A New Serotype-Specific Antigen, Designated Serotype g, from *Actinobacillus actinomycetemcomitans*

Masanori Saito

Nihon University Graduate School of Dentistry at Matsudo, Clinical Pathology, Matsudo, Chiba 271-8587, Japan

Correspondence to :

Masanori Saito

E-mail : saito.masanori@nihon-u.ac.jp

Abstract

During the isolation of *Actinobacillus actinomycetemcomitans* from periodontal pockets, a serologically untypeable strain, designated NUM-Aa 4039, was observed. Biochemical and serological identification and PCR-based analysis confirmed that NUM-Aa 4039 was a strain of *A. actinomycetemcomitans*. An antigen from NUM-Aa 4039 did not react with a to e serotype-specific antibodies in double-immunodiffusion experiments. DNA from the strain could not be amplified by PCR using specific PCR DNA primer of a to f serotype-specific O-polysaccharide gene cluster. The antigen was extracted from whole cells by autoclaving and purified by ion-exchange and gel filtration chromatography. The molecular weight was approximately 180,000. The new antigen was composed of glucose, rhamnose and N-acetyl-glucosamine. Double immunodiffusion and ELISA experiments with antigens and antibodies showed that the antigen had individual antigen determinant. I propose that untyped strain be assigned to a new *A. actinomycetemcomitans* serotype, designated serotype g.

Keywords :

A. actinomycetemcomitans, Serotype, Antigen, Juvenile periodontitis

Introduction

Actinobacillus actinomycetemcomitans, a gram-negative facultatively anaerobic coccobacillus, is implicated in the etiology of localized aggressive periodontitis (LAP), a severe form of periodontal disease, as well as non-oral infections, mostly bacterial endocarditis, and produces several potential virulence factors including leukotoxin and cytolethal distending toxin (1, 2). Serological investigations with polyclonal rabbit sera have led to the recognition of five *A. actinomycetemcomitans* serotypes, designated a to e (3-6). Recently, a new serotype, f, based on a serotype-specific O polysaccharide gene cluster was proposed (7). Among serotype a to e strains, serotype a and b strains are frequently isolated from LAP patients, serotype c strains are more common in nonoral infections and in healthy people, and serotype d and e strains are rare in all populations (3, 8-10). The serotype f strains are very similar in antigenicity to serotype b strains. Approximately 3 to 9% of *A. actinomycetemcomitans* isolates yet

remain untypeable with any of the a to e serotype-specific antisera (11).

The aim of the present study was to characterize an untypeable *A. actinomycetemcomitans* strain isolated from a patient with periodontitis, and to compare it with representative serotype a to f strains.

Materials and Methods

Bacterial strains and culture conditions

Actinobacillus actinomycetemcomitans ATCC29523 (serotype a), ATCC 43718 (b), ATCC 33384 (c), IDH781 (d), OMZ541 (e) and NUM-Aa 5005 (f) were used in this study. The new serotype strain, designated NUM-Aa 4039, was isolated from periodontal pockets 6 mm or greater in depth from an adult periodontal patient.

The strains were maintained on brain heart infusion (BHI, Difco Laboratories, Detroit, MI.) supplemented with 1% yeast extract (Difco) and 5% (v/v) rabbit blood and 1.5% agar (BHIY-blood). The organisms were cultured for 72h at 37 °C in 5% CO₂

incubator (Napco® Model 5400, Precision Scientific, IL, USA).

Biochemical analyses

The following biochemical tests were performed according to the procedures outlined by the manufacturer of the minitek system (BBL Microbiology systems, Cockeysville, MD): fermentation of glucose, fructose, mannose, maltose, raffinose, sucrose, lactose, cellobiose, trehalose, melibiose, arabinose, and salicin; and production of catalase.

PCR analysis

PCR analysis was performed directly on a single colony. A single colony was suspended in 25 µl of distilled water and boiled for 5 min, and 5 µl of the suspension was used as a template. The PCR running conditions and primers for the leukotoxin, species-specific 16S rRNA and O-PS gene clusters of *A. actinomycetemcomitans* (Table 1) in this study were described previously (7, 12, 13). PCR products were analyzed by 2.0% agarose gel electrophoresis. A 100

-bp DNA ladder (Takara Biomed., Otsu, Japan) was used as a molecular size marker.

Serological analyses

Serological analyses of various antigen and anti-serum preparations were conducted using the double immunodiffusion agar method (1). Rabbit antisera raised against reference strains were prepared as described previously (14, 15).

Preparation of a serotype-specific antigen

Rantz-Randall extracts were obtained by autoclaving dry cell suspension (300 mg/ml) from NUM-Aa 4039 in saline (16). The extracts were processed by two-column chromatography as suggested by Shibuya et al. (17). Briefly, the antigen was purified by DEAE Sephadex A-25 (Amasham Biosci. NJ) ion-exchange chromatography and gel filtration. An estimate of the molecular weight of the serotype-specific antigen was obtained by gel filtration using Sephacryl S-300 (1.5×130 cm; fractionation range of 20,000 to 400,000; Amersham). The gel filtration column was standardized with blue dextran and dextran T150, T10 (Pharmacia) and glucose. The polysaccharide content of antigen preparations was determined by a phenol-sulfuric acid method with glucose as the standard.

