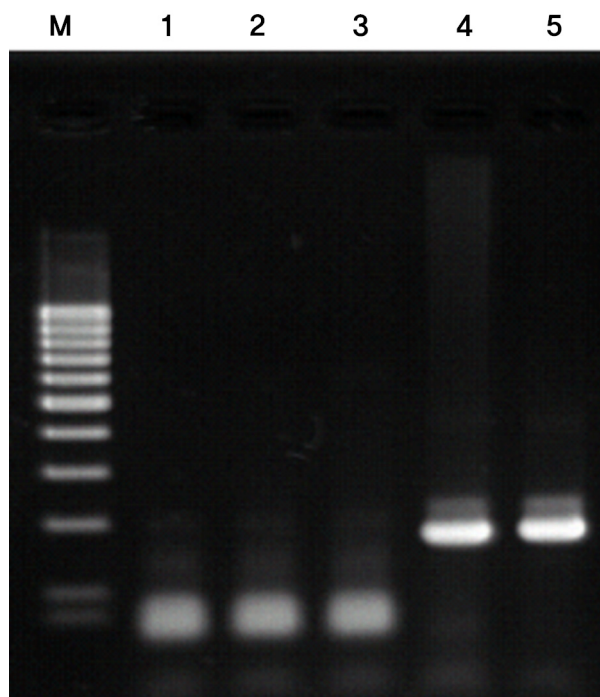


Fig. 2. The cross reactivity of BP primer set between *B. holmesii* and *B. pertussis*. Lane M, Molecular size marker (100 bp ladder); Lane 1, Negative control; Lane 2, *B. parapertussis*; Lane 3, *B. bronchiseptica*; Lane 4, *B. pertussis*; Lane 5, *B. holmesii*. The genomic DNAs of 4 *Bordetella* species were purified with QIAamp DNA Mini Kit and 10 ng of purified genomic DNAs were added to PCR reaction mixture. The temperature condition was 35 cycles of denaturation at 95°C for 5 sec and annealing/extension at 54°C for 10 sec. After reaction, the amplified target band was confirmed by 1.5% agarose gel electrophoresis.

parapertussis) was confirmed in this study. As shown in Fig. 2, positive bands were appeared in both lanes (lane 4 and 5) of *B. pertussis* and *B. holmesii*. However, *B. parapertussis* (lane 2) and *B. bronchiseptica* (lane 3) were not detected by BP primers set. Therefore, the use of BP primers set in PCR diagnosis of pertussis should be considered.

2. Design of PCR primers sets

In this study, we considered the 16S rDNA gene to develop new primer set for PCR-diagnosis of pertussis. For this, the 16S rDNA sequences of 4 major *Bordetella* species (*B. pertussis*, *B. holmesii*, *B. bronchiseptica* and *B. parapertussis*) were collected from NCBI GenBank and aligned by multiple alignment method to find sequence variations. As the result, there were no specific variable regions. This indicates that 16S rDNA

