Scientific Research Publishing

Open Journal of Stomatology, 2022, 12, 183-195
https://www.scirp.org/journal/ojst
ISSN Online: 2160-8717
ISSN Print: 2160-8709

One Step Multiplex PCR for Identifications at Subspecies Level of *Fusobacterium nucleatum* and *Fusobacterium necrophorum*

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How to cite this paper: Umezawa, K., Hayashi, S., Tsuzukibashi, O., Fukatsu, A., Uchibori, S., Fuchigami, M., Komine, C., Asano, T., Wakami, M., Kobayashi, T., Fukumoto, M. and Nomoto, T. (2022) One Step Multiplex PCR for Identifications at Subspecies Level of *Fusobacterium nucleatum* and *Fusobacterium necrophorum*. *Open Journal of Stomatology*, **12**, 183-195.
https://doi.org/10.4236/ojst.2022.126018

Received: May 19, 2022
Accepted: June 27, 2022
Published: June 30, 2022

Abstract

Purpose: *Fusobacterium nucleatum* is an opportunistic pathogen involved in periodontal diseases, extraoral infections, and colorectal cancer. *Fusobacterium necrophorum* causes a variety of necrotic infections. *F. nucleatum* and *F. necrophorum* are classified into five and two subspecies, respectively. Conventional identification methods were technically hard to distinguish each subspecies of two *Fusobacterium* species accurately. The purpose of the present study was to design primers to identify two medically important *Fusobacterium* species at the subspecies level, using one-step multiplex PCR. **Methods:** Polymerase chain reaction (PCR) primers were designed based on partial sequences of the 16S ribosomal RNA (16S rRNA) gene, RNA polymerase B (*rpoB*) gene, and DNA gyrase subunit B (*gyrB*) of each subspecies of *F. nucleatum* and *F. necrophorum*. **Results:** These primers were able to distinguish each subspecies of *F. nucleatum* and *F. necrophorum* and did not display cross-reactivity with representative *Fusobacterium* species other than *F. nucleatum* and *F. necrophorum*. **Conclusion:** Our developed one-step multiplex PCR method is accurate, specific, cost-effective, time-saving, and worked without requiring DNA extraction.

Keywords

Fusobacterium nucleatum, *Fusobacterium necrophorum*, One-Step Multiplex PCR

1. Introduction

Although the genus *Fusobacterium* was already described in 1922, a lot of new species have been found recently. This microorganism has traditionally included a variety of gram-negative bacilli that either had pointed or fusiform ends or that produced major amounts of butyric acid as an end product of metabolism [1] [2]. At present, the genus *Fusobacterium* comprises 33 species and 7 subspecies (http://www.bacterio.net/fusobacterium.html). This microorganism is gram-negative, non-spore forming, non-motile, pleomorphic, and obligate anaerobic bacilli, which inhabit the human oral cavity, oropharynx, upper respiratory, gastrointestinal, and female genitourinary tracts as part of the normal flora [3]. Nine species including *Fusobacterium nucleatum*, *Fusobacterium necrophorum*, *Fusobacterium ulcerans*, *Fusobacterium gonidiaformans*, *Fusobacterium mortiferum*, *Fusobacterium naviforme*, *Fusobacterium necrogenes*, *Fusobacterium russii*, and *Fusobacterium varium* cause human infections of the head and neck, chest, lung, liver, and abdomen [4]. The detection of *Fusobacterium* species in clinical specimens is important, as it may affect the prognosis and patient management. However, the identification by conventional biochemical methods can be difficult, since there are no characteristic biochemical properties to distinguish each subspecies.

F. nucleatum is the species most frequently isolated from humans. This microorganism is an opportunistic pathogen involved in various forms of periodontal diseases, extraoral infections, and colorectal cancer [5] [6], and is a highly heterogeneous species. Currently, *F. nucleatum* is classified into five subspecies, *i.e.*, subsp. *nucleatum*, subsp. *polymorphum*, subsp. *vincentii*, subsp. *fusiforme* and *subsp. animalis*. Five subspecies have been described on the basis of electrophoretic patterns of whole-cell proteins, in addition to DNA homology, and on the basis of the electrophoretic mobility of two enzymes and DNA homology. Various other techniques have been used to confirm or challenge the validity of the type strains. Morris *et al.* [7] grouped these subspecies by electrophoresis of 21 alloenzymes and found overlapping identifications and entirely different groupings. On the other hand, an arbitrarily primed PCR (AP-PCR) technique, which uses two primers, has demonstrated unique profiles for five subspecies [8]. Conrads *et al.* [9] examined the 16S-23S internal transcribed spacer regions (ITS) sequences of all currently defined *Fusobacterium* spp., along with several related taxons, to determine the inter- and intraspecies and subspecies relationships. Five subspecies of *F. nucleatum* were differentiated both from each other and as a category from the other closely related species. Different subspecies may vary in pathogenesis relating to different levels of disease activity [10] [11] [12]. *F. nucleatum* subsp. *nucleatum* is almost isolated from periodontal disease sites, whereas *F. nucleatum* subsp. *fusiforme* and *vincentii* are often isolated from healthy sites as normal flora [13]. *F. nucleatum* subsp. *animalis* and *polymorphum* are associated with pregnancy complications [11], and *F. nucleatum* subsp. *animalis* is also associated with inflammatory bowel disease [14]. Until now, molecular technologies have been the most effective and widely accepted tools

for subspecies identification [15] [16] [17]. At the subspecies level of *F. nucleatum*, the sequence divergences of 16S rRNA genes were only 0.6% to 1.9%, so full-length sequencing of 16S rRNA was desirable [16].

F. necrophorum is a normal inhabitant of the gastrointestinal, respiratory and genitourinary tracts of animals and humans [18] [19], and is associated with a variety of necrotic infections [20] [21] [22]. In humans, *F. necrophorum* primarily causes acute pharyngitis, thrombophlebitis and abscessation of the internal jugular vein, termed Lemierre's syndrome [18] [21] [23] [24]. *F. necrophorum* has two major subspecies, *i.e.*, subsp. *necrophorum* and subsp. *funduliforme* [25].

