Different frequencies of *Streptococcus anginosus* infection in oral cancer and esophageal cancer

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Multiple cancers frequently occur in the upper aerodigestive tract. The high incidence rate of multiple carcinomas in this region is often explained in terms of involvement of the same underlying risk factors. It has been reported that the oral bacterium *Streptococcus anginosus* (*S. anginosus*) is associated with esophageal, gastric, and pharyngeal cancer tissues. In this study, a highly specific quantification method for *S. anginosus* DNA using real-time PCR was established. We employed this assay to determine whether *S. anginosus* is also associated with oral cancer tissues. This precise quantification method revealed different degrees of infection with *S. anginosus* in esophageal cancer and oral cancer. We assayed 10 ng of genomic DNA from cancer tissues, and found that eight of 18 samples (44%) from the esophagus contained a detectable level (>10 fg) of *S. anginosus* DNA, whereas this was the case for only five of 38 samples (13%) of oral cancer. The quantity of *S. anginosus* DNA in the esophageal cancer tissues was significantly higher than in oral cancer. The maximum amount of *S. anginosus* DNA was approximately ten times higher in esophageal than in oral cancer tissues. In addition, none of the five different oral cancer sites (floor of the mouth, mandibular gingival, maxillary gingival, buccal mucosal, and tongue) showed significant signs of *S. anginosus* infection. On the other hand, most non-cancerous tissues of the esophagus and tongue showed an undetectable level of *S. anginosus*. These results suggest that *S. anginosus* is associated with esophageal cancer, but is not closely related with oral cancer. (Cancer Sci 2003; 94: 492–496)

Bacterial and viral infections are important factors in cancer development.[1)] It has been reported that *Helicobacter pylori* (*H. pylori*) is associated with gastritis, gastric atrophy, and gastric cancer.[2–4)] The presence of microorganisms in several kinds of human cancers was recently investigated, and *Streptococcus anginosus* (*S. anginosus*) DNA fragments were frequently found in DNA samples from esophageal cancer tissues, gastric cancer tissues, and dysplasia of the esophagus.[5, 6)] Viable *S. anginosus* was also recovered from esophageal cancer tissues (unpublished data). These results suggest that *S. anginosus* infection occurs at an early stage of esophageal cancer and is related to esophageal and gastric carcinogenesis. *S. anginosus* is classified as an oral bacterium and can be isolated from several parts of the body such as the oral cavity, gastrointestinal tract, and genitourinary tract. It is often associated with pyogenic infections, including endocarditis.[7–9)] *S. anginosus* DNA has also been found in head and neck squamous cell carcinomas[10)]; it was found much less frequently in non-cancerous tissues of the esophagus and was absent from the colon, lung, bladder, renal, and cervical cancer tissues.[6)] Therefore, it was suggested that *S. anginosus* DNA is associated with cancers in the upper digestive tract, although the involvement of *S. anginosus* infection in the carcinogenic process has not been clarified.

It is generally accepted that the upper aerodigestive tract is a region in which multiple primary cancers occur at a high rate. Squamous cell carcinoma of the oral cavity is often accompanied by other squamous cell carcinomas of the aerodigestive tract, such as oropharyngeal cancers or esophageal cancers.[11, 12)] The high incidence of multiple carcinomas in this region is often explained by the concept of field cancerization, which is based on the hypothesis that exposure to carcinogenic agents leads to independent carcinogenesis in epithelial cells at different sites in this region. Although little is known about this hypothetical etiology, many epidemiological studies have indicated some possible etiological factors, such as alcohol use.[13, 14)]

We investigated the presence of *S. anginosus* DNA in squamous carcinoma tissues of the oral cavity in this study and made comparisons with the esophagus. *S. anginosus* infection in cancer tissues of the upper digestive tract and the concept of field cancerization led us to consider *S. anginosus* to be one possible risk factor for cancer development and also led us to propose that *S. anginosus* infection occurs in oral cancer tissues as well as other sites in the upper digestive tract. Presently, neither the biochemical criteria nor PCR methods for identification of streptococci are sufficiently specific and reliable, because various oral *Streptococci* that are biochemically and phylogenetically similar to *S. anginosus* are dominant in the oral cavity, [15, 16)] and these species sometimes interfere with detection of *S. anginosus*. Therefore, in the present study, a quantitative, real-time PCR coupled with *Taq*Man chemistry, a highly sensitive and specific approach,[17)] was developed to assess *S. anginosus* status. Using this assay, *S. anginosus* DNA was accurately identified and quantified in oral and esophageal cancer tissues. The numerical values obtained were considered in relation to cancer development in the digestive tract.

