Detection of cfxA and cfxA2, the β-lactamase genes of Prevotella spp., in clinical samples from dentoalveolar infection by real-time PCR

Detection of *cfxA/A2*, the β-lactamase gene of *Prevotella,* in clinical samples from dentoalveolar infection by real-time PCR

Kaori Iwahara,[1] Tomoari Kuriyama,[2*] Satoshi Shimura,[3] David W. Williams[4],

Maki Yanagisawa,[1] Kiyomasa Nakagawa,[1,2] and Tadahiro Karasawa [3]

Department of Oral and Maxillofacial Surgery, Kanazawa University Graduate School of Medical Science [1], Department of Oral and Maxillofacial Surgery, Kanazawa University Hospital [2], and Department of Clinical Laboratory Science, Kanazawa University Graduate School of Medical Science,[3] Kanazawa, Japan, and Department of Oral Surgery, Medicine and Pathology, School of Dentistry, Cardiff University, Cardiff, United Kingdom[4]

Running title: Detection of β-lactamase from pus by real-time PCR

[*]Corresponding author: Dr Tomoari Kuriyama

Mailing address: Department of Oral and Maxillofacial Surgery, Kanazawa University Hospital. 13-1 Takara-machi, Kanazawa 920-8640, Japan.

Phone: (81) 762652444.

Fax: (81) 762344202

E-mail: tomkuriyama@ybb.ne.jp

ABSTRACT

Whilst most bacteria involved in dentoalveolar infection are highly susceptible to penicillin, some *Prevotella* strains exhibit resistant to this agent through the production of β-lactamase. The production of β-lactamase by *Prevotella* is in turn associated with the expression of the gene, *cfxA/A2*. The aim of the present study was to determine the prevalence of *cfxA/A2* in *Prevotella* strains using real-time PCR and to assess the performance of this molecular method for the direct detection of the gene in 87 clinical samples (pus and root canal exudates) from dentoalveolar infection. Production of β-lactamase by each isolate was determined using a nitrocefin disk. β-lactamase production was seen in 31% of *Prevotella* isolates, whilst all isolates of other species were β-lactamase negative. The penicillin resistance of isolates strongly correlated with the production of β-lactamase. Real-time PCR was found to detect the *cfxA/A2* gene from at least five cells per reaction mixture (5×10^3 cfu/ml of pus). Using real-time PCR, *cfxA/A2* was evident for all 48 β-lactamase-positive *Prevotella* strains. In contrast, neither β-lactamase-negative *Prevotella* (n=91) or non-*Prevotella* strains (n=31) were positive for the gene. In this study, 31 of the 87 samples yielded β-lactamase-positive *Prevotella*, and *cfxA/A2* was detected in all 31 samples. Of the 56 culture-negative samples, 8 (14%) were positive for *cfxA/A2* by the real-time PCR. This sensitive and specific molecular method offers a rapid clinical test for aiding in the selection of an appropriate antibiotic for treatment of dentoalveolar infection. Although penicillin remains largely effective in the treatment of dentoalveolar infection, ß-lactamase stable antibiotics should be considered in cases where ß-lactamase positive *Prevotella* are involved.

INTRODUCTION

Despite the improvement of dental health in developed countries, patients with dentoalveolar infections are still encountered. The majority of infections are associated with necrotic dental pulp tissue, although periodontal diseases and pericornitis may also provide a source of infection (2). Drainage, which can be achieved by tooth extraction, surgical incision or through root canal, is the most important factor in treatment of dentoalveolar infections (9, 11, 23, 24). In addition to drainage, systemic antibiotic can be prescribed to prevent the spread of infection and onset of serious complications (9, 11, 23, 24). Members of the penicillin group of antibiotics have long been the first-line treatment for dental infections because of their suitable antimicrobial spectrum, bactericidal activity, low incidence of adverse effects and cost-effectiveness (1, 9, 11, 12, 23, 24).

