

The Mouthwash: A Non-Invasive Sampling Method to Study Cytokine Gene Polymorphisms

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Background: We describe a simple, non-invasive mouthwash sampling method for rapid DNA isolation to detect cytokine gene polymorphisms. In the present paper, interleukin-1 β (*IL-1B*) and interleukin-1 receptor antagonist (*IL-1RN*) gene polymorphisms were studied.

Methods: Two mouthwash samples and blood samples were collected from 11 healthy individuals. The second mouthwash sample was stored for 7 days at room temperature. Polymerase chain reaction amplification was used to identify a bi-allelic polymorphism at position +3953 in the *IL-1B* gene and a variable number of tandem repeats (VNTR) polymorphism in the *IL-1RN* gene.

Results: Our results show that the typing of these cytokine gene polymorphisms using DNA isolated from mouthwash samples did not differ from those obtained by a phenol/chloroform isolation method from EDTA anti-coagulated blood. Moreover, reliable results from mouthwash samples were obtained after storage for at least 7 days at room temperature.

Conclusions: Mouthwash can be the method of choice to study gene polymorphisms in periodontitis and other chronic inflammatory diseases. *J Periodontol* 2000;71:1315-1318.

KEY WORDS

Cytokines; DNA/analysis; interleukin-1; mouthwashes; polymorphisms/genetic.

Pro-inflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and interferon-gamma (IFN- γ) are thought to be major pathological mediators in chronic inflammatory diseases including periodontitis.^{1,2} These cytokines are key regulators of the host response to a microbial challenge, and they are of special interest because of their bone metabolizing and inflammatory properties.^{3,4} Particularly, IL-1 has been reported to be a potent inducer of bone resorption in periodontitis.^{3,5}

Polymorphisms in the genes encoding IL-1 α (*IL-1A*)⁶, IL-1 β (*IL-1B*),⁷ and IL-1ra (*IL-1RN*)⁸ of the *IL-1* family have been described and an association between *IL-1* alleles and increased severity of chronic inflammatory diseases have been reported.^{6,9,10} Some cytokine gene polymorphisms may contribute to inter-individual variation in cytokine production upon microbial challenge.^{11,12} A previous investigation has demonstrated an association between a specific genotype of the *IL-1* gene family and severity of destructive periodontal disease in adult non-smokers.¹³

White blood cells are the main source of DNA for gene analysis. DNA isolation from anti-coagulated blood has, however, some disadvantages: donation is painful, medical supervision is needed, there is a risk of exposure to blood borne pathogens, and costs of DNA isolation are high. An alternative source of DNA may be a mouthwash, which is a painless, simple, non-invasive and rapid sampling method.^{14,15}

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The aim of the present study was to determine the suitability of the mouthwash method for the detection of cytokine gene polymorphisms. In addition, the effect of storage of the mouthwash samples at room temperature for 7 days on the polymerase chain reaction amplification was tested. For the purpose of this study, a bi-allelic polymorphism at position +3953 in the *IL-1B*⁷ gene and a variable number of tandem repeats (VNTR) polymorphism in the *IL-1RN* gene⁸ were studied.

MATERIALS AND METHODS

DNA Isolation From Mouthwash

Eleven healthy individuals gave informed consent to participate in the study. The individuals were not allowed to clean their teeth or to eat 30 minutes before sampling. Each individual rinsed out his/her mouth twice with 10 ml of 0.9% saline for 60 seconds. The first sample was stored at room temperature and processed 7 days later. The second sample was processed immediately. DNA was extracted according to the method of Lench et al.¹⁴ as modified by de Vries et al.¹⁵ Briefly, mouthwash samples were centrifuged at 300 × g for 10 minutes. The pellet was washed twice in 0.9% saline, resuspended in 100 µl of 50 mM NaOH, and boiled for 10 minutes. Samples were neutralized with 14 µl of 1 M Tris (pH 7.5) and centrifuged at 14,000 × g for 3 minutes. Supernatants were collected and stored at 4°C until polymerase chain reaction (PCR) analysis.

DNA Isolation From Peripheral Blood

Blood was collected by venous puncture. Genomic DNA was extracted from EDTA anti-coagulated peripheral blood using a standard proteinase K digestion and phenol/chloroform extraction procedure.

Polymorphisms in the IL-1 Gene Family

Restriction fragment length polymorphism (RFLP) at position +3953 in the *IL-1B* gene. The region that contains the *TaqI* polymorphic site within exon 5 of the *IL-1B* gene was amplified by PCR.⁷ The oligonucleotides 5' GTTGTCATCAGACTTTGACC 3' and 5' TTCAGTTCATATGGACCAGA 3' flanking this region were used as primers. Amplification was performed using a thermal cycler[†] according to the following parameters: 97°C for 90 seconds, 55°C for 90 seconds, and 74°C for 60 seconds for 3 cycles, followed by 32 cycles: 97°C for 30 seconds, 55°C for 30 seconds, and 74°C for 30 seconds. Finally, an incubation at 72°C for 10 minutes was followed by cooling to 4°C. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with 0.1% ethidium bromide. *TaqI* digestion of the 249-base pair (bp) fragment resulted in fragments that either remained intact (allele 2) or were cut in two fragments of 135 bp and 114 bp, respectively (allele 1). Fragments were analyzed by

Table 1.

Results of Genotype Frequencies

Genotypes	Mouthwash (n)	Blood (n)
<i>IL-1B TaqI</i>		
1.1	8	8
1.2	1	1
2.2	2	2
<i>IL-1RN VNTR</i>		
1.1	5	5
1.2	5	5
1.3	1	1

electrophoresis on 3% agarose gels containing 0.1% ethidium bromide.