Materials and Methods

Tumor samples. Eighteen esophageal carcinoma tissues and 6 non-cancerous tissues were obtained from patients at the National Cancer Center Hospital (Tokyo). Nineteen lingua carcinoma tissues, 5 mandibular and 4 maxillary gingival carcinoma tissues, 5 buccal carcinoma tissues, and 7 non-cancerous tissues of the tongue were obtained from patients at Yokohama City University Hospital (Yokohama). All of the surgical specimens were stained with Lugol's solution. They were then washed with PBS several times to remove surface-adherent bacteria, then frozen immediately in liquid nitrogen and stored at −80°C until use. Informed consent was obtained from all the patients. This study was approved by the ethical committees of the National Cancer Center and Yokohama City University.

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Bacterial strains. *S. anginosus* ATCC 33397, *S. intermedius* ATCC 27335, *S. constellatus* ATCC 27823, *S. mutans* LM 7, *S. sobrinus* AHT, *S. sanguis* ATCC 10556, *S. gordonii* ATCC 10558, *S. mitis* ATCC 6249, and *S. salivarius* ATCC 9759 were cultured and centrifuged. The resulting pellet was treated with 20 mg/ml lysozyme before DNA extraction.

DNA extraction. Genomic DNA was isolated from tissue samples and bacteria by a standard phenol-chloroform method or a filtration method. DNA content was determined spectrophotometrically.

Primers and PCR amplification. 16S rDNA sequences from 11 species, which cover the dominant oral streptococcal species groups,[15] were obtained from the GenBank database[18] and aligned by the computer program Clustal W.[19] *S. anginosus* ATCC 33397T was chosen from several *S. anginosus* strains for the alignment, since this strain and the ATCC 33397T-like strains are closely associated with infection.[20] Primers for amplification of 16S rDNA of *S. anginosus* were designed based on the sequence of *S. anginosus* ATCC 33397T in alignment. Sequences of primers were as follows: forward primers, F0 (5′-GAACGGGTGAGTAACGCGTAGGTA-3′), F1 (5′-CAAGTAGGACGCACAGTTTA-3′), F2 (5′-AAGTAGGACGCACAGTTTAT-3′), F3 (5′-CGTAGCTTGCTACACCATAG-3′), F4 (5′-GTAGCTTGCTACACCATAGA-3′); reverse primers, R0 (5′-AAGCATCTAACATGTGTTACATAC-3′), R1 (5′-AGCATCTAACATGTGTTACATA-3′), R2 (5′-AAGCATCTAACATGTGTTACAT-3′), R3 (5′-CAAGCATCTAACATGTGTTAC-3′). F0 and R0 are the same as St1 and St3, respectively, in our previous report.[6] PCR for amplification of 16S rDNA of *S. anginosus* was performed in a total volume of 25 μl containing 2 μM of each primer and 50 fg to 50 ng of template DNA. The thermal cycling conditions were 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 s with a decrease of 0.2°C per cycle, and extension at 72°C for 30 s.

Quantitative real-time PCR. Quantification of *S. anginosus* 16S rDNA was performed using real-time PCR based on *Taq*Man chemistry (ABI Prism 7700 Sequence Detection System, Applied Biosystems, Foster City, CA). The F1 and R3 primers were chosen for use in the quantification, and the *Taq*Man probe (5′-AGCTTGCTACACCATAGACTGTGAGTTGCGA-3′) was designed by the Primer Express 1.0 software package (Applied Biosystems) to perfectly complement the 16S rDNA gene of *S. anginosus* downstream of the forward primer. The *Taq*Man probe was labeled at the 5′ end with reporter dye (6-FAM) and at the 3′ end with quencher dye (TAMRA). The reaction mixture in a total volume of 25 μl contained *Taq*Man Universal PCR Master Mix (Ampli*Taq* Gold, Amperase uracil-*N*-glycosylase, Applied Biosystems), 200 nM of each primer, a 120 nM probe, and 5 μl of DNA solution. The thermal cycling conditions were set to activate AmpErase uracil-*N*-glycosylase at 50°C for 2 min and activate Ampli*Taq* Gold at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s and annealing and extension at 60°C for 1 min. Human DNA was quantified by real-time PCR with *Taq*Man β-actin control reagent (Applied Biosystems) according to the manufacturer's instructions.