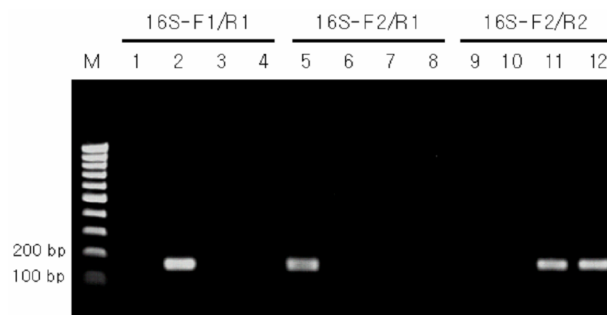


Fig. 3. The specific amplification of 16S rDNA using newly designed 16S primer sets. Lane M, Molecular size marker (100 bp ladder); Lane 1, 5, 9, *B. pertussis*; Lane 2, 6, 10, *B. holmesii*; Lane 3, 7, 11, *B. parapertussis*; Lane 4, 8, 12, *B. bronchiseptica*. The combined primer sets (Fig. 1) were tested using purified genomic DNA of 4 *Bordetella* species (*B. pertussis*, *B. holmesii*, *B. parapertussis* and *B. bronchiseptica*). 5 ng of purified genomic DNAs were used in PCR reaction and 2.5% dimethylsulfoxide (final concentration) was added to reaction mixture. The temperature condition also differently applied to primer sets. For 16S-F1/R1, 35 cycles of denaturation at 98°C for 10 sec and annealing/extension at 64°C for 15 sec were performed. For 16S-F2/R1 and 16S-F2/R2, the temperature condition was 35 cycles of denaturation at 98°C for 10 sec and annealing/ extension at 66°C for 15 sec. After reaction, the amplified target band was confirmed by 1.5% agarose gel electrophoresis.

sequence was highly conserved in *Bordetella* species (98.7% of sequence similarity). However, the two positions of 661 bp and 782 bp in alignment matrix showed specificity according to their species. We selected these two sites as the motif for PCR primer design (Fig. 1) and 4 new primers were designed. As shown in Fig. 1, the designed primers were different in only one nucleotide of their 3'-termini.

3. Specificity of designed 16S PCR primer sets

As mentioned above, the specificity of designed new PCR primer sets was examined to 4 *Bordetella* species. For specific amplification, the combinations of these 4 primers were applied. 16S-F1/16S-R1 set for *B. holmesii*, 16S-F2/16S-R1 set for *B. pertussis*, and 16S-F2/16S-R2 set for other *Bordetella* species (Fig. 1) were used. As the result, the specific amplification of *B. pertussis*, *B. parapertussis* and *B. bronchiseptica* using the combinatorial primers sets was successfully performed. As shown in Fig. 3, the positive PCR bands appeared in only each target species (Lane 2, Lane 5, Lane 11 and Lane 12). Therefore, it was confirmed that the designed PCR primer sets showed the specificity to

each *Bordetella* species.

16S-F2 and 16S-R1 primers were applied for *B. pertussis*. As shown in Fig. 3, the positive band was detected only in *B. pertussis* sample (Lane 5). However, we confirmed that the non-specific band was also detectable in the sample of *B. holmesii* under mild PCR condition. Therefore, stringent PCR condition was required in the use of 16S-F2 and 16S-R1 primers. For this reason, we added dimethylsulfoxide (DMSO) to reaction mixture until 2.5% of final concentration. As the result, 16S-F2/16S-R1 primers set specifically detected *B. pertussis* in this stringent condition.

4. Sensitivity of designed 16S PCR primer sets

The sensitivity of these 2 primer sets (BP and 16S)

for *B. pertussis* was estimated and compared. Under fixed PCR condition, the detection limit of target band was examined using purified genomic DNA. As shown in Fig. 4, when genomic DNA was used, the detection limit was 5 pg/reaction ((A)-lane 2). This estimated value was lower than the result of BP primer set (Figure 4-(B)). BP gene was detectable as low as 10 fg of genomic DNA ((B)-lane 6).

In the case of cell suspension, as shown in Fig. 5, the detection limit of 16S rDNA gene was $>10^5$ cells/mL ((A)-lane 5). This result is not quite different with the result of BP gene. The detectable PCR band for BP gene was also shown from the cell suspension of $>10^4$ cells/mL ((B)-lane 7). Therefore, these results indicated that 16S rDNA gene and BP gene showed similar