At present, *F. nucleatum* and *F. necrophorum* can be identified at the subspecies level by the sequence analysis of several target genes [26] [27], matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) [28], an AP-PCR method [8], and a PCR based typing assay [29]. However, because these methods are laborious, expensive, and time-consuming for use in the detection or discrimination of the clinical isolates of *F. nucleatum* and *F. necrophorum* at the subspecies level, epidemiological studies on the relationship between the subspecies of these microorganisms and various diseases are limited. Thus, a simple and more reliable assay for identifying *F. nucleatum* and *F. necrophorum* at the subspecies level is required. The purpose of the present study was to design primers to identify two medically important *Fusobacterium* species, *i.e.*, *F. nucleatum* and *F. necrophorum*, at the subspecies level, using one-step multiplex PCR.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

All bacterial strains were obtained from Japan Collection of Microorganisms (JCM; Japan). The following bacterial strains were used in the present study: *F. nucleatum* subsp. *vincentii* JCM 11023, *F. nucleatum* subsp. *nucleatum* JCM 8532, *F. nucleatum* subsp. *polymorphum* JCM 12990, *F. nucleatum* subsp. *animalis* JCM 1612, *F. nucleatum* subsp. *fusiforme* JCM 11024, *F. necrophorum* subsp. *funduliforme* JCM 3724, *F. necrophorum* subsp. *necrophorum* JCM 3718, *Fusobacterium equinum* JCM 11174, *Fusobacterium periodonticum* JCM 12991, *Fusobacterium simiae* JCM 17465, and *Fusobacterium canifelinum* JCM 17464. These strains were maintained by cultivating them on Anaerobic Blood Agar (CDC), that consists of a Tryptic soy agar (Becton, Dickinson and Co., Sparks, MD, USA) supplemented with vitamin K_1, hemin, L-cysteine, yeast extract, and sheep blood. These organisms were cultured at 37˚C for 48 h in an anaerobic jar with a gas pack system (AnaeroPack®, Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

2.2. Design of Species-Specific Primers for Seven Subspecies

Design of species-specific primers for five *F. nucleatum* subspecies and two *F.*

necrophorum subspecies was performed as described previously [30]. Briefly, the 16S rRNA gene sequences of *F. nucleatum* subsp. *animalis* (accession no. X55404), *F. nucleatum* subsp. *fusiforme* (X55403), *F. nucleatum* subsp. *nucleatum* (AB588016), *F. nucleatum* subsp. *polymorphum* (AF287812) and *F. nucleatum* subsp. *vincentii* (AJ006964), the DNA gyrase subunit B (*gyrB*) gene sequences of *F. nucleatum* subsp. *animalis* (accession no. HQ008306), *F. nucleatum* subsp. *fusiforme* (HQ008304), *F. nucleatum* subsp. *nucleatum* (HQ008294), *F. nucleatum* subsp. *polymorphum* (HQ008303), *F. nucleatum* subsp. *vincentii* (HQ008296), *F. necrophorum* subsp. *necrophorum* (AY370662) and *F. necrophorum* subsp. *funduliforme* (AY370667), and the RNA polymerase B (*rpoB*) gene sequences of *F. nucleatum* subsp. *animalis* (accession no. GQ274961), *F. nucleatum* subsp. *fusiforme* (GQ274960), *F. nucleatum* subsp. *nucleatum* (GQ274958), *F. nucleatum* subsp. *polymorphum* (GQ274957) and *F. nucleatum* subsp. *vincentii* (GQ274959) were obtained from the DNA Data Bank of Japan (DDBJ; https://www.ddbj.nig.ac.jp/services.html, Mishima, Japan), and a multiple sequence alignment analysis was performed with the CLUSTAL W program; *i.e.*, the 16S rRNA gene sequences of five *F. nucleatum* subspecies, the *gyrB* gene sequences of five *F. nucleatum* subspecies and two *F. necrophorum* subspecies, and the *rpoB* gene sequences of e five *F. nucleatum* subspecies were aligned and analyzed. Homology among the primers selected for each *F. nucleatum* and *F. necrophorum* subspecies and their respective 16S rRNA, *gyrB* and *rpoB* gene sequences was confirmed by a BLAST search.

2.3. Development of a One-Step Multiplex PCR Method Using Designed Primers

Bacterial cells were cultured in BHI supplemented with 0.5% yeast extract for 24 h, and 1 ml of the samples were then collected in microcentrifuge tubes and resuspended at a density of 1.0 McFarland standard (approximately 10^7 colony-forming units (CFU)/ml) in 1 ml of sterile distilled water. A total of 3.6 µl of the suspension was then used as a PCR template. The detection limit of PCR was assessed by serially diluting known numbers of bacterial cells in sterile distilled water and then subjecting each suspension to PCR. The multiplex PCR mixture contained 0.2 µM of each primer, 10 µl of 2 × MightyAmp Buffer Ver.3 (Takara Bio Inc., Shiga, Japan), 0.4 µl of MightyAmp DNA Polymerase (Takara), and 5 µl of the template in a final volume of 20 µl. PCR reactions were performed in a DNA thermal cycler (Applied Biosystems 2720 Thermal Cycler; Applied Biosystems, CA, USA). PCR conditions included an initial denaturation step at 98˚C for 2 min, followed by 30 cycles consisting of 98˚C for 10 s and 68˚C for 1 min. PCR products were analyzed by 2.0% agarose gel electrophoresis before being visualized by electrophoresis in 1 × Tris-borate-EDTA on a 2% agarose gel stained with ethidium bromide. A 100-bp DNA ladder (Takara Biomed, Shiga, Japan) was used as a molecular size marker. All experiments were performed in triplicate.