Statistical analysis. Differences in the quantity of *S. anginosus* DNA in the DNA samples from cancer tissues were statistically analyzed using the Mann-Whitney *U* test.

Results

Specificity and sensitivity of the primers for detecting *S. anginosus* DNA. Fig. 1 shows part of an alignment of the 16S rDNA of 11 species of *Streptococcus* and regions of the primers for detecting *S. anginosus*. Four forward and three reverse primers that were designed based on the sequence of *S. anginosus* ATCC 33397T in the variable region are shown above the alignment (F1–4, R1–3). F0 and R0 are previously reported primers.[6] All combinations of the 5 forward and 4 reverse primers were tested for specificity and sensitivity for detecting *S. anginosus*. Using 5 pg of *S. anginosus* DNA and primers F1 and R3 enabled strong amplification of a 160 bp DNA fragment, which is the expected size considering the position of the primers (Fig. 2A). However, only a faint band was detected for *S. salivarius*, even when using 50 000 pg of DNA as a template (Fig. 2A). A similar result with slightly weaker bands was obtained from *S.*

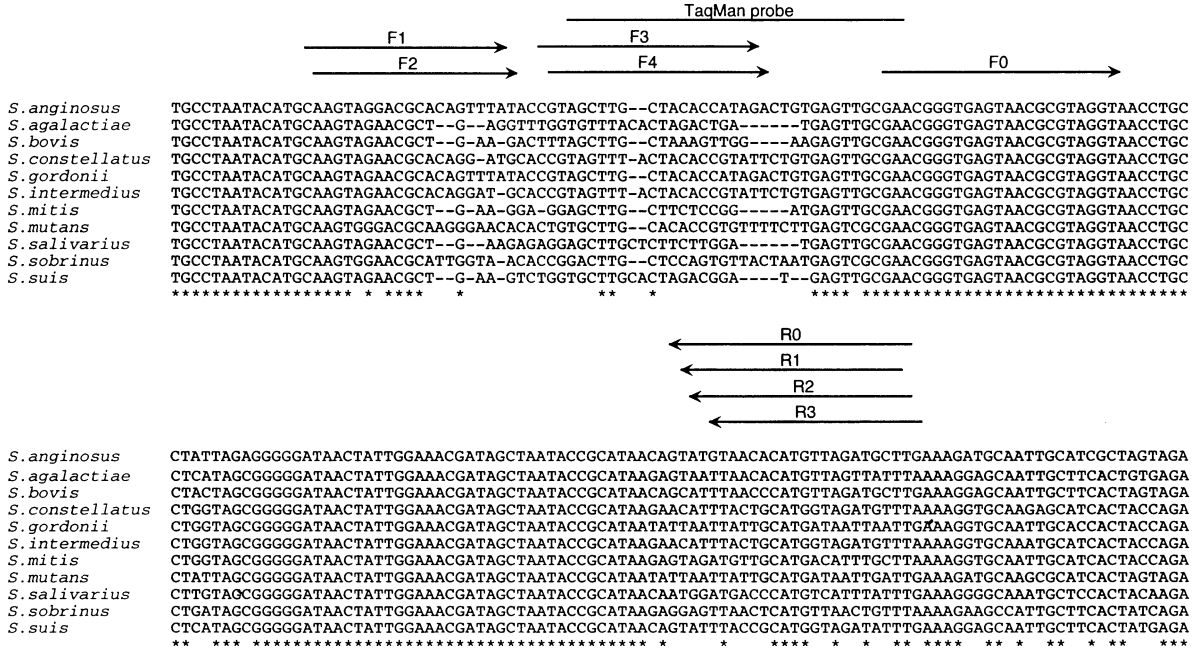

Fig. 1. Primers and *Taq*Man probe for specific detection of *S. anginosus* that were designed based on an alignment of the 5′ part of 16S rDNA from 11 species of *Streptococcus*. Arrows and the bar indicate primers and the *Taq*Man probe respectively. Stars represent identical nucleotides.

