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Research Article
Identification of HACEK Group Bacteria from Blood Samples of Patients with Infective Endocarditis by PCR-RFLP of the 16s rRNA Gene

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Abstract

Background and Objective: Identification of specific HACEK bacteria that inhabit the human oral cavity and cause infective endocarditis (IE) is difficult because conventional culture methods produce inconclusive results in cases of fastidious and slow-growing organisms. Although the study of a rapid and sensitive identification method for identification of HACEK bacteria based on polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene have been reported, the procedures were not revealed. Herein, in this study, the detail of the method's conditions and procedures were described and assessed its usefulness in analyzing clinical samples using eight clinical isolates from patients with IE. **Materials and Methods:** The bacteria were analyzed by the 16S rRNA gene PCR-RFLP method using *Hin*fI and *Msp*I. The isolate from patients were further subjected to species-specific identification with biochemical identification kits. **Results:** Seven isolates were identified as *Streptococcus intermedius* (2×), *Abiotrophia defective* (2×), *Granulicatella adiacens*, *Streptococcus salivarius* and *Staphylococcus epidermidis* using a biochemical identification kit (eighth was unidentified). The HACEK bacteria and the isolates were further subjected to PCR-RFLP analysis of the 16S rRNA gene. Typical restriction patterns were obtained by combination digestion with *Hin*fI and *Msp*I. The patterns of the unidentified isolate were same as those of *C. hominis*, thereby confirming the identification of the causative pathogen. The PCR-RFLP results of the other clinical isolates were identical to those with the identification kits. **Conclusion:** The PCR-RFLP analysis of the 16S rRNA gene is applicable for definitive diagnosis of HACEK group, fastidious growing bacteria and in samples with unidentifiable bacteria using conventional biochemical identification kits.

Key words: HACEK group bacteria, *Cardiobacterium hominis*, infective endocarditis, 16S rRNA gene, PCR-RFLP, identification, diagnosis

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The HACEK (*Haemophilus* species, *Aggregatibacter* species, *Cardiobacterium hominis*, *Eikenella corrodens* and *Kingella* species) group of bacteria consists of fastidious Gram-negative coccobacilli that inhabit the human oral cavity and have been identified as the causative pathogens of infective endocarditis (IE)[1]. Although the symptoms of IE caused by any member of the HACEK group are similar (i.e., fever, splenomegaly, embolic phenomena and a new or changing murmur), susceptibility to antibiotics varies among the different species[2-4]. Therefore, it is important to identify the causative organism to arrive to an accurate diagnosis and chose an appropriate therapy. However, identification of specific HACEK bacterium is known to be rather difficult and the results of conventional culture methods are occasionally inconclusive because the organisms are fastidious and slow-growing with closely related phenotypic characteristics[5,6].

Data of the 16S rRNA gene sequences of numerous bacteria have been recently accumulated to identify pathogens in clinical samples[7-10]. Polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) analysis based on the 16S rRNA gene is used to identify suspected bacterial pathogens whose specific genes have not yet been cloned[11,12]. Therefore, the PCR-RFLP method may also be beneficial to differentiate closely related bacterial species without the need of species-specific primers or DNA sequencing[13-16]. Although the review article[17] of a rapid and sensitive identification method based on PCR-RFLP analysis of the 16S rRNA gene for the identification of HACEK group bacteria were reported previously, the experimental conditions and procedures were not described in detail in the previous article. It may be difficult for other researchers to accurately understand and interpret the results.

This study was the first report described in detail for identification method of PCR-RFLP analysis of the 16S rRNA gene for HACEK group bacteria. Therefore, the present study enables other researchers to perform the procedure of HACEK group bacteria identification. In addition, the effectiveness of PCR-RFLP of the 16S rRNA gene in the analysis of blood samples from patients with IE was investigated. In this regard, the PCR-RFLP results from clinical isolates including fastidious slow growing bacteria were identical to those from identification kits. Furthermore, the PCR-RFLP patterns of the isolates that could not be confirmed with biochemical identification kits were identical to those of *C. hominis* of the HACEK group bacteria and the 16S rDNA sequences of the isolates matched that of *C. hominis*. Therefore, the present method could be applicable for the identification of clinical isolates, including difficult to identify by biochemical identification, from IE patients.

MATERIALS AND METHODS

This study project was conducted at Iwate Medical University (Shiwagun, Japan) during the period 2003-2010.