3. Results

3.1. Primer Design

Fourteen specific primers covering the upstream regions of the 16S rRNA, *gyrB* and *rpoB* gene sequences of five *F. nucleatum* subspecies and two *F. necrophorum* subspecies were designed in the present study (Figures 1-4). The specific forward primers were designated as FNAF for *F. nucleatum* subsp. *animalis*, FNFF for *F. nucleatum* subsp. *fusiforme*, FNNF for *F. nucleatum* subsp. *nucleatum*, FNPF for *F. nucleatum* subsp. *polymorphum*, FNVF for *F. nucleatum* subsp. *vincentii*, FNecNF for *F. necrophorum* subsp. *necrophorum*, and FNecFF for *F. necrophorum* subsp. *funduliforme*, whereas the specific reverse primers were designated as FNAR for *F. nucleatum* subsp. *animalis*, FNFR for *F. nucleatum* subsp. *fusiforme*, FNNR for *F. nucleatum* subsp. *nucleatum*, FNPR for *F. nucleatum* subsp. *polymorphum*, FNVR for *F. nucleatum* subsp. *vincentii*, FNecNR for *F. necrophorum* subsp. *necrophorum*, and FNecFF for *F. necrophorum* subsp. *funduliforme*. The amplicon sizes of *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *animalis*, *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *fusiforme*, *F. nucleatum* subsp. *vincentii*, *F. necrophorum* subsp. *necrophorum*, and *F. necrophorum* subsp. *funduliforme* were 101 bp, 260 bp, 535 bp, 756 bp, 912 bp, 164 bp, and 451 bp, respectively.

3.2. Multiplex PCR

3.2.1. Detection Limit

Our multiplex PCR method for identifying and detecting five *F. nucleatum* subspecies and two *F. necrophorum* subspecies, *i.e.*, *F. nucleatum* subsp. *animalis*, *F. nucleatum* subsp. *fusiforme*, *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *vincentii*, *F. necrophorum* subsp. *necrophorum* and *F. necrophorum* subsp. *funduliforme* successfully amplified DNA fragments of the expected size for each species (Figure 5 and Figure 6). The detection limit was assessed in the presence of titrated bacterial cells, and

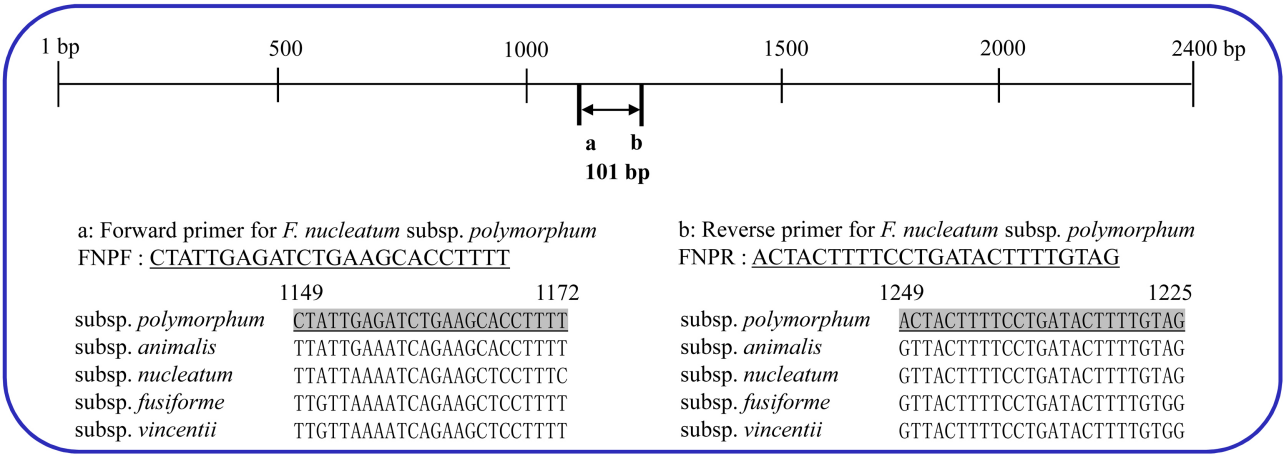

Figure 1. Locations and sequences of species-specific primers for the *gyrB* gene of *F. nucleatum* subsp. *polymorphum*. The nucleotide sequence of each primer has been underline.