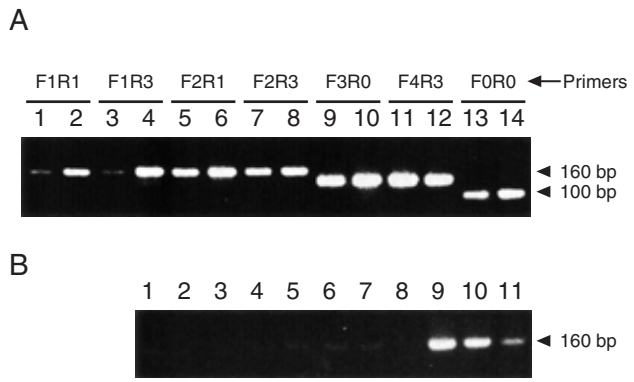

Fig. 2. Test of PCR primer sets for specific detection of *S. anginosus* DNA. A, 5 pg of *S. anginosus* DNA (lanes 2, 4, 6, 8, 10, 12, 14) and 50 000 pg of *S. salivarius* DNA (lanes 1, 3, 5, 7, 9, 11, 13) were amplified with the primer sets shown above the bars. B, 50 pg of DNA from *S. salivarius* (lane 1), *S. mutans* (lane 2), *S. sobrinus* (lane 3), *S. sanguis* (lane 4), *S. gordonii* (lane 5), *S. mitis* (lane 6), *S. constellatus* (lane 7), *S. intermedius* (lane 8), or *S. anginosus* (lane 9), and 5 pg and 0.5 pg of *S. anginosus* DNA (lane 10 and lane 11) amplified with the primer set F1 and R3.

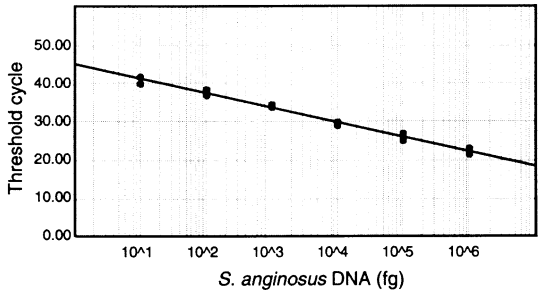

Fig. 3. Standard curve for *Taq*Man PCR. 10^1 to 10^6 fg of DNA of *S. anginosus* were assayed by real-time PCR. The threshold cycle number, which corresponds to the PCR cycle number at which the fluorescence signal exceeded the detection threshold, was plotted against each standard DNA. Correlation coefficient=0.989.

anginosus DNA using the F1 and R1 primers (Fig. 2A). In contrast, PCR using the remaining combinations of primers demonstrated that rDNA fragments are equally amplified in *S. anginosus* and *S. salivarius* samples when a concentration of *S. salivarius* DNA 10 000 times greater was used (Fig. 2A). This suggests that large amounts of *Streptococcus* might yield bands similar to that of *S. anginosus* in PCR analysis even when using specific primers for *S. anginosus*. We also examined amplification of a DNA fragment in other streptococcal strains using the F1 and R3 and the F1 and R1 primer sets, but observed no strong bands with either (Fig. 2B). Therefore, we concluded that F1 and R3, as well as F1 and R1, are highly specific and sensitive primers for detecting *S. anginosus*.

Real-time PCR for quantifying *S. anginosus* DNA. Absolute quantification of DNA is based on generating a standard curve with external standards. To this end, 10^0 to 10^6 fg of *S. anginosus* DNA in a series of 10-fold dilutions was assayed by real-time PCR. The standard curve was created by plotting the C_t number, the cycle number at which the fluorescence signal crossed the detection threshold, against each DNA concentration tested spectrophotometrically. The amounts of DNA plotted against the C_t values were linear over the range of 10^1 to 10^6 fg (r>0.989, Fig. 3), indicating that *S. anginosus* DNA can be properly quantified within this range. The quantities of *S. anginosus* DNA in test samples were then derived from the C_t numbers and the standard curve. The specificity of this assay for *S. anginosus* was determined using other *Streptococcus* species, including those which have the greatest similarities with *S. anginosus* in the regions of the primers and probe, based on the GenBank data base. When 10^6 fg of genomic DNA from the seven *Streptococcus* species were assayed, the calculated values derived from the C_t numbers were lower than the detectable level, 10^1 fg (data not shown). These results demonstrate that this system properly quantifies not only *S. anginosus*, but also other species.

