Considerations in Evaluating the Applicability of Universal Detection of Oral Pathogens

Akihiro Yoshida et al. (4), recently published an interesting paper describing the development of a 5' fluorogenic nuclease-based real-time PCR assay for quantitative detection of the oral pathogens *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. The authors provide the description of a set of primers and probe for amplification and detection of a broad range of bacteria based on highly conserved regions in the 16S rRNA gene. Alignment of the forward and reverse primers and probe sequences, however, showed mismatches at the 5' as well as the 3' ends of the 16S rRNA sequences with different oral pathogens (Fig. 1). Most likely these mismatches interfere with the amplification of *P. gingivalis*. In addition, according to Yoshida et al., the primers and probe sequences were based on earlier published data (2). We noticed that except for the probe sequence, the primer sequences presented in the publication of Greisen et al. (2) are located 172 and 254 bases, respectively, upstream and 180 bases downstream from the site indicated by Yoshida et al.

We have tested this universal probe and primer set with one nonoral bacterium and six oral bacteria: *Escherichia coli*, *P. gingivalis*, *A. actinomycetemcomitans*, *Tannerella forsythensis* (formerly *Bacteroides forsythus*), *Micromonas micros* (formerly *Peptostreptococcus*), *Prevotella intermedia*, and *Fusobacterium nucleatum*. Growth of the bacteria, preparation of serial dilutions of pure cultures, DNA isolation, and real-time PCR amplification were performed as described previously (1).

The *E. coli* amplification signals consistently showed an increasing C_T range (i.e., decreasing amplification signal) between 15.9 and 26.3, which corresponded to 4.95×10^7 to 4.95×10^4 CFU equivalents/PCR. All the oral bacteria, however, showed a C_T range between 29.5 and 33.5, which is at the level of the negative control signal ($C_T = 29.5$). For *A. actinomycetemcomitans*, an amplification signal was obtained only with a high DNA concentration, corresponding to 10,000 CFU/ml, and the signals obtained with lower DNA concentrations were at the level of the negative control. To validate the DNA isolation, a real-time PCR with a specific primer-probe combination for *E. coli* (3) and *P. gingivalis* (1) was used. The amplification signals obtained with the specific PCR on the same DNA confirmed that the amount of DNA present in the dilution corresponded to the amount isolated.

In summary, the results with the eubacterium real-time PCR confirm the mismatches in the alignment but are in contradiction with the results on the same strains presented by Yoshida et al. In our laboratory, the primer and probe set described by Yoshida is not universal, does not amplify *P. gingivalis*, *T. forsythensis*, *M. micros*, *P. intermedia*, or *F. nucleatum*, and amplifies *A. actinomycetemcomitans* only at a high DNA concentration. We conclude that the universal real-time probe and primer set published by Yoshida et al. to detect *A. actinomycetemcomitans* and *P. gingivalis* does not seem applicable for sensitive detection of oral bacterium species belonging to the major bacterial periodontal pathogens.

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Khalil Boutaga

Arie Jan van Winkelhoff

Department of Periodontology

Section of Oral Microbiology

Academic Center for Dentistry

Amsterdam, The Netherlands

Christina M. J. E. Vandenbroucke-Grauls

Paul H. M. Savelkoul*

Medical Microbiology and Infection Control

VU University Medical Center

Amsterdam, The Netherlands

*Phone: 31204440552

Fax: 31204440473

E-mail: p.savelkoul@vumc.nl

Authors' Reply

Dr. Boutaga et al. commented on the universal primers and probes that we used for the real-time PCR assay that we described previously (4). They stated that our universal primers and probe set are not applicable for the sensitive detection of *A. actinomycetemcomitans* and *P. gingivalis*. They pointed out that Greisen et al. (2) published the sequence that we used as a universal primer and that many more 16S rRNA gene sequences are now available in various databases. Using these data, they showed that there were three or four mismatches with the 24-base 5' primer Uni152-F.

However, the 3' sequence of Uni152-F matched all the bacteria listed in Fig. 1. The essential point is the correspondence of the 3' sequence of the universal primer to the sequence of the template DNA. We do not think that mismatches of three or four nucleotides in the middle of one primer have a serious effect. We subsequently reexamined the real-time PCR assay using five of the oral bacteria that they examined (all except *P. micros*) and *E. coli*. The bacteria studied were *P. gingivalis* W83, *A. actinomycetemcomitans* TN-1, *T. forsythensis* (formerly *Bacteroides forsythus*) ATCC 43037, *F. nucleatum* ATCC 10953, and *P. intermedia* ATCC 25611. This time, we used LightCycler FastStart DNA Master Hybridization probes and LightCycler (Roche Diagnostics GmbH, Penzberg, Germany) for the real-time PCR assay, instead of the 2x TaqMan Universal PCR Master and ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, Calif.). The real-time PCR conditions were as described in our report (4). Although we used different kits for the real-time PCR assay, we con-

FIG. 1. Alignment of the 16S rRNA sequence region described by Yoshida et al., including six oral bacteria compared to the *E. coli* sequence. The locations of the universal primers (Uni152-F and Uni220-R) and the probe (Uni177-T) are identical to the *E. coli* sequence as indicated. Strains: 1, DH5 α ; 2, W83; 3, ATCC 29523; 4, ATCC 43037; 5, ATCC 33270; 6, ATCC 25611; 7, ATCC 10953. Dots, nucleotides identical to those in the *E. coli* sequence; dashes, deletions.

firmed that the universal primer pairs and probes worked in all of these bacteria (data not shown).

As previously reported (3), several studies have found mismatches with the universal primers (1). As an aside, is a “completely universal” primer possible? How can we prove the universality of primers? We believe that a completely universal primer is impossible, but a “broad-range” primer is possible. The calculation of the relative amount of bacteria using a universal primer is theoretically correct, but the design of a universal primer is very difficult. However, is calculation of the percentages of bacteria using a universal primer meaningless? We believe that the comparative detection of bacteria using a universal primer is very meaningful as one index of the relative amount.

Of course, the accuracy of the relative amounts of bacteria is very important. However, nobody has established a truly accurate method of counting bacteria. The most important thing is to improve the accuracy of the counting procedure in small steps. Boutaga’s suggestion is useful information for improving the accuracy of bacterial counting methods.

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Akihiro Yoshida*
Department of Preventive Dentistry
Kyushu Dental College
Kitakyushu 803-8580, Japan

Nao Suzuki
Department of Oral Microbiology
Ozu University
Koriyama, Japan

Yoshio Nakano
Miki Kawada
Department of Preventive Dentistry
Kyushu University Faculty of Dental Science
Fukuoka, Japan

Takahiko Oho
Department of Preventive Dentistry
Graduate School of Medical and Dental Sciences
Kagoshima University
Kagoshima, Japan

*Phone: 81 93 582 1131, ext. 2103
Fax: 81 93 5917736
E-mail: akihiro@kyu-dent.ac.jp

Ed. Note: The Yoshida group has just published two more related articles (A. Yoshida, N. Suzuki, Y. Nakano, M. Kawada, T. Oho, and T. Koga, *J. Clin. Microbiol.* **41**:4438–4441, 2003; N. Suzuki, A. Yoshida, T. Saito, M. Kawada, and Y. Nakano, *J. Clin. Microbiol.* **42**:2255–2257, 2004). The discussion here may apply to these two articles as well.