Bacterial strains and culture conditions: The following laboratory strains were used for analysis: *A. aphrophilus* (formerly *H. aphrophilus*), ATCC 33389^T; *A. actinomycetemcomitans*, ATCC 33384^T; *C. hominis*, ATCC 12826^T; *E. corrodens*, ATCC 23834^T and *K. kingae*, ATCC 23330^T. Eight clinical strains previously isolated from blood samples collected from patients with IE were also used with the consent of the Ethics Committee of the Iwate Medical University (approval No. 01283). The laboratory HACEK group bacteria and the clinical isolates were cultured and maintained anaerobically on HK agar (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan) plates supplemented with or without 5% sheep blood.

Biochemical tests for identification of the isolates: The BD BBLCRYSTAL (Becton Dickinson and Company, Franklin Lakes, NJ, USA) and RapID NH (Remel Inc., Norcross, GA, USA) systems were used for conventional identification of the bacterial strains.

PCR-RFLP: The DNA from the HACEK bacteria and clinical isolates was extracted as described previously[12]. The PCR was performed with the bacterial genomic DNA using primer pair (sense) 5′-AGA GTT GAT CAT GGC TCA G-3′ and (antisense) 5′-AAG TCG TAA CAA GGT AAC C-3′ corresponding to the *Escherichia coli* 16S rRNA gene. Each reaction was performed using a thermal cycler (Takara Bio Inc., Shiga, Japan) with the following cycling profile: A DNA denaturation step at 94°C for 120 sec followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 48°C for 30 sec and extension at 72°C for 30 sec. The purified PCR products were then digested with 4 U of either *Hin*fI (New England Biolabs, Japan Inc., Tokyo, Japan) or *Msp*I (New England Biolabs) for 1.5 h at 37°C, then separated on a 1.8% agarose gel and photographed under UV light.

DNA sequence data: The GenBank and DNA Data Bank of Japan accession numbers of the 16S rRNA genes used in this study were as follows: *A.* (H.) *aphrophilus*, M75041; *A. actinomycetemcomitans*, M75039; *C. hominis*, M35014; *E. corrodens*, M22512; *K. kingae*, M22517; *Streptococcus intermedius*, AF104671; *Streptococcus salivarius*, X58320; *Abiotrophia defectiva*, D50541; *Granulicatella adiacens*, D50540 and *Staphylococcus epidermidis*, L37605. The complete 16S rRNA gene sequences of these bacteria were processed using the Genetyx multialignment software program (Genetyx Corp., Tokyo, Japan).

RESULTS

Identification of bacteria from clinical isolates using biochemical identification kits: Seven strains (from No. 2-8 in Table 1) from eight samples collected from patients with IE were identified using biochemical identification kits as *Streptococcus intermedius* (two samples), *Abiotrophia defective* (two samples), *Granulicatella adiacens* (one sample), *Streptococcus salivarius* (one sample) and *Staphylococcus epidermidis* (one sample), while one isolate (No. 1 in Table 1) could not be identified using the kit.

Identification of HACEK and the unidentified isolate by PCR-RFLP analysis of the 16S rRNA gene: The strain that could not be confirmed using the biochemical identification kit was further subjected to PCR-RFLP analysis of the 16S rRNA genes. The 16S rRNA genes from all HACEK group bacteria and the unidentified isolate by biochemical tests were successfully amplified as demonstrated by the approximate 1,500 bp lengths of the PCR products (Fig. 1a). Typical restriction patterns following digestion of the PCR products with *Hin*fI and *Msp*I are shown in Fig. 1b and c, respectively. The estimated sizes of the fragment and the deduced sizes of the restriction fragments generated by digestion of the PCR products with the *Hin*fI and *Msp*I restriction enzymes of the

Table 1: Identification of the eight clinical isolates from endocarditis patients using biochemical tests

No. of Isolates	Identification
1	Not identified
2	*Streptococcusintermedius*
3	*Streptococcus intermedius*
4	*Abiotrophia defectiva*
5	*Abiotrophia defectiva*
6	*Granulicatella adiacens*
7	*Streptococcus salivarius*
8	*Staphylococcus epidermidis*