Quantities of *S. anginosus* DNA in tumor tissue samples. The amount of *S. anginosus* DNA in 38 oral and 18 esophageal cancer tissues was assessed by real-time PCR (Table 1). Ten nanograms of genomic DNA from the esophageal cancer tissues were assayed. Eight of 18 samples (44%) contained detectable levels of *S. anginosus* DNA. Five of these yielded over 10^2 fg, and the maximum found was 1.9×10^3 fg. Assuming that 1 cell of *S. anginosus* contains about 5 fg of genomic DNA and 1 human cell contains about 0.006 ng of genomic DNA, it was estimated that approximately 0.2 cell of *S. anginosus* was present per 1 human cell at the highest level. In contrast, only 5 of 38 oral cancer tissue samples (13%) showed detectable levels of *S. anginosus* DNA, and only 1, derived from the tongue, had over 10^2 fg. One other sample, of lingua cancer, contained a detectable level of *S. anginosus* DNA. Two of 5 samples from the floor of the mouth, and 1 of 5 from the buccal mucosa, yielded detectable levels of *S. anginosus* DNA. Neither mandibular nor maxillary gingival cancer samples contained a detectable level of *S. anginosus* DNA. Statistical analysis indicated that the difference between the quantities of *S. anginosus* DNA in esophageal and oral cancer tissues was significant (P<0.01), assuming that the samples with undetectable levels of *S. anginosus* DNA were all at the same level, 10 fg.

Quantities of *S. anginosus* DNA in non-cancerous tissue samples. Quantities of *S. anginosus* DNA were determined in DNA samples from non-cancerous tissues. Five out of 6 esophageal and all 7 lingua normal tissues showed an undetectable level of *S. anginosus* DNA (Table 2).

Quantities of human DNA in tumor tissue samples. Quantities of human DNA were determined in DNA samples from tissues by real-time PCR. Each sample yielded a similar value to the whole genomic DNA measured spectrophotometrically (data not shown). These results indicated that the genomic DNA isolated from tissue samples was mostly human DNA.

Discussion

We determined the amounts of *S. anginosus* in oral and esophageal cancer tissues by quantitative real-time PCR. The method that we established enables very sensitive and specific detection of *S. anginosus* DNA. The level of sensitivity is 10 fg of *S. anginosus* DNA, and the other phylogenetically closely related species presently classified did not interfere with the quantification.

The conventional PCR method was employed in detecting *S. anginosus* DNA in esophageal, gastric, and head and neck cancer tissues,[6, 10] although its accuracy is not as good as real-time PCR. It was also reported that gingival smears from cancer patients contain *S. anginosus*.[10] In addition, epithelial tissues in the oral cavity and the esophagus are exposed to similar kinds of oral bacteria through the saliva. Therefore, we expected that *S. anginosus* would be present to the same degree in oral cancer tissues as in other upper digestive tract cancers. However, quantitative real-time PCR revealed a low frequency and small amounts of *S. anginosus* DNA in oral cancer tissues. Genomic DNA from tissue samples was mostly human DNA. When 10 ng of genomic DNA from the tissue samples was assayed, the maximum value of *S. anginosus* DNA in esophageal cancer tis-