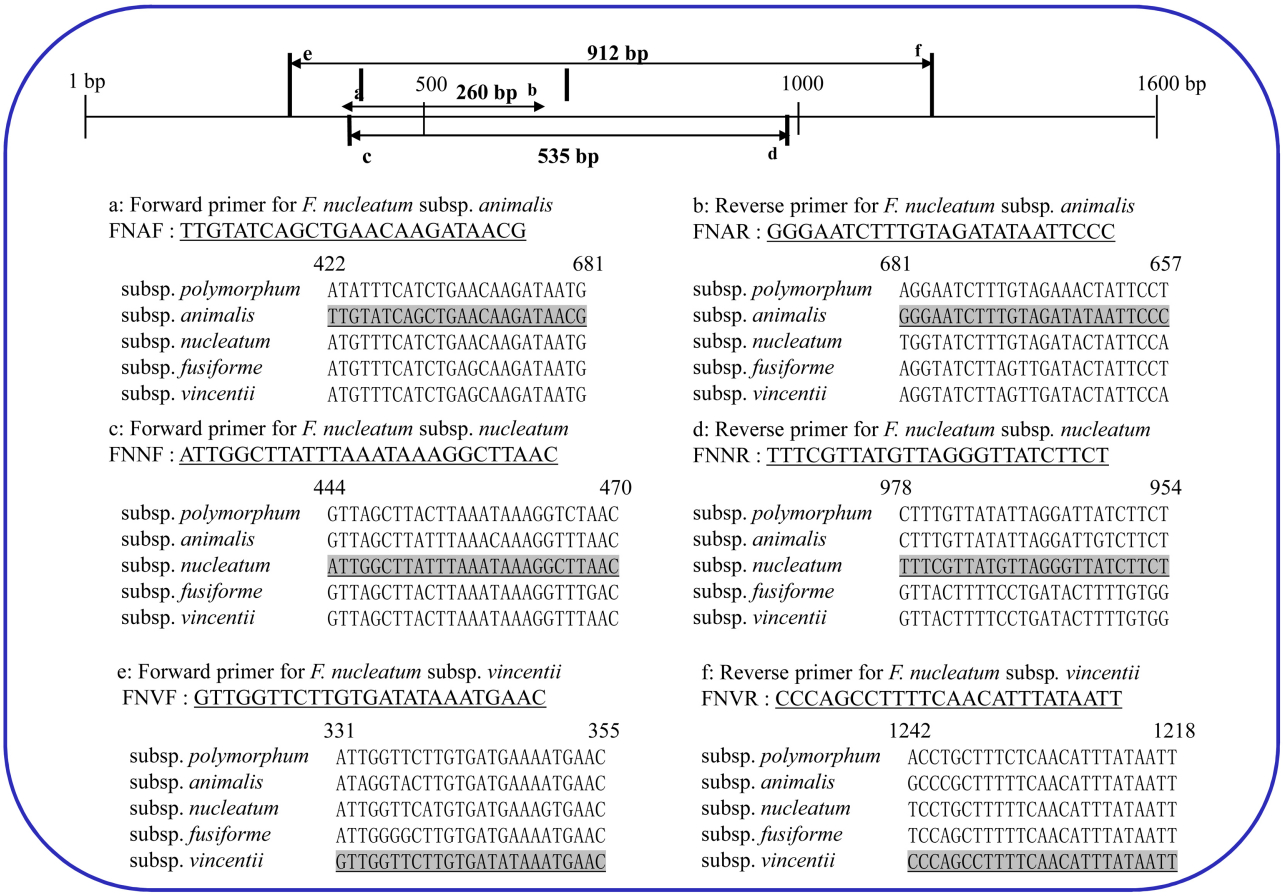

Figure 2. Locations and sequences of species-specific primers for the *rpoB* gene of *F. nucleatum* subsp. *animalis*, subsp. *nucleatum* and subsp. *vincentii*. The nucleotide sequence of each primer has been underline.

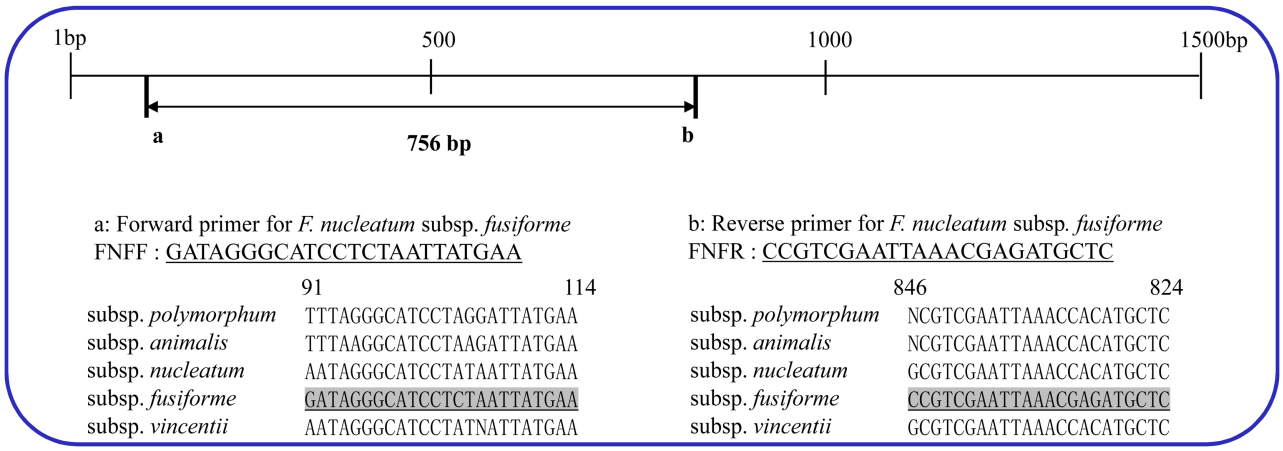

Figure 3. Locations and sequences of species-specific primers for the 16S rDNA of *F. nucleatum* subsp. *fusiforme*. The nucleotide sequence of each primer has been underline.

the sensitivity of the PCR assay was between 5 × 1 and 5 × 10 CFU per PCR template (5.0 μl) for the *F. nucleatum* subsp. *vincentii*-specific primer set with strain JCM JCM 11023, the *F. nucleatum* subsp. *nucleatum*-specific primer set with strain JCM 8532, the *F. nucleatum* subsp. *polymorphum*-specific primer set

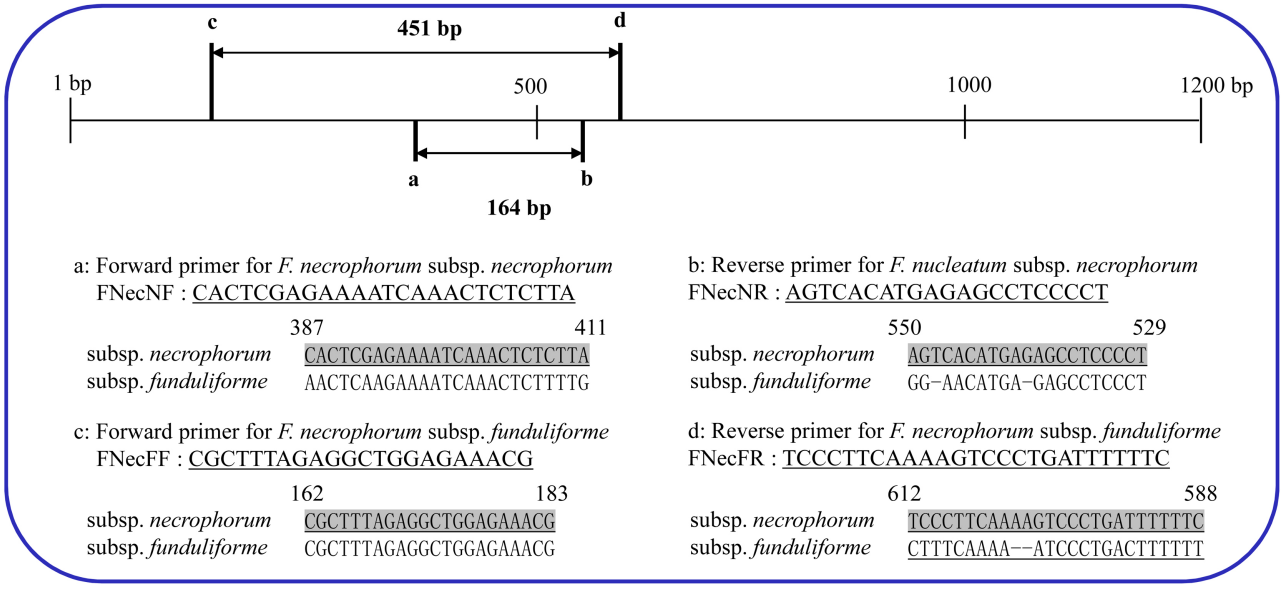

Figure 4. Locations and sequences of species-specific primers for the *gyrB* gene of *F. necrophorum* subsp. *necrophorum* and subsp. *funduliforme*. The nucleotide sequence of each primer has been underline.