RapID NH or BD CRYSTAL was used for identification of the bacterial strains

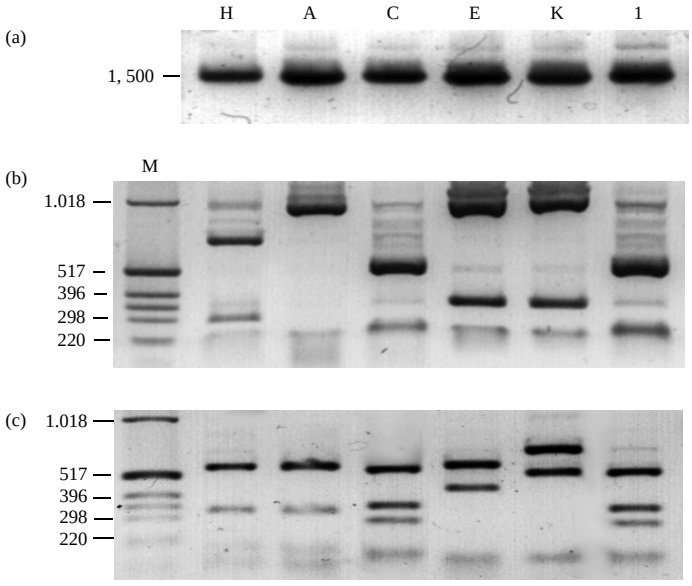

Fig. 1(a-c): PCR-RFLP analysis of 16S rRNA genes. (a) HACEK group bacterial 16S rRNA genes were amplified from purified genomic bacterial DNA and a clinical isolate. The PCR products were separated by electrophoresis on a 0.9% agarose gel and purified. Then, the aliquots of the purified PCR products were digested with 4 U of (b) *Hin*fI and (c) *Msp*I. Lane H: *A.* (H.) *aphrophilus* (ATCC 33389), Lane A: *A. actinomycetemcomitans* (ATCC 33384), Lane C: *C. hominis* (ATCC 12826), Lane E: *E. corrodens* (ATCC 23834), Lane K: *K. kingae* (ATCC 23330), Lane 1: Clinical isolate unidentified by biochemical identification kit, M (marker): 1 kb ladder. Numbers to the left are in base pairs

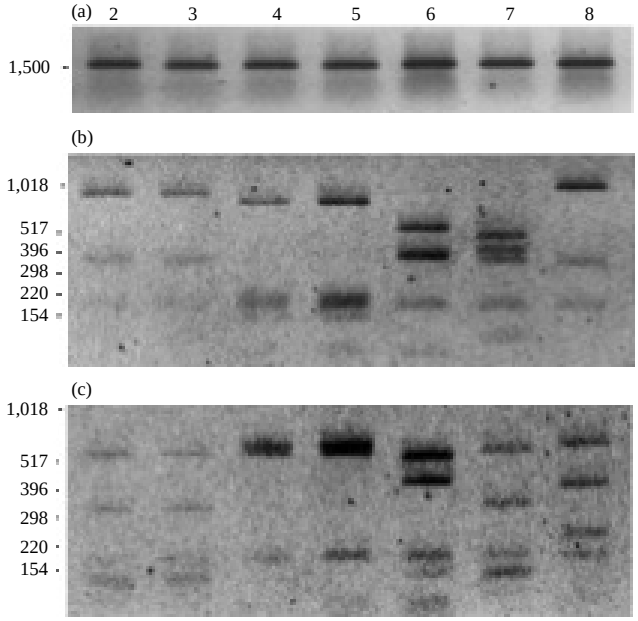

Fig. 2(a-c): PCR-RFLP analysis of clinical isolates. (a) Purified genomic DNA from isolates was used to amplify 16S rRNA genes. The PCR products were separated by electrophoresis on a 0.9% agarose gel and purified. Then, the aliquots of the purified PCR products were digested with 4 U of (b) *Hin*fI and (c) *Msp*I. Lanes 2-8: Clinical isolates from blood samples of IE patients. Numbers to the left are in base pairs

Table 2: Estimated and deduced sizes of DNA fragment of the 16S rRNA gene PCR products cleaved with *Hin*fI and *Msp*I

	Estimated sizes cleaved with		Deduced sizes cleaved with	
Strain	*Hin*fI (bp)	*Msp*I (bp)	*Hin*fI (bp)	*Msp*I (bp)
A. (*H.*). *aphrophilus* ATCC 33389	660, 280, 160	550, 310, 170	663, 275, 155, 121, 120[a]	545, 309, 168, 130, 127, 109
A. actinomycetemcomitans ATCC 33384	940, 210, 130	550, 320, 210,170	939, 209, 127, 120	546, 315, 211, 169, 126, 110
C. hominis ATCC 12826	510, 490, 220	490, 320, 250,140	508, 491, 208, 153, 109	490, 305, 242, 130, 124 110
E. corodens ATCC 23834	1000, 340	550, 410,130	1000, 337	547, 408, 130, 126, 110
K. kingae ATCC 23330	1000, 340	660, 330,170	1000, 327	657, 324, 169, 130, 124
Isolate No. 1	510, 490, 220	490, 320, 250, 140		

