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Rapid identification of oral isolates of *Aggregatibacter actinomycetemcomitans* obtained from humans and primates by an ultrafast super convection based polymerase chain reaction

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Abstract

Aggregatibacter actinomycetemcomitans is a Gram negative oral bacterium associated with localized aggressive periodontitis (LAP). Detection of *A. actinomycetemcomitans* in clinical samples is routinely done by PCR. Our aim was to develop a rapid and reliable PCR method that can be used as a chair-side tool to detect *A. actinomycetemcomitans* in clinical samples. Sensitivity and specificity assessment was performed on buccal and plaque samples obtained from 40 adolescents enrolled in an ongoing LAP study by comparing 20 *A. actinomycetemcomitans*-positive subjects and 20 who were negative. In a second study, *A. actinomycetemcomitans* presence was tested in oral samples from eighty-six primates that included rhesus monkeys, chimpanzees, marmosets, tamarins and baboons. All samples were processed for detection of *A. actinomycetemcomitans* by means of culture, conventional PCR (cPCR) and rapid PCR (rPCR) using a Super Convection based AmpXpress thermal cycler (AlphaHelix, Sweden). For human samples, culture, cPCR and rPCR showed perfect agreement. Using this method *A. actinomycetemcomitans* was detected in 27 of 32 rhesus monkeys, 4 of 8 chimpanzees and 1 of 34 marmosets. Rapidity of AmpXpress thermal cycler, combined with Ready-To-Go PCR beads (GE Life sciences), a quick DNA extraction kit (Epicentre Biotechnologies, Madison, Wisconsin, USA) and a bufferless fast agarose gel system, made it possible to obtain results on *A. actinomycetemcomitans* detection within 35 min. We conclude that AmpXpress fast PCR can be conveniently used as a chair-side tool for rapid detection of *A. actinomycetemcomitans* in clinical samples.

Keywords

A. actinomycetemcomitans; Localized aggressive periodontitis; rapid PCR; Super convection

1. Introduction

Aggregatibacter actinomycetemcomitans is a Gram negative coccobacillus implicated in localized aggressive periodontitis (Zambon, 1985; Christersson, 1993). *A. actinomycetemcomitans* colonizes the oral cavities of humans (Slots, 1976; Socransky and

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Haffajee, 1992) and non-human primates (Eke et al., 1993) and belongs to the HACEK group of organisms believed to be associated with a number of systemic diseases including infective endocarditis (Das et al., 1997; Ellner et al., 1979; Paturel et al., 2004).

In addition to conventional culture based methods used to identify *A. actinomycetemcomitans*, PCR is now a well-established and widely used technique (Flemmig et al., 1995; Tran and Rudney, 1999). A large number of reports exist in the literature describing PCR based identification of *A. actinomycetemcomitans* (Flemmig et al., 1995; Goncharoff et al., 1993; Kim et al., 2005). While several studies have aimed at identifying only *A. actinomycetemcomitans* in the specimen, others have identified additional oral bacteria apart from *A. actinomycetemcomitans* e.g., using multiplex PCR (Tran and Rudney, 1999). Furthermore, 16S rDNA has been used as the target in PCR in many studies, but other genes such as lktA (Flemmig et al., 1995; Goncharoff et al., 1993; Tonjum and Haas, 1993) have also been used to identify *A. actinomycetemcomitans*. PCR based detection of bacteria in clinical specimens is sensitive and specific (Ficarra and Eversole, 1992; Olive, 1989). Conventional PCR, however, is time-consuming more often than not. This is mainly due to poor heat transfer on conventional PCR machines, resulting in longer time required to complete the reaction. In a clinical study setting during field screening of patients, generally samples are collected and brought to the laboratory where samples are processed and PCR performed for *A. actinomycetemcomitans* identification. The overall time for conventional PCR can vary from 2 to 4 h to overnight (Kramer and Coen, 2001). It is advantageous when conducting a screening examination to identify subjects who harbor *A. actinomycetemcomitans* at chair side within a short time period so that they can be informed that they are carriers of this potentially pathogenic organism. Extended time periods required for conventional PCR are inconvenient and can result in loss of subject interest and participation in ongoing studies. Therefore, rapid attainment of data at chair side during screening examinations could provide a great advantage and should improve recruitment of subjects. In our approach to develop a rapid PCR method for the detection of *A. actinomycetemcomitans*, we utilized samples from *A. actinomycetemcomitans*-positive and negative subjects who were involved in a longitudinal study of the relationship of A. *actinomycetemcomitans* to the initiation of localized aggressive periodontitis. In addition, oral samples from several primate species were used to compare culture to conventional PCR and to a new Super Convection rapid PCR technique. In this report we demonstrate that the new ultrafast PCR technique can be conveniently used as a chair-side tool for rapid *A. actinomycetemcomitans* detection.

2. Materials and methods

2.1. Bacterial culture

Buccal and plaque samples were suspended in *A. actinomycetemcomitans* Growth Medium (AAGM) broth [trypticase soy broth with 0.8% glucose (8 g/l), 0.6% yeast extract (6 g/l) and 0.4% sodium bicarbonate (4 g/l), 75 μg/ml bacitracin and 5 μg/ml vancomycin] and brought to the laboratory for processing. In some cases, plaque and buccal samples were collected using cytology brushes, and then the brushes were stabbed in half-strength AAGM agar in small glass vials before being sent to our laboratory. Once samples reached the laboratory,

serial 10-fold dilutions were made and spread on AAGM plates. After 3 days of incubation at 37 °C and 10% CO_2, *A. actinomycetemcomitans* colonies were preliminarily identified by colony morphology and catalase positivity. Presumptive *A. actinomycetemcomitans* colonies were subcultured from each sample. Human *A. actinomycetemcomitans* isolate IDH781 and *Aggregatibacter aphrophilus* ATCC® 33389™, a phylogenetic relative of *A. actinomycetemcomitans*, were also grown on AAGM as above.