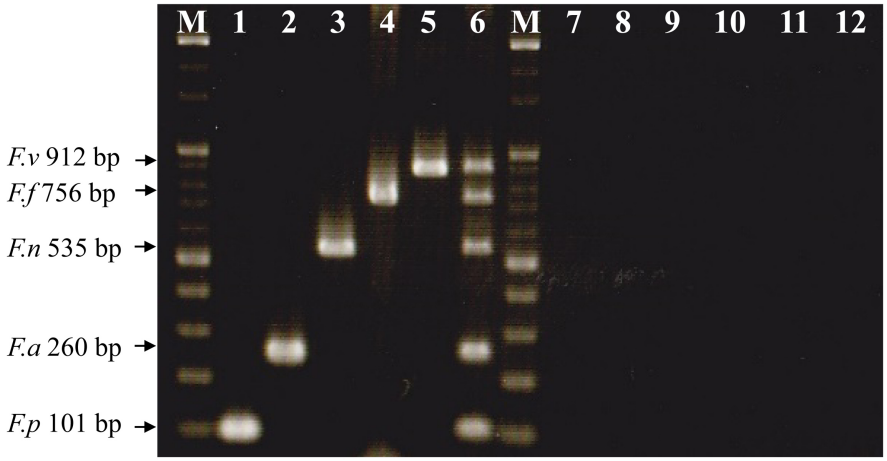

Figure 5. Specificity of the multiplex PCR assays for *F. nucleatum* subspecies. The primer mixture contained FNPF, FNPR, FNAF, FNAR, FNNF, FNNR, FMFF, FNFR, FNVF and FNVR. Lanes: 1, *F. nucleatum* subsp. *polymorphum* JCM 12990; 2, *F. nucleatum* subsp. *animalis* JCM 11025; 3, *F. nucleatum* subsp. *nucleatum* JCM 8532; 4, *F. nucleatum* subsp. *fusiforme* JCM 11024; 5, *F. nucleatum* subsp. *vincentii* JCM 11023; 6, Mixture of *F. nucleatum* subsp. *polymorphum*, subsp. *animalis*, subsp. *nucleatum*, subsp. *fusiforme* and subsp. *vincentii*; 7, *F. necrophorum subsp. necrophorum* JCM 3718; 8, *F. necrophorum subsp. fundliforme* JCM 3717; 9, *F. equinum* JCM 11174; 10, *F. periodonticum* JMC 12001; 11, *F. simiae* JCM 17465; 12, *F. canifelinum* JCM 17464. M, molecular size marker (100-bp DNA ladder).

with strain JCM 12990, the *F. nucleatum* subsp. *animalis*-specific primer set with strain JCM 1612, the *F. nucleatum* subsp. *fusiforme*-specific primer set with strain JCM 11024, the *F. necrophorum* subsp. *funduliforme*-specific primer set with strain JCM 3724 and the *F. necrophorum* subsp. *necrophorum*-specific primer set with strain JCM 3718 (data not shown).

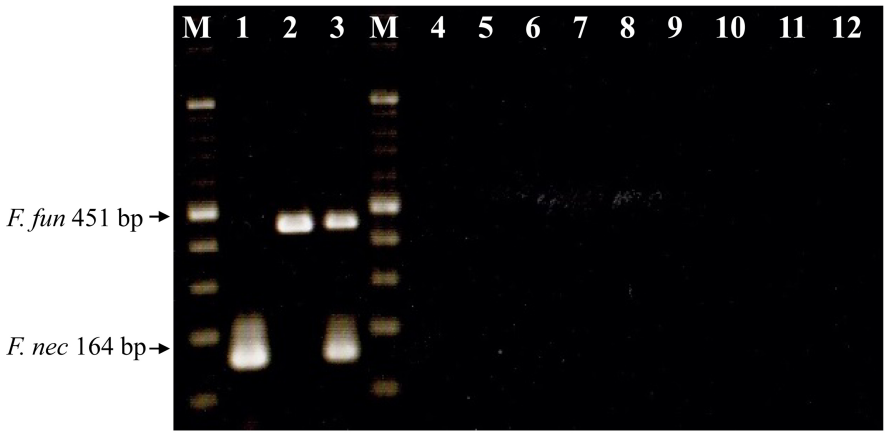

Figure 6. Specificity of the multiplex PCR assays for *F. necrophorum* subspecies. The primer mixture contained FNecNF, FNecNR, FNecFF and FNecFR. Lanes: 1, *F. necrophorum subsp. necrophorum* JCM 3718; 2, *F. necrophorum subsp. fundliforme* JCM 3717; 3, Mixture of *F. necrophorum subsp. necrophorum* and *subsp. fundliforme*; 4, *F. nucleatum* subsp. *polymorphum* JCM 12990; 5, *F. nucleatum* subsp. *animalis* JCM 11025; 6, *F. nucleatum* subsp. *nucleatum* JCM 8532; 7, *F. nucleatum* subsp. *fusiforme* JCM 11024; 8, *F. nucleatum* subsp. *vincentii* JCM 11023; 9, *F. equinum* JCM 11174; 10, *F. periodonticum* JMC 12001; 11, *F. simiae* JCM 17465; 12, *F. canifelinum* JCM 17464. M, molecular size marker (100-bp DNA ladder).