***IL-1RN VNTR* polymorphism.** The region within the second intron of the *IL-1RN* gene that contains variable numbers of a VNTR of 86 bp was amplified by PCR. The oligonucleotides 5' CTCAGCAACACTCCTAT 3' and 5' TCCTGGTCTGCAGGTAA 3' flanking this region were used as primers.¹⁶ Amplification was performed using a thermal cycler.[†] The PCR conditions were 94°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 56°C for 1 minute, and elongation at 72°C for 1 minute. Finally, an elongation at 72°C for 5 minutes was followed by cooling to 4°C. The PCR products of 410 bp (allele 1 = four repeats of the 86-bp region), 240 bp (allele 2 = two repeats), 500 bp (allele 3 = five repeats), 325 bp (allele 4 = three repeats) and 595 bp (allele 5 = six repeats) were analyzed by electrophoresis on a 2% agarose gel stained with 0.1% ethidium bromide.

RESULTS

No DNA isolation failure occurred. It was possible to analyze PCR products from all mouthwashes and blood DNA isolates from 11 individuals. The oral rinses could be stored for 7 days at room temperature with reliable results of the *IL-1B* and *IL-1RN* genotypes.

The results of the *IL-1B* +3953 and *IL-1RN* VNTR gene polymorphisms assays from mouthwash and from blood DNA isolates were identical for each individual as summarized in Table 1. Figure 1 shows the *IL-1B* +3953 genotypes from 2 individuals and its detection in 3 different DNA isolates: mouthwash at the day of sampling, mouthwash 7 days after sampling and storage at room temperature, and white blood cells.

DISCUSSION

Sampling of venous blood to study cytokine gene polymorphisms is impractical in a large epidemiological survey and in clinical settings. Therefore, the pos-

[†] Perkin-Elmer 9700, Applied Biosystems, Foster City, CA.

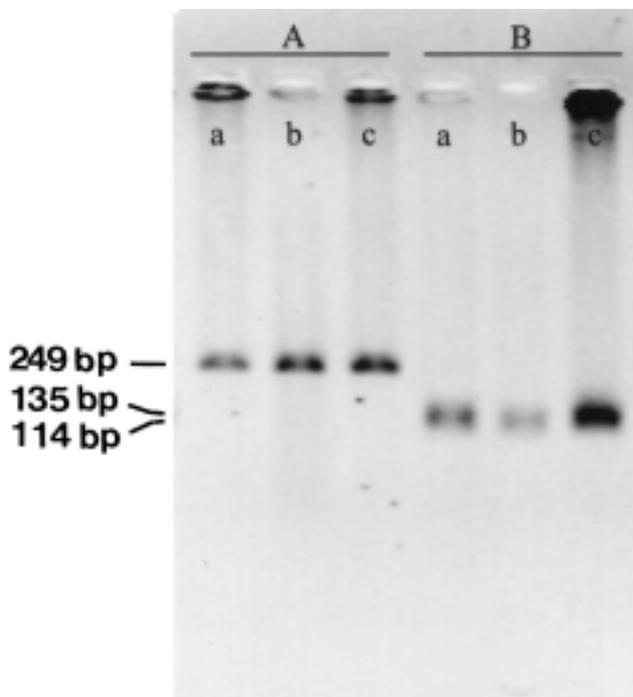


Figure 1.

Photograph of a 2% agarose gel showing analysis of the IL-1B gene polymorphism (TaqI RFLP at position +3953) in 2 individuals. DNA from each individual was isolated from mouthwash at the day of sampling (a), from mouthwash 7 days after sampling and storage at room temperature (b), and from white blood cells (c). Individual A genotype 2.2 and individual B genotype 1.1.

sibility of a simple non-invasive mouthwash sampling method used in other genetics studies was investigated.¹⁷⁻¹⁹ In the present study, the mouthwash method was found to be reproducible in detecting polymorphisms in *IL-1B* and *IL-1RN* cytokine genes.

Pro-inflammatory (IL-1 α , IL-1 β) and anti-inflammatory cytokines (IL-1ra) have been suggested to be important in the regulation of intestinal inflammation.^{9,16,20} Polymorphisms in the *IL-1* gene family have been reported to be associated with several chronic inflammatory diseases such as intestinal and periodontal diseases^{13,21,22} as well as a prognostic factor in multiple sclerosis.¹⁰

In the present study, results from DNA obtained from mouthwash and blood samples reached 100% agreement in the 11 subjects studied. However, we cannot rule out the possibility that in a larger study group this correlation might be less; e.g., because of inhibiting factors and contaminating DNA, originating from oral bacteria. Homologous DNA sequence of contaminating DNA might give rise to an amplification product of equal length to that derived from human genomic DNA. However, in accordance with results of de Vries et al.¹⁵ we did not find any evidence for this. In the analysis of other genes the presence of homologous

DNA sequences from other than human origin should be ruled out.

The high efficiency in the detection of cytokine gene polymorphisms using DNA isolates from mouthwash allows screening of a large number of individuals. Different conditions such as temperature and storage were tested to validate this technique. Storage of the samples for 7 days at 4°C or at room temperature did not affect the reliability of the detection of the gene polymorphisms.

This mouthwash method does not only allow a large-scale sampling in dental practices but also offers the possibility to mail the samples to a diagnostic laboratory without taking special precautions related to duration or temperature. Moreover, mouthwash samples were also successfully used in our laboratory for the detection of other cytokine gene polymorphisms.

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