16S rDNA PCR detection of *Actinobacillus actinomycetemcomitans* from periodontitis-free plaque

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Introduction

Actinobacillus actinomycetemcomitans, a gram-negative capnophilic coccobacillus is a well known periodontopathogen and has strong implications in human periodontitis especially aggressive forms of the disease (Slots and Ting, 1999). *A. actinomycetemcomitans* prevalence in subgingival plaque of healthy young individuals is not high (Sirinian *et al*, 2002; Rudney *et al*, 2003), while the prevalence of this microbe in diseased individuals varies (Doungudomdacha *et al*, 2001; Lee *et al*, 2003; Takeuchi *et al*, 2003). Overall, low quantity of the pathogen in diseased subgingival plaque was reported (Doungudomdacha *et al*, 2001). Researchers have used various methods for isolating or studying this periodontopathogen associated with periodontal health and disease. These have included: selective culture (Slots, 1982), commercial immuno-assay (Boyer *et al*, 1996), indirect immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA, Loesche *et al*, 1992), DNA probe (Ximenez-Fyvie *et al* 2000) and PCR (Ashimoto *et al*, 1996). The PCR detection method is currently considered to be the most sensitive (Doungudomdacha *et al* 2001). It is only by culture, however, that the individual periodontopathogen strains could be isolated and be further studied regarding factors or pathogenic potentials. Highly sensitive detection protocol assisting isolation of *A. actinomycetemcomitans* strains associated with periodontal health or diseasae, therefore, would be desirable for researchers studying periodontal disease caused by different strains of the microbes. This study was conducted to test the utility, if any, of the 16S rRNA PCR detection method (Ashimoto *et al*, 1996) in assisting cultural isolation and identification of *A. actinomycetemcomitans* from subgingival plaque samples of young periodontally healthy adults.

Materials and Methods

Subjects selection and clinical examination

Fifty 20-24 year-old dental students were recruited: i) free of any signs of periodontal diseases, i.e. no probing pocket depth (PPD) ≥ 4 mm, no radiographic signs of alveolar bone loss around any teeth; ii) all first molars and incisors to be present and intact, with no ≥ 2 surfaces restoration nor root canal treatment; iii) free of any systemic disease. Full mouth plaque (Pl%) and bleeding on probing (BOP%) scores were recorded prior to examination.

Sample site selection, Sampling and culture

- One site from 16 & 26 and one more site from 36 or 46 by tossing a coin
- MB (1), ML (2), DB (3), DL (4) site selected by throwing a dice
- Sampling by sterile paper points
- Culture in TSBV agar

A. actinomycetemcomitans 16S rDNA PCR detection/identification

- Plaque detected - (Ashimoto *et al*, 1996)
- Identification – MicroSeq 500 16S rDNA based bacterial identification and bicochemical test as per Kilian (1976)

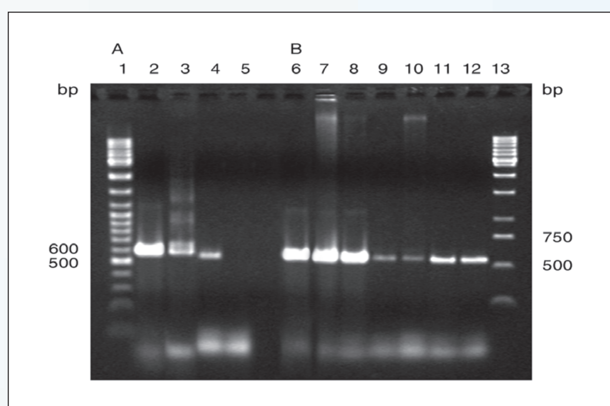

Figure 1 16S rDNA PCR detection (Ashimoto *et al*, 1996) of *A. actinomycetemcomitans*. Panel A: signals from subgingival plaque samples. Lane 1: DNA ladder mix molecular size standard (MBI Fermentas); lane 2: *A. actinomycetemcomitans* ATCC 29523 strain PCR amplification by 16S rDNA universal primers (602 bases amplicon); lane 3: 16S rDNA PCR amplicon from a subgingival plaque sample using universal primers; lanes 4 and 5: 16S rDNA PCR amplicon (557 bases) by *A. actinomycetemcomitans* primers showing positive (lane 4) and negative (lane 5) signal. Panel B: signals from TSBV isolates PCR amplification with 16S rDNA *A. actinomycetemcomitans* primers. Lane 6: *A. actinomycetemcomitans* ATCC 29523 strain; lanes 7 and 8: *A. actinomycetemcomitans* isolates (3 and 7b, respectively); lanes 9-12: *Campylobacter showae* (isolate 6), *Haemophilus paraphrophilus* (isolate 4), *Haemophilus segnis* (isolate 5b) and *Neisseria subflava* (isolate 1), respectively; lane 13: 1 kb DNA ladder molecular size standard (MBI Fermentas). Note that i) signal intensities for all 16S rDNA *A. actinomycetemcomitans* primers PCR positive plaque samples were more or less the same; ii) weak positive PCR amplicon identified from some non-*A. actinomycetemcomitans* isolates (lanes 9-12); iii) identity of all isolates was confirmed by partial 16S rDNA gene sequencing (Applied Biosystem) and biochemical tests.