3.2.2. Assay of Representative Candida Species Other than Eight Medically Important Species

As representative *Fusobacterium* species other than *F. nucleatum* and *F. necrophorum* subspecies targeted in this study, *Fusobacterium equinum*, *Fusobacterium periodonticum*, *Fusobacterium simiae* and *Fusobacterium canifelinum* were subjected to PCR using the designed primer sets. No amplicons were produced from any of representative *Fusobacterium* species other than *F. nucleatum* and *F. necrophorum* (**Figure 5** and **Figure 6**).

4. Discussion

To develop a PCR-based technique more applicable for clinical use than conventional PCR, we established a one-step multiplex PCR system for identifying and detecting simultaneously two medically important *Fusobacterium* species, using only two PCR tube per sample. A multiplex-PCR method is a rapid tool that allows for the simultaneous amplification of more than one sequence of target DNA in a single reaction, thereby saving time and reagents [31]. The most significant problem with regard to this method is the possibility of hybridization among the different sequences of primers. Bi *et al.* reported a PCR strategy allowing the identification at the subspecies level of *F. nucleatum* as same as the present study [29]. This PCR method was based on the amplification of the fragments from the subspecies marker genes in a single PCR. However, because this method was not able to identify *F. nucleatum* subsp. *fusiforme* and adopted a conventional PCR, it was not suitable for routine clinical laboratory identification of *Fusobacterium* species.

The phylogenetic differences are not only expressed in the sequence information itself but also by the different lengths of amplificons and, in some cases, in the formation of distinct band patterns (in gel electrophoresis) resulting from variations among the rrn operons in the same strain [32] [33]. The taxonomy of fusobacterial species and some related genera and species is still a scientific riddle, especially with respect to the five controversial subspecies in *Fusobacterium nucleatum* [34] and the two in *Fusobacterium necrophorum* [35]. In the present study, we designed species-specific primers with the already mentioned means, for the identification at the subspecies level of two medically important *Fusobacterium* species, *i.e.*, *F. nucleatum* and *F. necrophorum*, with a PCR method. These primers were able to distinguish each *Fusobacterium* species at the subspecies level and did not display cross-reactivity with representative *Fusobacterium* species other than the two species targeted in this study. Moreover, we developed a one-step multiplex PCR method with the ability to identify and differentiate two medically important *Fusobacterium* species at the subspecies level using only each one PCR tubes per sample. Species-specific primers for seven subspecies were designed based on the sequences of 16S rRNA, *gyrB* and *rpoB* gene. The protein-coding genes that have been tested for the assessment of microbial diversity include the genes for DNA gyrase subunit B (*gyrB*) [36], RNA polymerase subunit B (*rpoB*) [37], the TU elongation factor (*tuf*) [38], the 60 kDa chaperonin protein (*cpn*60) [39]. The *gyrB* and *rpoB* genes have a number of potential advantages. Previous reports have shown these markers to be suitable for phylogenetic analyses, as it provides a better resolution at species level than the 16S rRNA gene [40].

Our multiplex PCR method is easy because the use of MightyAmp DNA Polymerase Ver.3 (Takara) means that DNA extraction may be avoided, and the subspecies identification and detection using this method only takes approximately 2 hours. Thus, the method described herein will allow the prevalence at the subspecies level of *F. nucleatum* and *F. necrophorum* and their involvement in the various infections, to be fully clarified in future studies.

5. Conclusion

Our developed one-step multiplex PCR method is accurate, specific, cost-effective, time-saving, and worked without requiring DNA extraction. It is predicted that our multiplex PCR method can be used in routine practice in clinical microbiology laboratories to identify *F. nucleatum* and *F. necrophorum* at the subspecies level.

Authors and Affiliations

Umezawa K, Hayashi S, Tsuzukibashi O, Fukatsu A, Fuchigami M, Uchibori S, Komine C and Asano T corrected the data. Umezawa K, Tsuzukibashi O, Wakami M, Kobayashi T and Fukumoto M drafted and wrote the manuscript. The concept of this manuscript was devised by Umezawa K. All authors read and

approved the final manuscript.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Holt, J.G. and Krieg, N.R., Eds. (1984) Bergey's Manual of Determinative Bacteriology. Williams and Wilkins, Baltimore, MD, 631-637.

[2] Jousemies-Somer, H.R. (1997) Recently Described Clinically Important Anaerobic Bacteria: Taxonomic Aspects and Update. *Clinical Infectious Diseases*, **25**, S78-S87. https://doi.org/10.1086/516227

[3] Long, S., Prober, C., Schouls, L. and Fischer, M. (2017) Principles and Practice of Pediatric Infectious Diseases. 5th Edition, Elsevier, Amsterdam, 1015-1017.

[4] Citron, D.M. (2002) Update on the Taxonomy and Clinical Aspects of the Genus *Fusobacterium*. *Clinical Infectious Diseases*, **35**, S22-S27. https://doi.org/10.1086/341916

[5] Allen-Vercoe, E., Strauss, J. and Chadee, K. (2011) *Fusobacterium nucleatum*: An Emerging Gut Pathogen? *Gut Microbes*, **2**, 294-298. https://doi.org/10.4161/gmic.2.5.18603

[6] Han, Y.W. (2015) *Fusobacterium nucleatum*: A Commensal-Turned Pathogen. *Current Opinion in Microbiology*, **23**, 141-147. https://doi.org/10.1016/j.mib.2014.11.013

[7] Morris, M.L., Andrews, R.H. and Rogers, A.H. (1997) Investigations on the Taxonomy and Systematics of *Fusobacterium nucleatum* Using Alloenzyme Electrophoresis. *International Journal of Systematic and Evolutionary Microbiology*, **47**, 103-110. https://doi.org/10.1099/00207713-47-1-103

[8] George, K.S., Reynolds, M.A. and Falkler Jr., W.A. (2007) Arbitrarily Primed Polymerase Chain Reaction Fingerprinting and Clonal Analysis of Oral *Fusobacterium nucleatum* Isolates. *Oral Microbiology and Immunology*, **12**, 219-226. https://doi.org/10.1111/j.1399-302X.1997.tb00382.x