The antimicrobial susceptibility of bacteria involved in the infection is a primary factor affecting the likely outcome of antibiotic therapy (9, 10, 24). The presence of penicillin-resistant bacteria is implicated as the cause of clinical failure of treatment in some cases of oral purulent infection (10). As a consequence, information determining whether penicillin-resistant bacteria are involved is important in predicting the effectiveness of treatment with penicillin for dentoalveolar infection. Although antibiotic sensitivity can be determined from standard cultural microbiological analysis, this generally takes several days due to the slow growth of fastidious anaerobic bacteria. Since infection can spread rapidly and cause severe complications such as sepsis and obstruction of the airway, such a delay can prove problematic and undesirable.

The introduction of polymerase chain reaction (PCR)-based techniques has resulted in the development of tests that can detect specific pathogens and genes directly

and rapidly from clinical samples. Indeed, conventional PCR has already become an important tool in clinical diagnostic and research laboratories. Recently, a more rapid, sensitive and reproducible PCR has been described and is termed real-time PCR (18). This approach also allows the quantitative assessment of target nucleic acid.

Dentoalveolar infection usually involves bacteria residing in the oral cavity. In particular, strict anaerobes such as *Peptostreptococcus*, *Prevotella* and *Fusobacterium*, and oral streptococci are the predominant isolates from the infection (9, 11-15, 23, 24). It has been demonstrated that the majority of these bacteria are highly susceptible to penicillin whilst some *Prevotella* strains have recently been reported as being resistant (9, 11-17, 24). Penicillin-resistance of *Prevotella* is closely associated with the production of β-lactamase, which is an enzyme that degrades β-lactam agents (12-15, 24). β-lactamase produced by *Prevotella* has the property of class A/group 2e β-lactamases, which hydrolyzes most penicillins and broad-spectrum cephalosporins but are inactive to penicillin combined with clavulanic acid and imipenem (3). Although the genetic basis of β-lactamase-production by *Prevotella* has not been clarified completely, it has been demonstrated that the *cfxA2* gene is associated with β-lactamase production (3, 6, 19). This gene shares 98% identity with *cfxA*, the structural gene of a β-lactamase produced by *Bacteroides vulgatus* (19).

The aim of this study was to determine the prevalence of *cfxA/A2* in *Prevotella* strains using real-time PCR and to assess the performance of this approach for direct gene detection in clinical samples from dentoalveolar infection.

MATERIALS AND METHODS

Clinical samples. A total of 87 clinical samples were obtained from patients with

dentoalveolar infection (53 males, 34 females; mean age 48.1 years) attending the Oral and Maxillofacial Surgery Clinic of Kanazawa University Hospital between September 2001 and March 2005. Each of the samples obtained were recovered from distinct and individual patients. Seventy-two pus samples (yellow thin pus, 50; blood-like pus 22) were taken by aspiration from the abscess using a disposable syringe with an 18-G needle. Each sample was stored at -80°C prior to analysis by real-time PCR, with a portion immediately used for cultural bacterial examination. The remaining 15 samples were pus-like exudates from the root canal of the tooth involved. These samples were obtained using three sterilized paper points (size: #25). Each sample held within the paper points was suspended in 100 µl of saline by vortexing vigorously for 20 s. A 20-µl volume of the suspension was cultured and the remainder stored at -80°C prior to DNA extraction.

Bacteriological culture examination. Samples were inoculated aerobically and microaerophilically on Brucella HK agar (Kyokuto, Tokyo, Japan) containing 5% (v/v) sheep blood for 48 h at 37°C. Culture of strict anaerobes was on Brucella HK agar with 5% (v/v) sheep blood, which was incubated in an anaerobic atmosphere for up to seven days at 37°C. Duplicate plates were prepared containing paromomycin (75 mg/L, Pfizer, Tokyo, Japan) and vancomycin (2.5 mg/L, Shionogi, Osaka, Japan) in order to selectively isolate strictly anaerobic Gram-negative bacilli. Isolates were identified by conventional methods (20). Bacterial growth was recorded to determine the approximate number of bacteria in each sample. After identification, a colony of each isolate was stored at –80°C.