Results

Table 1 Samples showing positive *Actinobacillus actinomycetemcomitans* 16S rDNA PCR (Ashimoto *et al*, 1996) in plaque and TSBV isolates[a]

Subject		Clinical Parameter		Sample site				Microbiology				
											Identification	
Sex	*Age*	*PI (%)*	*BOP (%)*	*Location*	*PD (mm)*	*CAL (mm)*	*BOP*	*Isolate*	*TVC (CFU per paper point)*	*TSBV isolates: 16S rDNA PCR using Aa primers*[b]	*Aa*[c]	*Non-Aa*[c]
M	21	26	22	26MP	1	1	+	1	$2.2X10^6$	w+		*Nerisseria subflava* (AF479578.1)
F	21	10	6	36MP	3	3	-	2	$1.8X10^4$	w+		*Nerisseria subflava* (AF479578.1)
F	23	16	16	16DL	2	2	+	3	$4.1X10^5$	+	(AF355094.1)	
F	22	6	22	36MP	2	2	+	4	$2.0X10^4$	w+		*Haemophilus paraphrophilus* (HPA290766)
F	22	13	15	16DL	2	2	-	5a	$1.1X10^4$	w+		*Nerisseria subflava* (AF479578.1)
				36MB	3	3	+	5b	$3.7X10^4$	w+		*Haemophilus seginis* (AF355094.1)
M	20	29	27	16ML	2	2	-	6	$3.5X10^4$	w+		*Campylobacter showae* (LO6975.1)
M	24	34	52	16MB	2	2	+	7a	$1.4X10^5$	w+		*Haemophilus seginis* (HSE290764)
				46MB	2	2	+	7b	$4.6X10^5$	+	(ACNRRNAL)	
F	20	34	72	26DB	2	2	+	8a	$7.2X10^5$	+	(ACNRRNAJ)	
				46DL	3	3	-	8b	$4.8X10^5$	+	(AF355094.1)	

[a] All paper point samples (n = 94) were positive towards amplification by 16S rDNA universal primers; samples showing negative signal to *A. actinomycetemcomitans* 16S rDNA primers PCR were not included (n = 83)
[b] w+ = weak positive
[c] *Aa* = *A. actinomycetemcomitans*; NCBI sequence accession number in parenthesis.

Figure 2 Sequence analysis of PCR products from isolates showing positive *A. actinomycetemcomitans* 16S rDNA PCR reaction. Also shown is the 557 bases sequence of *A. actinomycetemcomitans* ATCC 29523 (accession number AY 362885). Please refer to Table 1 for identification of individual isolates. The two *A. actinomycetemcomitans* and three *N. subflava* isolates of the same MicroSeq assession numbers were considered as same strains. Percentage similarity of *A. actinomycetemcomitans* isolates at the region of interest(∗) with reference sequence ranges from 69% - 91%. Percentage similarity(∗) between cross reacting isolates and *A. actinomycetemcomitans* ranges from 67% - 80%.

Conclusion

1. The prevalence and percentage proportion of the periodontopathogen *A. actinomycetemcomitans* were low in subgingival plaque of periodontal healthy young Chinese individuals.
2. The Ashimoto *et al* (1996) 16S rDNA PCR protocol appeared to be specific, useful and convenient for detection of *A. actinomycetemcomitans* in plaque samples.
3. Clear evidence was shown in the current study indicating that the Ashimoto protocol could also amplify false positive signals from non-*A. actinomycetemcomitans* isolates such as *Campylobacter showae, Haemophilus paraphrophilus, Haemophilus segnis,* and *Neisseria subflava* which possess conserved 16S rDNA sequences of interest hence reducing the positive predictive value of the PCR detection method.

Reference

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