Enzyme-linked immunosorbent assays (ELISAs)

The procedure used for ELISAs was suggested by Kaplan et al. (7) with some modifications. Antigens used in this experiment were 200- and 100-fold dilutions of column-purified serotype-specific antigen and Rantz Randall extract from *Escherichia coli*. The serotype specific antibodies were diluted 1000-fold with blocking buffer. The secondary antibody, goat anti-rabbit immunoglobulin G alkaline phosphatase-conjugate (Funakoshi Co.), was diluted 2000-fold with blocking buffer.

Sugar composition

The column-purified polysaccharide antigen was hydrolyzed with 2N trifluoroacetic acid for neutral sugars and 4N HCl for amino sugars at 100 °C for 6h.

Table 1. *A. actinomycetemcomitans* serotype-specific PCR assay

Primer pairs*(5'-3')	amplicon length in bp
16SrRNA (<i>A. actinomycetemcomitans</i>)	557
f: AAA CCC ATC TCT GAG TTC TTC TTC	
r: ATG CCA ACT TGA CGT TAA AT	
Leukotoxin	285
TT15: TCG CGA ATC AGC TCG CCG	
TT16: GCT TTG CAA GCT CCT CAC C	
O-PS gene cluster	
serotype b P11: TCT CCA CCA TTT TTG AGT GG	333
serotype c P12: GAA ACC ACT TCT ATT TCT CC	268
serotype f P13: CCT TTA TCA ATC CAG ACA GC	232
universal primer for serotype b, c, f	
P14: ARA AYT TYT CWT CGG GAA TG	
serotype a P15: TGG GTC ATG GGA GGT ACT CC	293
P16: GCT AGG AAC AAA GCA GCA TC	
serotype d P17: TGG AAC GGG TAT GGG AAC GG	411
P18: GGA TGC TCA TCT AGC CAT GC	
serotype e P19: ATT CCA GCC TTT TGG TTC TC	311
P20: TGG TCT GCG TTG TAG GTT GG	

*R=A or G; Y=C or T; W=A or T

The hydrolysate was evaporated to dry and dissolved with water. Neutral sugars in the hydrolysates were analyzed by HPLC (LC-9A, Shimadzu Co.) using a TSK-gel Sugar AXG column (150×4.6 mm; Toso Co.). Amino sugars were analyzed with an amino acid analyzer (L-8500, Hitachi Co.).

Results

Biochemical analyses of NUM-Aa 4039

The strain NUM-Aa 4039 was gram-negative, coccobacillus and facultative. It fermented glucose, fructose, mannose maltose, and raffinose, but not sucrose, lactose, cellobiose, trehalose, melibiose, arabinose or salicin, and produced catalase.

Fig.1. PCR-amplification of DNA of *A. actinomycetemcomitans* strains using leukotoxin (285bp) and 16S rRNA (557bp). Lane 1, ATCC43718; 2, NUM-Aa 4039; M, 100 bp ladder.

Fig.2. PCR amplification of DNA of *A. actinomycetemcomitans* strains using O-PS (serotype a-f) primers. Primers used in this experiment are: a, serotype a; b, serotype b; c, serotype c; d, serotype d; e, serotype e; and f, serotype f. Lane 1, ATCC29523 (serotype a); 2, ATCC43718 (b); 3, ATCC33384 (c); 4, IDH781 (d); 5, OMZ541 (e); 6, NUM-Aa 5005 (f); 7, NUM-Aa 4039; M, 100 bp ladder.

PCR analyses

Leukotoxin and species-specific products, 285 bp and 557 bp, respectively corresponding to *A. actinomycetemcomitans* were observed in tested strains, ATCC43718 and NUM-Aa 4039 (Fig. 1). Fig. 2 shows the gel of the electrophoresis pattern of PCR products using the serotype-specific primers. The serotype-specific PCR-amplified products of a through f were 293, 333, 268, 411, 311, 232-bp fragments, respectively. Each serotype strain has a specific product. However, NUM-Aa 4039 was not amplified by any of the primers.

Double immunodiffusion analysis for antigenic sites of serotype-specific polysaccharides

Fig. 3 shows a double immunodiffusion precipitin reaction pattern obtained with anti-NUM-Aa 4039 serum tested against serotype-specific polysaccharides. The anti-NUM-Aa 4039 serum gave a single band with the corresponding NUM-Aa 4039 antigen but did not react with any of the antigens to the other serotypes. Each serotype antibody reacted predominantly with the corresponding antigen (Fig. 3).

Fig. 3. Double immunodiffusion analysis of the antigens in the autoclave extract of *A. actinomycetemcomitans* with serotype-specific antibodies. Well 1, ATCC29523 (serotype a); 2, ATCC43718 (b); 3, ATCC33384 (c); 4, IDH781 (d); 5, OMZ541 (e); 6, NUM-Aa 5005 (f); 7, NUM-Aa 4039; A, anti-a; B, anti-b; C, anti-c; D, anti-d; E, anti-e; F, anti-NUM-Aa 4039.