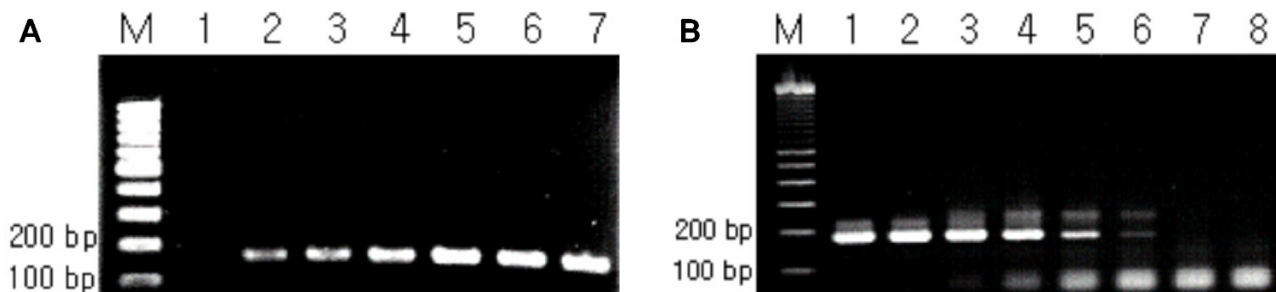


Fig. 4. Comparison of the sensitivity for detection of *B. pertussis* using purified genomic DNA by 16S and BP primer sets. (A) Sensitivity of 16S primer for detection of *B. pertussis*. Lane M, Molecular size marker (100 bp ladder); Lane 1, : 1 pg; Lane 2, 5 pg; Lane 3, 10 pg; Lane 4, 50 pg; Lane 5, 100 pg; Lane 6, 500 pg; Lane 7, 1 ng. (B) Sensitivity of BP primer for detection of *B. pertussis*. Lane M, Molecular size marker (100 bp ladder); Lane 1, 1 ng; Lane 2, 100 pg; Lane 3, 10 pg; Lane 4, 1 pg; Lane 5, 0.1 pg; Lane 6, 0.01 pg; Lane 7, 0.001 pg; Lane 8, Negative control. The sensitivity of 16S and BP primer sets were estimated using various concentration of purified *B. pertussis* genomic DNA. As indicated in this fig, 0.001 pg – 1 ng of purified genomic DNA was tested. After reaction, 10 μ L of reactants were electrophoresed on 1.5% agarose gel and the amplified band was observed under UV illuminator.

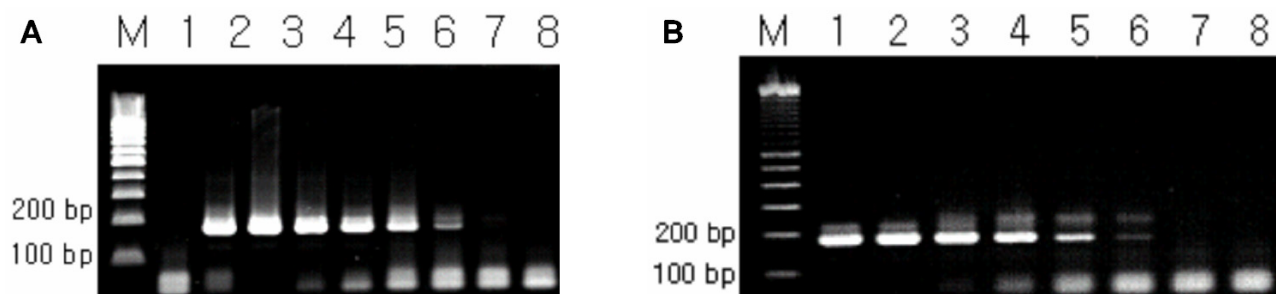


Fig. 5. Comparison of the sensitivity for detection of *B. pertussis* using cell suspension by 16S and BP primer sets. (A) Sensitivity of 16S primer for detection of *B. pertussis*. Lane M, Molecular size marker (100 bp ladder); Lane 1, 10^{10} cells/mL; Lane 2, 10^9 cells/mL; Lane 3, 10^8 cells/mL; Lane 4, 10^7 cells/mL; Lane 5, 10^6 cells/mL; Lane 6, 10^5 cells/mL; Lane 7, 10^4 cells/mL. (B) Sensitivity of BP primer for detection of *B. pertussis*. Lane M, Molecular size marker (100 bp ladder); Lane 1, Negative control; Lane 2, 10^4 cells/mL; Lane 3, 10^5 cells/mL; Lane 4, 10^6 cells/mL; Lane 5, 10^7 cells/mL; Lane 6, 10^8 cells/mL; Lane 7, 10^9 cells/mL; Lane 8, 10^{10} cells/mL. sensitivity of 16S and BP primer sets were estimated using various concentrations of cell suspensions. As indicated in this fig, 10^4 – 10^{10} cells/mL of cell suspensions were heated in boiled water for 5 min and centrifuged at 15,000 rpm for 5 min. After centrifugation, 1 μ L of supernatant was added to PCR reaction mixture as template. The amplified PCR band was observed under UV illuminator after electrophoresis on 1.5% agarose gel.