[9] Conrads, G., Claros, M.C., Citron, D.M., Tyrell, K.L., Merriam, V. and Goldstein, E.J. (2002) 16S-23S rDNA Internal Transcribed Spacer Sequences for Analysis of the Phylogenetic Relationships among Species of the Genus *Fusobacterium*. *International Journal of Systematic and Evolutional Microbiology*, **52**, 493-499. https://doi.org/10.1099/00207713-52-2-493

[10] Bolstad, A.I., Jensen, H.B. and Bakken, V. (1996) Taxonomy, Biology, and Periodontal Aspects of *Fusobacterium nucleatum*. *Clinical Microbiology Reviews*, **9**, 55-71. https://doi.org/10.1128/CMR.9.1.55

[11] Han, Y.W. and Wang, X. (2013) Mobile Microbiome: Oral Bacteria in Extra-Oral Infections and Inflammation. *Journal of Dental Research*, **92**, 485-491. https://doi.org/10.1177/0022034513487559

[12] Dahya, V., Patel, J., Wheeler, M. and Ketsela, G. (2015) *Fusobacterium nucleatum* Endocarditis Presenting as Liver and Brain Abscesses in an Immunocompetent Patient. *The American Journal of the Medical Sciences*, **349**, 284-285. https://doi.org/10.1097/MAJ.0000000000000388

[13] Gharbia, S.E., Shah, H.N., Lawson, P.A. and Haapasalo, M. (1990) The Distribution

and Frequency of *Fusobacterium nucleatum* Subspecies in the Human Oral Cavity. Molecular *Oral Microbiology*, **5**, 324-327. https://doi.org/10.1111/j.1399-302X.1990.tb00434.x

[14] Han, Y.W., Shen, T., Chung, P., Buhimschi, I.A. and Buhimschi, C.S. (2009) Uncultivated Bacteria as Etiologic Agents of Intra-Amniotic Inflammation Leading to Preterm Birth. *Journal of Clinical Microbiology*, **47**, 38-47. https://doi.org/10.1128/JCM.01206-08

[15] Strauss, J., White, A., Ambrose, C., McDonald, J. and Allen-Vercoe, E. (2008) Phenotypic and Genotypic Analyses of Clinical *Fusobacterium nucleatum* and *Fusobacterium periodonticum* Isolates from the Human Gut. *Anaerobe*, **14**, 301-309. https://doi.org/10.1016/j.anaerobe.2008.12.003

[16] Kim, H.S., Lee, D.S., Chang, Y.H., Kim, M.J., Koh, S., Kim, J., Seong, J.H., Song, S.K., Shin, H.S., Son, J.B., Jung, M.Y., Park, S.N., Yoo, S.Y., Cho, K.W., Kim, D.K., Moon, S., Kim, D., Choi, Y., Kim, B.O., Jang, H.S., Kim, C.S., Kim, C., Choe, S.J. and Kook, J.K. (2010) Application of *rpoB* and Zinc Protease Gene for Use in Molecular Discrimination of *Fusobacterium nucleatum* Subspecies. *Journal of Clinical Microbiology*, **48**, 545-553. https://doi.org/10.1128/JCM.01631-09

[17] Gonzales-Marin, C., Spratt, D.A. and Allaker, R.P. (2013) Maternal Oral Origin of *Fusobacterium nucleatum* in Adverse Pregnancy Outcomes as Determined Using the 16S-23S rRNA Gene Intergenic Transcribed Spacer Region. *Journal of Medical Microbiology*, **62**, 133-144. https://doi.org/10.1099/jmm.0.049452-0

[18] Hagelskjaer, K.L. and Prag, J. (2000) Human Necrobacillosis, with Emphasis on Lemierre's Syndrome. *Clinical Infectious Diseases*, **31**, 524-532. https://doi.org/10.1086/313970

[19] Nagaraja, T.G., Narayanan, S.K., Stewart, G.C. and Chengappa, M.M. (2005) *Fusobacterium necrophorum* Infections in Animals: Pathogenesis and Pathogenic Mechanisms. *Anaerobe*, **11**, 239-246. https://doi.org/10.1016/j.anaerobe.2005.01.007

[20] Brazier, J.S., Hall, V., Yusuf, E. and Duerden, B.I. (2002) *Fusobacterium necrophorum* Infections in England and Wales 1990-2000. *Journal of Medical Microbiology*, **51**, 269-272. https://doi.org/10.1099/0022-1317-51-3-269

[21] Navas, E., Martinez-San, J.M., Garcia-Villanueva, M. and de Blas, A. (1994) Brain Abscess with Intracranial Gas Formation: Case Report. *Clinical Infectious Diseases*, **19**, 219-220. https://doi.org/10.1093/clinids/19.1.219

[22] Razonable, R.R., Rahman, A.E. and Wilson, W.R. (2003) Lemierre Syndrome Variant: Necrobacillosis Associated with Inferior Vena Cava Thrombosis and Pulmonary Abscesses after Trauma-Induced Leg Abscesses. *Mayo Clinic Proceeding*, **78**, 1153-1156. https://doi.org/10.4065/78.9.1153

[23] Brazier, J.S. (2006) Human Infections with *Fusobacterium necrophorum*. *Anaerobe*, **12**, 165-172. https://doi.org/10.1016/j.anaerobe.2005.11.003

[24] Riordan, T. and Wilson, M. (2004) Lemierre's Syndrome: More Than a Historical Curiosa. *Postgraduate Medical Journal*, **80**, 328-334. https://doi.org/10.1136/pgmj.2003.014274

[25] Shinjo, T., Fujisawa, T. and Mitsuoka, T. (1991) Proposal of Two Subspecies of *Fusobacterium necrophorum* (Flugge) Moore and Holdeman: *Fusobacterium necrophorum* Subsp. *necrophorum* Subsp. Nov., Nom. Rev. (ex Flugge 1886), and *Fusobacterium necrophorum* Subsp. *funduliforme* Subsp. Nov., Nom. Rev. (ex Halle 1898). *International Journal of Systematic and Evolutionary Microbiology*, **41**, 395-397. https://doi.org/10.1099/00207713-41-3-395