Column chromatography of the new serotype-specific antigen

The autoclaved extracts from NUM-Aa 4039 strain were fractionated on DEAE-Sephadex A-25 into flow-through, carbohydrate-rich fractions, which reacted with anti-NUM-Aa 4039 serum, by a double immunodiffusion agar method to determine the NUM-Aa 4039 antigen. Subsequent chromatography using Sephacryl S-300 for gel filtration was performed. Selected samples from the tubes representing the profile were tested against anti-NUM-Aa 4039 serum (Fig. 4). Elution profile was coincident in position with serotype-specific activities. The molecular weight of the purified antigen was estimated to be approximately 180,000.

Fig. 4. Elution profile of the new serotype-specific antigen from *A. actinomycetemcomitans* NUM-Aa 4039 on Sephacryl S-300. Vo, Void volume; Vt, total bed volume.

Fig. 5. Reactivity of NUM-Aa 4039 antibody with various antigens from *A. actinomycetemcomitans* as measured by ELISA. Negative control wells contained the Rantz and Randall extract from *Escherichia coli*. The mean values and SD for triplicate wells are shown.

Cross-reactivities of the new serotype-specific antigen with anti-a to -e and -NUM-Aa 4039 sera with ELISA

To demonstrate that the new antigen NUM-Aa 4039 is antigenically distinct from antigen a to e was tested in an ELISA using each antibody. The NUM-Aa 4039 antigen and its antibody showed strong specificity. The cross-reactivities with serotype a to e antibodies and NUM-Aa 4039 antigen were not observed (Fig. 5).

Sugar content of the new serotype-specific antigen

An analysis of the sugar content of the column-purified antigen was performed. The antigen was found to be mainly composed of glucose, rhamnose and N-acetyl-glucosamine (Table 2). Talose, fucose and N-acetyl-galactosamine, which composed a previously reported (7, 17, 18) serotype-specific determinant of *A. actinomycetemcomitans*, were not detected.

Table 2. Sugar composition of the column purified-antigen from NUM-Aa 4039 strain.

Sugar	Molar ratio, %
Glucose	38.5
Rhamnose	26.6
Xylose	7.3
Galactose	5.5
Mannose	4.6
Arabinose	1.8
Talose	0
N-Acetylglucosamine	12.8
N-Acetylgalactosamine	2.8

Discussion

Immunological studies of pathogenic bacteria have been highly valuable in elucidating microorganisms and specific microbial components involved in the etiology of infectious diseases. In view of the importance of *A. actinomycetemcomitans* in human periodontal disease, it was considered essential to establish the antigenic relationship between serotype-specific antigenicity and different types of periodontal disease. *A. actinomycetemcomitans* colonization of periodontal pockets may play facilitated by cell-surface carbohydrates (3) and serotype antigens may be a part (1). Microbial surface structures play a critical role in the initiation of infectious diseases. Among the surface components, a serotype-specific polysaccharide of *A. actinomycetemcomitans* is known to be a potent mediator of alveolar bone resorption, which is one of the major symptoms of periodontitis (3, 10). Serotype-specific antigens may be more pathogenic at periodontal sites and inhibit phagocytosis. *A. actinomycetemcomitans* strains are serologically classified into 6 distinct serotypes. The serotype-defining determinant of the antigens responsible for serotypes a to f has been proposed to be the polysaccharide side chain of the lipopolysaccharide (7, 18, 19).

Previous studies have led to the recognition of six serotypes of *A. actinomycetemcomitans*, designated a to f. However, 3% of isolates remained untypeable and are found in 5% of subjects (5). Results of immunodiffusion test and PCR in the present study

revealed that *A. actinomycetemcomitans* NUM-Aa-4039 differs from serotypes a to f, and should be designated a serotype g strain. The molecular weight of serotype-specific antigen from NUM-Aa 4039 was estimated to be approximately 180,000 which was similar to those of serotype a to c antigens (17). The serotype-specific antigenic determinants are D-talose for serotype a, D-fucose, L-rhamnose and N-acetyl-galactosamine for serotype b, L-talose for serotype c, D-glucose, D-mannose and L-rhamnose for serotype d, L-rhamnose and N-acetyl-glucosamine for serotype e, and L-rhamnose and N-acetyl-galactosamine for f (7, 17, 18). The new serotype-specific antigen was composed of glucose, rhamnose and N-acetyl-glucosamine. Thus, *A. actinomycetemcomitans* strains of all serotypes commonly produce polysaccharide antigens consisting of 6-deoxyhexoses. The 6-deoxyhexoses found in *A. actinomycetemcomitans* strains of all serotypes are closely related biosynthetically (17). The antigenic determinant of NUM-Aa 4039 remains to be identified. In this study I identified a seventh serotype of *A. actinomycetemcomitans*, serotype g.

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