β-lactamase production and antimicrobial susceptibility. The nitrocefin disk (Cefinase®; Becton Dickinson, Cockeysville, MA) was used to determine whether

strains were positive for β-lactamase production (12-14). Susceptibility of 242 randomly chosen clinical isolates (*Prevotella*, 139; *Fusobacterium*, 33; *Peptostreptococcus*, 23; *Campylobacter*, 13; *Porphyromonas*, 12; *Corynebacterium*, 4; *Veillonella*, 7; *Streptococcus*, 5; *Bacteroides*, 3; *Bifidobacterium*, 1; *Gemella*, 1; *Lactobacillus*, 1) to penicillin G was determined by a disk diffusion method according to the National Committee for Clinical Laboratory standards (NCCLS) (22). In addition, minimum inhibitory concentrations (MICs) of *Prevotella* isolates for amoxicillin (Astellas, Tokyo, Japan) and amoxicillin/clavulanate (GlaxoSmithKline, Middlesex, UK) were determined by the agar dilution method recommended by the NCCLS (21). *Bacteroides fragilis* ATCC 25285 and *Bacteroides thetaiotaomicron* ATCC 29741 were used as quality control strains in each test. The resistance breakpoint for amoxicillin and amoxicillin/clavulanate was determined to be 2 and 16 μg/ml, respectively, based on the NCCLS guidelines (21).

Extraction of DNA from bacteria. Tested strains were subcultured on the Brucella HK agar containing 5% (v/v) sheep blood for 48 h at 37°C, and resulting colonies suspended in distilled water (100 μl) at a concentration equivalent to a MacFarland 1.5 standard. The suspension was heated to 96°C for 10 min, chilled to 4°C for 5 min, and the cell debris removed by centrifugation. The supernatant was used as template for PCR.

Extraction of DNA from clinical samples. A 50-μl volume of pus was suspended in 50 μl of distilled water and treated as described above. For root canal exudates, the suspension (100 μl) was centrifuged at 12000×*g* for 10 min and the supernatant (80 μl) discarded. The concentrate (20 μl of the remainder) was then used for DNA extraction. This concentration procedure was also employed for blood-like

pus samples.

Real-time PCR. The primers and the TaqMan probe were designed from *cfxA* and *cfxA2* gene sequences (GenBank accession nos. U38243 and AF11810, respectively) using Primer Express software version 2 (Applied Biosystems, Foster, CA). The nucleotide sequence of the forward primer was 5'-GCGCAAATCCTCCTTTAACAA-3' (KAG309), and the reverse primer sequence was 5'-ACCGCCACACCAATTTCG-3' (KAG310). The sequence of the real-time PCR probe was 5'-TGATAGCATTTCTCAAATTGTCTCAGCTTGTCC-3' (cfxA *Taq* Pr). The cfxA *Taq* Pr was 5' end-labeled with 6-carboxyfluoscein (FAM) as the reporter dye and 3' end-labeled with 6-carboxytetramethylrhodamine (TAMRA) as the quencher. The primers and probe were selected from a region with 100% nucleotide identity between *cfxA* and *cfxA2*. Although amino acid substitutions of *cfxA/A2* have been reported (6), the target region for PCR-amplification has not been found to differ in sequence. The real-time PCR was performed in a 25-μl final volume containing 12.5 μl of Premix Ex *Taq* (Perfect Real Time; Takara, Kyoto, Japan), 2 μl of DNA template, 0.5 μl of 10 μM KAG309 and KAG310 primers and 0.5 μl of 10 μM cfxA *Taq* Pr probe at a final concentration of 0.2 μM, 0.5 μl of ROX Reference Dye (×50) and 8.5 μl of distilled water. All reactions were run on an ABI Prism 7000 sequence detection system (Applied Biosystems) in triplicate with the following cycling parameters; 95°C for 10 s, followed by 40 cycles of 95°C for 10 s and 60°C for 31 s.