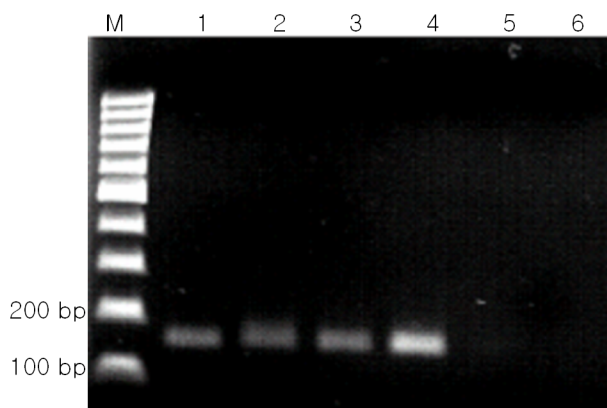


Fig. 6. Application of 16S PCR primer (16S-F2/R1) to clinical specimens. Lane M, Molecular size marker (100 bp ladder); Lane 1-4, Pertussis positive specimens; Lane 5-6, Pertussis negative specimens. The clinical specimens were nasopharyngeal aspirates collected from pertussis-like patients. 0.5 mL of the collected specimens was heated in boiled water for 5 min and centrifuged at 15,000 rpm for 5 min. After centrifugation, 1 μ L of supernatant was added to PCR reaction mixture as template. The amplified PCR band was observed under UV illuminator after electrophoresis on 1.5% agarose gel. All positive and negative specimens were already confirmed by BP primer set.

detection range under practical diagnostic condition.

5. Application to clinical specimens

Applicability of new 16S primers to clinical specimens were estimated. The specimens were the nasopharyngeal aspirates collected from the suspected patients pertussis in 2006. These specimens were already confirmed positive for *B. pertussis* by BP primer PCR and culture. As shown in Fig. 6, all positive specimens were also confirmed positive by 16S PCR primer (Lane 1-4 in Fig. 6). Therefore, the newly developed 16S PCR primer sets were also applicable to the clinical specimens.

DISCUSSION

According to the WHO guide for *Bordetella* species diagnosis(1), the cross reactivity of BP primer to *B. holmesii* was already described. However, BP primer set is still used in most diagnostic laboratories because of its high sensitivity and specificity. Moreover, uncertainty of *B. holmesii* for human infection and carriage was also the reason. However, pathogenicity and carriage activity of *B. holmesii* for human were also proved recently(14,15). For these reasons, there is need to de-

velop new primer sets for discrimination of these two *Bordetella* species at PCR diagnostic step.

For this, we considered the 16S rDNA gene. As already known, 16S rDNA gene is most frequently used taxonomic marker in all Bacteria and Archaea(18). Firstly, we confirmed the sequence similarity of 16S rDNA gene to estimate their usefulness for *B. pertussis*. The group mean similarity of *Bordetella* Genus was 98.7% and it was higher than overall mean similarity of Alcaligenaceae Family (93.6%) containing *Bordetella* Genus. This indicates that 16S rDNA sequence is highly conserved to each Genus of Alcaligenaceae Family and this gene is a useful taxonomic marker to discriminate each Genus of Alcaligenaceae Family.

Currently, 9 *Bordetella* species have been reported in NCBI Taxonomy database (*B. ansorpii*, *B. avium*, *B. bronchiseptica*, *B. hinzii*, *B. holmesii*, *B. parapertussis*, *B. petrii*, *B. trematum*, *B. pertussis*). Among these species, the detectable species from human respiratory specimens were *B. bronchiseptica*, *B. hinzii*, *B. holmesii*, *B. parapertussis* and *B. pertussis*(19-21). Because *B. hinzii* was reported as rarely isolated species from immunocompromised(20) and cystic fibrosis patients(22), we did not considered it as major *Bordetella* species isolated from normal respiratory specimens. However, discrimination of *B. pertussis* and *B. hinzii* would be also possible by using new 16S primer sets (Fig. 1).