[a]Deduced sizes of fragments of <99 bp are not provided

HACEK bacteria (at least 100 bp long) are listed in Table 2. The estimated sizes of the fragments of HACEK group bacteria were nearly the same as the deduced sizes. The estimated sizes of the DNA fragments of the clinical isolate No. 1 unidentified using the biochemical test were 510, 490 and 220 kb with *Hin*fI and 490, 320, 250 and 140 kb with *Msp*I. The sizes were almost identical to those of *C. hominis* (Table 2) and the isolate was identified using sequence analysis of the 16S rRNA gene (data not shown; GenBank accession No. M3514). Consequently, the isolate was identified by PCR-RFLP of the 16S rRNA gene as *C. hominis*.

Identification of isolates by PCR-RFLP analysis of the 16S rRNA gene: The results of the 16S rRNA gene PCR-RFLP analyses of the seven clinical isolates (from No. 2-8) and the estimated sizes of the fragments are shown in Fig. 2 and Table 3. Based on the deduced sizes of the restriction fragments (Table 4), all seven isolates were identified by the estimated sizes of the DNA fragments (Table 3). Namely, the estimated sizes of the clinical isolates No. 2 and 3 were 880, 350 and 180 kb with *Hin*fI and 560, 320, 160 and 130 kb with *Msp*I. The sizes were almost identical to those of *S. intermedius* (Table 4). And those of sizes of No. 4 and 5 were 800, 200 and 170 kb with *Hin*fI and 600, 580 and 170 kb with *Msp*I. The sizes were almost identical to those of *A. defective* (Table 4). Furthermore, those of sizes of No. 6 were 550, 400 and 200 kb with *Hin*fI and 530, 420, 180 and 140 kb with *Msp*I. The sizes were almost identical to those of *G. adiacens* (Table 4). In addition, those of sizes of No. 7 were 500, 400, 350 and 190 kb with *Hin*fI and 560, 320, 210 and 160 kb with *Msp*I. The sizes were almost identical to those of *S. salivarius* (Table 4). Further, the sizes of No. 8 were

Table 3: Estimated sizes of DNA fragments of the 16S rRNA gene PCR products cleaved with *Hinf*I and *Msp*I for seven clinical isolates from endocarditis

	Estimated sizes cleaved with		
Isolate No.	*Hinf*I (bp)	*Msp*I (bp)	Putative pathogen estimated by the restriction profile
2	880, 350, 180	560, 320, 160, 130	*Streptococcus intermedius*
3	880, 350, 180	560, 320, 160, 130	*Streptococcus intermedius*
4	800, 200, 170	600, 580, 170	*Abiotrophia defectiva*
5	800, 200, 170	600, 580, 170	*Abiotrophia defectiva*
6	550, 400, 200	530, 420, 180, 140	*Granulicatella adiacens*
7	500, 400, 350, 190	560, 320, 210, 160	*Streptococcus salivarius*
8	1000, 350, 190	620, 390, 230, 170	*Staphylococcus epidermidis*

Table 4: Deduced sizes of DNA fragments of the 16S rRNA gene PCR products cleaved with *Hinf*I and *Msp*I the pathogens associated with infective endocarditis

Strain	Deduced sizes cleaved with *Hinf*I (bp)	Deduced sizes cleaved with *Msp*I (bp)
Streptococcus intermedius ATCC27335	891, 344, 172[a]	563, 317, 163, 127, 125, 120
Streptococcus salivarius ATCC7073^T	486, 412, 336, 172	561, 316, 211, 163, 125, 113
Abiotrophia defectiva ATCC41976^T	783, 185, 173, 172, 133	606, 564, 163, 111
Granulicatella adiacens ATCC41975^T	545, 362, 225, 163, 146	537, 407, 164, 116
Staphylococcus epidermidis ATCC14990	978, 337, 172	608, 388, 211, 156

[a]Deduced sizes of fragments of <99 bp are not provided

1,000, 350 and 190 kb with *Hinf*I and 620, 390, 230 and 170 kb with *Msp*I. The sizes were almost identical to those of *S. epidermidis* (Table 4). The PCR-RFLP results from clinical isolates were identical to those from the identification kits.