Analytical specificity and sensitivity (detection limit). Ten CfxA/A2 β-lactamase-positive and 10 β-lactamase-negative *Prevotella* strains, which had been stored in our laboratory, were examined to confirm specificity of real-time PCR. β-lactamase production by all these strains had previously been confirmed using the

nitrocefin disk, antimicrobial susceptibility tests and the presence of *cfxA/A2* by PCR as described previously (4) using primers (5'-GCAAGTGCAGTTTAAGATT-3' and 5'-GCTTTAGTTTGCATTTTCATC-3'). In addition to these *Prevotella* strains, clinical isolates of *Porphyromonas gingivalis* (n=12), *Peptostreptococcus micros* (n=20), *Fusobacterium nucleatum* (n=36), *Campylobacter gracilis* (n=7) and *Veillonella* sp. (n=6) were also examined. All these strains were β-lactamase negative.

Quantified dilutions of four β-lactamase-positive *Prevotella* strains (*P. intermedia*, 2; *P. melaninogenica*, 1; *P. buccae*, 1) were prepared by measuring the number of colony forming units (cfu). A randomly selected pus sample was confirmed to be *cfxA/A2*-negative and β-lactamase-negative using the real-time PCR, conventional PCR (4), and culture examination. A 20-μl of each bacterial dilution was mixed with same volume of the pus. Bacterial DNA extraction was done as described above.

Ethics for study. This study was approved by the Ethics Committee of Kanazawa University Graduate School of Medical Science.

RESULTS

Growth of bacteria from samples. Bacterial growth was found in all clinical samples. Half of the thin pus samples, 4 of 22 blood-like pus samples and 14 of 15 pus-like exudates from root canal yielded heavy bacteria growth under anaerobic conditions. The remaining samples produced limited bacterial growth.

Bacteriology, β-lactamase production and penicillin disk susceptibility. The results of cultural bacteriological examination are presented in Table 1. *Prevotella*, *Peptostreptococcus*, *Fusobacterium* and *Campylobacter* were predominantly isolated from the infections. Of the *Prevotella* isolates, 48 (31%) were found to be positive for

β-lactamase and furthermore, there were no non-*Prevotella* β-lactamase-positive isolates detected. The penicillin disk susceptibility data was 100% concordant with the results of β-lactamase production test (data not shown).

Analytical specificity and sensitivity of real-time PCR. Real-time PCR confirmed the presence of the *cfxA/A2* gene for all 10 β-lactamase-positive and none of the 10 β-lactamase-negative *Prevotella* strains. This gene was not detected by real-time PCR in any strains of *Porphyromonas*, *Peptostreptococcus*, *Fusobacterium*, *Campylobacter* and *Veillonella*. Using 'spiked' pus, real-time PCR revealed a detection limit of 5×10^3 bacterial cfu/ml, corresponding to 5 cfu/reaction mixture, for three *Prevotella* strains tested. In one *Prevotella* strain, the detection limit was 1×10^3 bacterial cfu/ml of pus.

Prevalence of *cfxA/A2* in *Prevotella* species and the MIC for amoxicillin/clavulanate. Although a total of 155 *Prevotella* isolates were recovered in this study, 16 strains that were stored by freezing, were not subsequently recovered by culture for the PCR study. Therefore, to determine the prevalence of *cfxA/A2* in *Prevotella*, a total of 139 clinical strains were examined. In this study, 48 (100%) of the β-lactamase-positive *Prevotella* were found to be positive for *cfxA/A2* by real-time PCR (Table 2). In contrast, all β-lactamase-negative strains were negative for the PCR. The β-lactamase-positive strains were highly susceptible to amoxicillin/clavulanate although resistance to amoxicillin was recorded for all of the strains (Table 2). β-lactamase-negative strains exhibited low MICs to both amoxicillin and amoxicillin/clavulanate with only one strain identified as having low level resistance to amoxicillin (MIC, 2 μg/ml).

Direct detection of *cfxA/A2* from clinical pus samples by real-time PCR.

Table 3 presents the agreement between cultural examination and PCR with respect to detection of β-lactamase-positive bacteria. Cultural bacteriological examination revealed that 31 (36%) of 87 clinical samples had β-lactamase-positive strains. Using the real-time PCR, *cfxA/A2* was detected for all these 31 β-lactamase-positive samples. Additionally, the real-time PCR also detected the resistance gene in 8 (14%) of the 56 samples deemed negative for β-lactamase by culture. Of these eight samples, six yielded β-lactamase-negative *Prevotella*, and no *Prevotella* isolates were recovered from the remaining two samples.