Although *B. ansorpii* and *B. trematum* were also isolated from human originated specimens(23,24), they did not relate with human respiratory infection. Therefore, 4 *Bordetella* species (*B. ansorpii*, *B. petrii*, *B. trematum*, *B. avium*) were excluded from diagnostic targets in this study.

Especially, we also confirmed the specific primer set (16S-F1 and 16S-R1) for *B. holmesii* in this study (Fig. 3, Lane (2)). Until now, biochemical tests and cellular fatty acids analysis were mainly performed as identification tests for *B. holmesii*(14). In the molecular methods, DNA hybridization technique and 16S rDNA sequence analysis were performed(21,25). However, there is no method for rapid identification of *B. holmesii*. IS481 based BP primer set for *B. pertussis* is used in *B. holmesii* detection(10,12,13). Therefore, there

is need to develop PCR primer of *B. holmesii* for rapid identification. Although recA gene was recently reported as target gene for specific PCR of *B. holmesii*(26), other specific primer or target gene were not reported. As the result, the confirmed PCR primer set for *B. holmesii* in this report will be applicable to clinical diagnostic tests.

As shown in Fig. 4, 16S primer set showed 100 times lower sensitivity than BP primer set. It is attributed in the copy numbers of these two related genes. The BP primer was originated in IS481 repetitive sequence. It was known that there were approximately 80-100 copies of IS481 region in a bacterial cell(12,27). However, for the 16S rDNA gene in the case of Tohama I strain, 3 copies per genome were present(28). Therefore, the major reason of reduced sensitivity of 16S primer was inferred as this different copy number.

As the conclusion, the specific PCR primers sets for *B. pertussis*, *B. holmesii*, *B. parapertussis* and *B. bronchiseptica* were developed based on 16S rDNA gene and their specific amplification of target strain was successfully confirmed in this study. Although the sensitivity was relatively low as compared to BP primers set, these new primer sets are applicable to clinical specimens and especially useful to discriminate *B. holmesii* and *B. pertussis*.

요 약

목 적 : 백일해의 원인균인 *B. pertussis*는 성장속도가 늦기 때문에 PCR 검출방법은 백일해 진단의 초기단계에서 유용하다. 현재 BP primer가 PCR 검출에 광범위하게 사용되나 BP primer는 최근에 *B. holmesii*와의 교차반응성이 보고되었다. 따라서 PCR 단계에서 *B. pertussis*와 *B. holmesii*의 감별이 필요하다. 이러한 이유로 본 논문에서는 16S rDNA sequence를 기반으로 하는 진단용 primer를 새로 고안하였고, 그 효용성을 측정하였다.

재료 및 방법 : 특이적 PCR primer들은 다양한 *Bordetella* 속의 16S rDNA 유전자들의 정렬된 서열 집합체로부터 고안되었다. 고안된 primer의 특이도는 임상적으로 중요한 4개 *Bordetella* 종 (*B. pertussis*, *B. holmesii*, *B. parapertussis*, *B. bronchiseptica*)을 사용하여 평가하였다. 민감도 또한 평가하였고 그 결과를 BP primer와 비교하였다.

결 과 : 결과적으로, 새롭게 개발된 primer는 *B. holmesii*를 포함하는 다른 *Bordetella* 종들로부터 *B. pertussis*를 성공적으로 감별하였다. 민감도분석에서는 *B. pertussis*에 대한 16S-F2/16S-R1 primer의 검출한계는 5 pg의 genomic DNA와 10^5 cells/mL의 세포부유액까지 였다. 또한 임상검체에 대해서 BP primer set와 동일한 결과가 확인되었다.

결 론 : 본 연구에서 오직 *B. pertussis*에 대해 특이적인 PCR primer를 16S rDNA를 기반으로 하여 개발하였고, 이는 *B. holmesii*와의 교차반응성을 나타내지 않았다. 또한 본 연구에서 새롭게 개발된 primer의 임상검체에 대한 적용성도 확인되었다.

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