[26] Ye, X., Wang, R., Bhattacharya, R., Boulbes, D.R., Fan, F., Xia, L., Adoni, H., Ajami,

N.J., Wong M.C., Smith, D.P., Petrosino J.F., Venable, S., Qiao, W., Baladandayuthapani, V., Maru, D. and Ellis, L.M. (2017) *Fusobacterium nucleatum* Subspecies *animalis* Influences Proinflammatory Cytokine Expression and Monocyte Activation in Human Colorectal Tumors. *Cancer Prevention Research*, **10**, 398-409. https://doi.org/10.1158/1940-6207.CAPR-16-0178

[27] Smith, G.R. and Thornton, E.A. (1997) Classification of Human and Animal Strains of *Fusobacterium necrophorum* by Their Pathogenic Effects in Mice. *Journal of Medical Microbiology*, **46**, 879-882. https://doi.org/10.1099/00222615-46-10-879

[28] Nie, S., Tian, B., Wang, X., Pincus, D.H., Welker, M., Gilhuley, K., Lu, X., Han, Y.W. and Tang, Y.W. (2015) *Fusobacterium nucleatum* Subspecies Identification by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry. *Journal of Clinical Microbiology*, **53**, 1399-1402. https://doi.org/10.1128/JCM.00239-15

[29] Bi, D., Zhu, Y., Gao, Y., Li, H., Zhu, X., Wei, R., Xie,, R., Wei, Q. and Qin, H. (2021) Newly Developed PCR-Based Method Revealed Distinct *Fusobacterium nucleatum* Subspecies Infection Patterns in Colorectal Cancer. *Microbial Biotechnology*, **14**, 2176-2186. https://doi.org/10.1111/1751-7915.13900

[30] Fukatsu, A., Tsuzukibashi, O., Suzuki, H., Asaka, K., Ono, Y., Fuchigami, M., Kobayashi, T., Uchibori, S., Takahashi, Y., Komine, C., Konishi, Y., Ogura, Y., Omori, H., Wakami, M., Murakami, H. and Fukumoto, M. (2021) One-Step Multiplex PCR for Simultaneous Detection and Identification of Eight Medically Important *Candida* Species. *Open Journal of Stomatology*, **11**, 14-24. https://doi.org/10.4236/ojst.2021.111002

[31] Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H. (1997) Multiplex PCR: Critical Parameters and Step-by-Step Protocol. *BioTechniques*, **23**, 504-511. https://doi.org/10.2144/97233rr01

[32] Christensen, H., Moller, P.L., Vogensen, F.K. and Olsen, J.E. (2000) Sequence Variation of the 16S to 23S rRNA Spacer Region in *Salmonella enterica*. *Research of Microbiology*, **151**, 37-42. https://doi.org/10.1016/S0923-2508(00)00129-7

[33] Iteman, I., Rippka, R., Tandeau de Marsac, N. and Herdman, M. (2000) Comparison of Conserved Structural and Regulatory Domains within Divergent 16S rRNA-23S rRNA Spacer Sequences of Cyanobacteria. *Microbiology*, **146**, 1275-1286. https://doi.org/10.1099/00221287-146-6-1275

[34] Gharbia, S.E. and Shah, H.N. (1992) *Fusobacterium nucleatum* Subsp. *fusiforme* Subsp. Nov. and *Fusobacterium nucleatum* Subsp. *animalis* Subsp. Nov. as Additional Subspecies within *Fusobacterium nucleatum*. *International Journal of Systematic and Evolutionary Microbiology*, **42**, 296-298. https://doi.org/10.1099/00207713-42-2-296

[35] Okwumabua, O., Tan, Z., Staats, J., Oberst, R.D., Chengappa, M.M. and Nagaraja, T.G. (1996) Ribotyping to Differentiate *Fusobacterium necrophorum* Subsp. *necrophorum* and *F. necrophorum* Subsp. *funduliforme* Isolated from Bovine Ruminal Contents and Liver Abscesses. *Applied and Environmental Microbiology*, **62**, 469-472. https://doi.org/10.1128/aem.62.2.469-472.1996

[36] Poirier, S., Rue, O., Peguilhan, R., Coeuret, G., Zagorec, M., Champomier-Verges, M.C., Loux, V. and Chaillou, S. (2018) Deciphering Intra-Species Bacterial Diversity of Meat and Seafood Spoilage Microbiota Using *gyrB* Amplicon Sequencing. A Comparative Analysis with 16S rDNA V3-V4 Amplicon Sequencing. *PLOS ONE*, **13**, e0204629. https://doi.org/10.1371/journal.pone.0204629

[37] Roux, S., Enault, F., Bronner, G. and Debroas, D. (2011) Comparison of 16S rRNA

and Protein-Coding Genes as Molecular Markers for Assessing Microbial Diversity (Bacteria and Archaea) in Ecosystems. *FEMS Microbiology Ecology*, **78**, 617-628. https://doi.org/10.1111/j.1574-6941.2011.01190.x

[38] Ghebremedhin, B., Layer, F., Konig, W. and Konig, B. (2008) Genetic Classification and Distinguishing of Staphylococcus Species Based on Different Partial Gap, 16S rRNA, *hsp60*, *rpoB*, *sodA*, and *tuf* gene sequences. *Journal of Clinical Microbiology*, **46**, 1019-1025. https://doi.org/10.1128/JCM.02058-07

[39] Links, M.G., Dumonceaux, T.J., Hemmingsen, S.M. and Hill, J.E. (2012) The Chaperonin-60 Universal Target is A Barcode for Bacteria That Enables De Novo Assembly of Metagenomic Sequence Data. *PLOS ONE*, **7**, e49755. https://doi.org/10.1371/journal.pone.0049755

[40] Adekambi, T., Drancourt, M. and Raoult, D. (2009) The *rpoB* Gene as A Tool for Clinical Microbiologists. *Trends in Microbiology*, **17**, 37-45. https://doi.org/10.1016/j.tim.2008.09.008