Table 1. *S. anginosus* load in esophageal and oral cancer tissues

Oral cancer				Esophageal cancer	
Case	*S. anginosus* DNA[1)] (fg/10 ng total DNA)	Case	*S. anginosus* DNA[1, 2, 3)] (fg/10 ng total DNA)	Case	*S. anginosus* DNA[1, 2)] (fg/10 ng total DNA)
Floor of the mouth		Tongue		Esophagus	
1	ND	20	ND	39	ND
2	ND	21	ND	40	$9.5\ (1.3)\times10^{1}$
3	ND	22	ND	41	ND
4	$1.5\ (0.3)\times10^{1}$	23	ND	42	$3.0\ (2.0)\times10^{1}$
5	$1.1\ (0.2)\times10^{1}$	24	ND	43	$1.3\ (0.2)\times10^{2}$
		25	ND	44	ND
Mandibular gingival		26	$1.9\ (0.7)\times10^{1}$	45	ND
6	ND	27	ND	46	ND
7	ND	28	ND	47	ND
8	ND	29	ND	48	$1.9\ (0.9)\times10^{1}$
9	ND	30	$2.0\ (0.6)\times10^{2}$	49	$2.6\ (0.5)\times10^{2}$
10	ND	31	ND	50	ND
		32	ND	51	$2.7\ (0.7)\times10^{2}$
Maxillary gingival		33	ND	52	$1.5\ (0.8)\times10^{2}$
11	ND	34	ND	53	ND
12	ND	35	ND	54	ND
13	ND	36	ND	55	ND
14	ND	37	ND	56	$1.9\ (0.6)\times10^{3}$
		38	ND		
Buccal mucosa					
15	ND				
16	ND				
17	$3.0\ (1.3)\times10^{1}$				
18	ND				
19	ND				

1) Mean number of triplicate experiments and standard deviation in parenthesis.
2) 10 ng DNA extracted from tissue samples were used.
3) ND: $<1.0\times10^{1}$.

sues was $1.9\ (0.6)\times10^{3}$ fg, but in oral cancer tissues it was ten times lower [$2.0\ (0.6)\times10^{2}$ fg]. The quantity of *S. anginosus* DNA was found to be significantly lower in oral cancer tissues than in esophageal cancer tissues ($P<0.01$). The frequency of *S. anginosus* DNA also differed in that the percentage of subjects with a detectable level of *S. anginosus* DNA (>10 fg) was about three times lower for oral cancer tissues (13%) than for esophageal cancer tissues (44%). In addition, five different oral cancer sites were examined and none yielded high frequencies of *S. anginosus* infection or contained large amounts of DNA. Two of 5 cancer tissues from the floor of the mouth did contain detectable levels of *S. anginosus* DNA, but the values were not as high as in esophageal cancer. On the other hand, both the normal esophageal and the normal oral tissue samples gave similar results, i.e., undetectable or only a low level of *S. anginosus* DNA.

In our previous report,[6)] 14 of 15 (93%) esophageal cancers showed positive. In another report,[10)] 4 of 10 (40%) head and neck cancers showed positive when they used our original primers, while 100% of head and neck cancers showed positive with other primers for detecting *S. anginosus*. In our previous report, 100 ng of cancer tissue DNA was used for 35 cycles of PCR. In this study, only 10 ng of DNA was used. *S. anginosus* DNA was then quantified within the range of linear amplification. This explains why there is a discrepancy between our present results and our previous report.

With the present method, further study such as comparison of *S. anginosus* DNA content of cancer tissues of different parts of the body or comparison of *S. anginosus* DNA content between ulcerated (usually with inflammation) and non-ulcerated portions of cancer tissues should be performed so that the pathophysiology of *S. anginosus* in cancer development may be better understood. Investigating bacteria other than *S. anginosus* involved in cancers of the upper digestive tracts is also important. A real-time PCR system can not be used to investigate the roles of various kinds of bacterial species simultaneously, because suitable specific primers or probes are not available. Therefore, we have to perform PCR using universal primers to detect a variety of bacteria and then sequence DNA clones in the tissue samples. Unfortunately, a comprehensive detection system has still not been developed. Thus, we can not state whether or not other bacterial species may also be involved in esophageal and oral cancer. However, it appears that *S. anginosus* increases specifically in esophageal cancer tissues, and we speculate that there is a strong association of this bacterium with esophageal cancer.

Table 2. *S. anginosus* load in normal tissues of esophagus and tongue

Tongue		Esophagus	
Case	*S. anginosus* DNA[1, 2, 3)] (fg/10 ng total DNA)	Case	*S. anginosus* DNA[1, 2, 3)] (fg/10 ng total DNA)
57	ND	64	ND
58	$1.5\ (0.7)\times10^{1}$	65	ND
59	ND	66	ND
60	ND	67	ND
61	ND	68	ND
62	ND	69	ND
63	ND		

1) Mean number of triplicate experiments and standard deviation in parenthesis.
2) 10 ng DNA extracted from tissue samples were used.
3) ND: $<1.0\times10^{1}$.

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