DISCUSSION

This study described in detail PCR-RFLP analysis of the 16S rRNA gene for the identification of HACEK group as well as fastidious and slow growing bacteria that are frequently isolated from blood samples of patients with IE[18-22]. Furthermore, the method has revealed that it could be remarkably applicable for identification of clinical isolate from IE patients including difficult to identify by biochemical identification. Although, the review article[17] that a method for the identification of HACEK group bacteria by 16S rRNA gene PCR-RFLP was previously reported, the description of the method in the article was insufficient to allow others to perform the experiments, complicating the interpretation of the results. As a result, in this paper, the conditions for the PCR of the 16S rRNA gene, procedures for RFLP analysis and results showing the digest patterns are described here in fine details to make the PCR-RFLP method and its advantages clearly understandable. The results in this study have showed that typical restriction patterns produced by combined digestion with *Hinf*I and *Msp*I could identify each species of HACEK bacteria as same as previous article and eight clinical isolates based on the estimated sizes of the restriction fragments de novo. Furthermore, the results from the seven of the eight clinical isolates were identical to those obtained using the conventional biochemical identification kits, suggesting that the PCR-RFLP method is suitable for the identification of bacterial pathogens in clinical samples collected from patients with IE. Notably, the digestion pattern of the one strain that could not be identified using a biochemical identification kit was the same as that for *C. hominis*, suggesting that *C. hominis* can exclusively be identified by 16S rRNA gene PCR-RFLP.

The Gram-positive cocci, viridans streptococci and staphylococci, are the most frequently identified pathogens in IE (up to 50% of cases)[23,24]. These organisms are usually cultivable and thus can be easily identified by conventional identification methods, leading to a relatively straightforward diagnosis. However, IE is suspected in some patients even with negative blood culture analysis results of the HACEK group, *Abiotrophia* and *Granulicatella* or unknown even with no prior use of antimicrobial agents[25-27]. In such cases, the diagnosis and specific pharmaceutical therapy could be delayed. Accordingly, the etiologic agents of IE should be identified as soon as possible. The PCR-RFLP analysis of the 16S rRNA gene is rapid and highly sensitive and can applied for the identification of clinical isolates. In addition, the PCR-RFLP method, in this study, is a remarkable tool that could identify from clinical blood isolates not only strains unidentifiable by biochemical identification kits but also nutritional variants and fastidious growing streptococci such as *A. defectiva* and *G. adiacens*. These bacteria are presumed to be responsible for many incidences of "culture-negative" IE[28,29]. While there are many PCR-RFLP methods that target bacteria that are easy to culture[30-32], the proposed PCR-RFLP method is highly sensitive and could also be suitable in identifying bacterial pathogens in clinical samples from patients with "culture-negative" IE.

Time-of-flight mass spectrometry and 16S rRNA sequencing are additional methods for HACEK group bacteria identification, although these methods are limited by cost and equipment availability[33,34]. Furthermore, direct PCR detection of bacteria from blood samples requires primers for each species[35-37]. The16S rRNA gene PCR-RFLP analysis in this study has the added advantage of being able to identify Gram-negative and fastidious microorganisms whose species-specific genes have not yet been cloned because it can be performed without species-specific primers.

Taken together, the present PCR-RFLP method, but not previous review, with the restriction enzymes *Hin*fI and *Msp*I described in detail enable researchers to perform this procedure of HACEK group bacteria identification. And it applicable in the definitive diagnostic identification of HACEK group and fastidious slow-growing streptococci in of blood samples from patients with IE, the procedure also facilitates the identification of pathogenic bacteria which cannot be differentiated via conventional biochemical identification kits. Therefore, the method proposed in this study provides useful information for immediate diagnosis and treatment of IE.

CONCLUSION

The PCR-RFLP of the 16S rRNA gene is a rapid and sensitive identification method that does not require species-specific primers or DNA sequencing analysis. The method revealed typical restriction patterns of HACEK group bacteria as well as clinical isolates from IE with the enzymes *Hin*fI and *Msp*I. In addition, the method was used to accurately identify *C. hominis* from the isolate that could not be identified using a biochemical identification kit. Therefore, this method could be extensively applied for identification of bacteria that are difficult to identify using conventional biochemical identification kits or fastidious slow-growing streptococci.

SIGNIFICANCE STATEMENT

This study describes in detail how the 16S rRNA gene PCR-RFLP method can help to identify HACEK and fastidious slow-growing bacteria in clinical isolates from IE patients. Further, the method is widely practical for isolates that are difficult to analyze with conventional biochemical kits. The method provides beneficial information to the clinician or clinical technologist for making immediate diagnostic or definite therapeutic decisions for IE. In addition, the findings in the data collected in this evaluation of the method could be applicable to molecular epidemiology of IE.

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