DISCUSSION

Due to improvement of culture and sampling techniques, recent studies demonstrate that dentoalveolar infections consist predominantly of strict anaerobes (9, 11-15, 23, 24). Our bacteriological investigation supports the results of these studies.

The nitrocefin method is capable of detecting ß-lactamases produced by almost all bacterial species and has been commonly used in clinical laboratories (20). In this study, the detection of ß-lactamase was limited to *Prevotella* (Table 1). Other studies have revealed that ß-lactamase-positive strains of facultative anaerobic bacteria such as staphylococci and non-*Prevotella* anaerobes are also isolated from dental infections (4, 7, 16). However, even in these previous studies, the incidence of such bacteria and the prevalence of ß-lactamase-positive strains in these species was very low. Consequently, with regard to ß-lactamase production, *Prevotella* would appear be the most important pathogen in dentoalveolar infection.

It has been reported that *cfxA/A2* occurs in 100% of β-lactamase-positive *Prevotella* strains from American and Norwegian patients with periodontal disease (8). French

investigators have also demonstrated a 100% prevalence of the resistance gene in β-lactamase-positive *Prevotella* (6). Our results are in good agreement with these studies and indicative that almost all β-lactamase-positive *Prevotella* strains are positive for *cfxA/A2*. This would further suggest that detection of *cfxA/A2* by the molecular method is a useful indicator in determining β-lactamase producing *Prevotella* strains. In addition, although β-lactamase-positive *Prevotella* are considered to produce CfxA/A2 variants with minor nucleotide substitutions (6), the real-time PCR parameters, including the primers and probe employed in the present study appeared to detect almost all types of *cfxA/A2* with high specificity and sensitivity.

There are various chromosomally-encoding and plasmid-mediated genes associated with β-lactamase production (8). However, it has been demonstrated that no *Prevotella* strain harbors TEM, SHA, OHA, AmpC and CF β-lactamase genes (8), and very few strains are positive for *cepA/cblA* (4). Together with these reports, our results of *cfxA/A2* prevalence and MICs in *Prevotella* isolates would support that *Prevotella* β-lactamases are class A/group 2e β-lactamases (3) and that CfxA/A2 is the most important and widely distributed β-lactamase produced by *Prevotella*.

In this study, PCR-positive results were obtained in 100% of clinical samples that were positive for β-lactamase by culture (Table 3). In contrast, 14% of the culture-negative samples were positive by PCR. If culture is considered the gold standard, it can be calculated that the diagnostic sensitivity and specificity of the real-time PCR is 100% and 86%, respectively. Thus indicating the high performance of the real-time PCR for direct detection of β-lactamase-positive *Prevotella* from clinical samples. Nevertheless, the question about the significance of culture-negative PCR-positive samples and clinical relevance could be raised. As it is likely that

real-time PCR is more sensitive for the detection target bacteria compared with cultural examination, it is perhaps not surprising that the PCR detected the resistance gene from the samples containing low numbers of β-lactamase-positive *Prevotella*. It would also be possible that the resistance gene would be detected by PCR from β-lactamase-positive *Prevotella* exhibiting a highly fastidious growth on the culture media. In these circumstances, the improved sensitivity of the real-time PCR would be clinically advantageous.

In clinical laboratories, routine bacteriological investigation generally includes semi-quantitation by evaluating bacterial growth on agar. It has previously reported that multiple strains of the same species can be isolated from the same individual (5), and indeed, in this study, some clinical samples yielded β-lactamase-positive and -negative strains of *Prevotella* species. It was therefore difficult to determine whether the cycle threshold (*Ct*) value accurately reflected the number of *cfxA/A2*-positive bacteria in sample. However, we confirmed that *Ct* value was roughly correlated with the count of colonies in *cfxA/A2*-positive samples that yielded a single phenotype of suspected *Prevotella* colonies (data not shown). This suggests the applicability of real time PCR for quantitative detection of β-lactamase-positive *Prevotella* in the clinical samples although further study is necessary.

Penicillin, and in particular amoxicillin, has been the first-line antibiotic in the treatment of acute dentoalveolar infection (1, 9, 11, 23, 24). However, the incidence of penicillin resistance has brought into question the appropriateness of penicillin in the management of dental infections (24). In this study, only *Prevotella* exhibited resistance to penicillin, and the penicillin resistance was completely concordant with β-lactamase production confirming the penicillin resistance mechanism of *Prevotella* (9,

14, 24). These results support the continuance of penicillin use as the first-line antibiotic therapy, and the proposal that *Prevotella* remains a highly significant pathogen when consideration is made concerning treatment with penicillin (25).

The present study has highlighted the high performance of real-time PCR for the detection of *cfxA/A2* in clinical samples of dentoalveolar infection. This molecular method could provide a useful and rapid clinical test for aiding the selection of antibiotic for therapy. Some antibiotics such as amoxicillin/clavulanate, cefmetazole, clindamycin and metronidazole have been demonstrated to be effective for ß-lactamase-positive *Prevotella* (12, 14, 16, 17). These should be considered in circumstances where involvement of β-lactamase-positive *Prevotella* is indicated. Since *Prevotella* is also implicated in other oral and non-oral infections such as periodontitis, osteomyelitis, sinusitis and sepsis (4, 7, 23), this molecular diagnostic method may also prove of value in aiding treatment these infections.

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TABLE 1. Identification and β-lactamase production by isolates from dentoalveolar infection

Species	No. of β-lactamase-positive strains / No. of total isolates (%)
Prevotella intermedia/nigrescens	12 / 39 (31)
Prevotella melaninogenica	9 / 21 (43)
Prevotella denticola	1 / 9 (11)
Prevotella loescheii	5 / 23 (22)
Prevotella oralis	8 / 26 (31)
Prevotella buccae	8 / 24 (33)
Prevotella oris	5 / 12 (42)
Prevotella bivia	0/ 1
Fusobacterium nucleatum/necrophorum	0 / 66
Peptostreptococcus micros	0 / 55
Peptostreptococcus prevotii	0 / 4
Peptostreptococcus anaerobius	0 / 2
Porphyromonas gingivalis	0 / 16
Porphyromonas endodontalis	0 / 3
Porphyromonas asaccharolytica	0 / 3
Campylobacter gracilis	0 / 33
Campylobacter rectus	0 / 10
Bacteroides capillosus	0 / 7
Gemella morbillorum	0 / 3
Propionibacterium acnes	0 / 1
Streptococcus sp.	0 / 27
Corynebacterium sp.	0 / 14
Veillonella sp.	0 / 13
Staphylococcus sp.	0 / 4
Lactobacillus sp.	0 / 3
Eubacterium sp.	0 / 2
Capnocytophaga sp.	0 / 2
Neisseria sp.	0 / 2
Actinomyces sp.	0 / 1
Bifidobacterium sp.	0 / 1
Unidentified anaerobic Gram-negative bacilli	0 / 12
Unidentified aerobic Gram-negative bacilli	0 / 12

TABLE 2. Prevalence of *cfxA/A2* and antimicrobial susceptibility in *Prevotella* strains isolated from dentoalveolar infection

	No. of tested strain	No. of *cfxA/A2* positive strain	Amoxicillin			Amoxicillin/clavulanate		
			MIC_{50}	MIC_{90}	Resistance (%)[a]	MIC_{50}	MIC_{90}	Resistance (%)[a]
β-lactamase-positive isolates	48	48	>64	>64	100	2	4	0
β-lactamase-negative isolates	91	0	0.06	0.25	1	0.06	0.25	0

[a] Resistance rate. Breakpoint for amoxicillin and amoxicillin/clavulanate resistances was 2 and 16 μg/ml, respectively.

TABLE 3. *cfxA/A2* detection from clinical pus samples by real-time PCR

Culture result	No. of sample	No. of sample (%)	
	sample	*cfxA/A2* positive	*cfxA/A2* negative
β-lactamase-positive	31	31 (100)	0
β-lactamase-negative	56	8 (14)[a]	48 (86)

[a] Two of the eight samples did not yield *Prevotella* isolates by culture.