2.2. Purification of DNA

DNA from the buccal/plaque samples from humans and primates, and genomic DNA from *A. actinomycetemcomitans* isolates was purified using DNeasy® blood and tissue kit from Qiagen (QIAGEN Sciences, Germantown, Maryland, USA). Briefly, the samples were treated with a lysis buffer and proteinase-K overnight at 56 °C, followed by extraction and purification of DNA using Qiaquick spin columns (Qiagen). DNA from buccal samples from subjects with or without LAP were extracted using QuickExtract™ DNA extraction kit from Epicentre Biotechnologies (Madison, Wisconsin, USA). The swab samples were suspended first in a quick DNA extract solution and heated at 65 °C for 6 min and then the tubes were transferred to 98 °C and incubated for 2 min. The extracted DNA was stored at —20 °C.

2.3. Human sampling and analysis

Buccal samples from 40 subjects (29 females and 11 males, mean age = 14 year) enrolled in an ongoing LAP study (20 *A. actinomycetemcomitans*-positive subjects and 20 *A. actinomycetemcomitans*-negative) were used for detecting *A. actinomycetemcomitans* by both cPCR and rPCR. All volunteers gave consent, using a form that was reviewed and approved by the Institutional Review Board (IRB) of the University of Medicine & Dentistry of New Jersey (UMDNJ).

2.4. Primate sampling

Monkey samples were collected from the North East Regional Primate Research Center (NEPRC) at Harvard University, Southwest National Primate Research Center (SNPRC), Yerkes Regional Primate Research Center at Emory University and Laboratory Animal Services facility at Rutgers University. All monkeys (Table 2) had an intact dentition and were housed in separate cages. Prior to sampling all primates were anesthetized using ketamine hydrochloride (15 mg/kg) and a supplement of isoflurane. Buccal mucosa of the monkeys was sampled with sterile wooden tongue depressors. Plaque samples were collected using autoclaved dental scalers. The samples were suspended in AAGM broth and processed for bacterial culture as described above. Sample collection from primates was approved by the Institutional Animal Care and Use Committees of the UMDNJ, Harvard University and Rutgers University.

2.5. Super convection rapid PCR

Table 1 shows primers and amplicon sizes. For all PCR reactions, Ready-To-Go beads (GE HealthCare Biosciences, Buckinghamshire, UK) were used. When each bead was reconstituted to 25 μl final volume, the concentration of each dNTP was 200 μM in 10 mM Tris–HCl (pH 9), 50 mM KCl and 1.5 mM $MgCl_2$. Primers were used at a concentration of

0.5 μM and the amount of template DNA was 50–100 ng per reaction. PCR reactions were performed on the rapid PCR machine AmpXpress (Alpha Helix Molecular Diagnostics AB, Sweden). Rapid PCR is facilitated by a centrifugation based convection technology used in the instrument (Martensson et al., 2006). A typical thermal profile consisted of an initial denaturation of 94 °C for 1 min followed by 30 cycles of 94 °C for 0 s, 55 °C for 6 s and 72 °C for 7 s. No final elongation was required.

2.6. Conventional PCR

A Techne TC-412 PCR machine (Techne Inc. Burlington, NJ, USA) was used. All PCR reagents were the same as described above for rapid PCR. Except for variable annealing temperatures for different primer pairs, the temperature profile was as follows: Initial denaturation 94 °C for 10 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min.

2.7. Agarose gel electrophoresis

PCR products were visualized by electrophoresis through a 2% agarose gel after adding the dye EZ-Vision™ Three (Amresco, Ohio, USA) to the entire PCR product, i.e., 25 μl. In rPCR experiments, a rapid and bufferless agarose gel system that completes in 6 min at 250 V (Febe bufferless agarose gel; Biokeystone Co, California, USA) was used. The gels were then exposed to UV light on a trans illuminator and pictures were taken by the attached Kodak DC290 camera.

3. Results

3.1. Validation of the efficacy of rPCR

In order to test the reliability of the Super Convection rPCR, we performed PCR for lktA of *A. actinomycetemcomitans* strains in parallel both on the AmpXpress machine as well as the conventional PCR machine. Fig. 1A shows that all *A. actinomycetemcomitans* strains tested produced an expected 262-bp band of similar intensity from both PCR machines. Sensitivity of the super convection rPCR was also compared with that of the conventional PCR. Genomic DNA from a serial 10-fold dilutions of *A. actinomycetemcomitans* IDH781 was used in the same PCR reaction as above using *A. actinomycetemcomitans*-specific *lktA* primers. It was possible to amplify the fragment at a bacterial concentration as low as 10^3/ml using rPCR, similar to cPCR (Fig. 1B). Comparison of rPCR with cPCR in terms of time requirements is schematically shown in Fig. 2. The time requirement for each step of either PCR method was established after running at least 3 experiments. Regardless of using a quick DNA extraction method and a fast agarose gel electrophoresis system, the cPCR takes approximately 2 h, while rPCR requires as little as 35 min to obtain results (Fig. 2).

3.2. Efficacy of super convection AmpXpress PCR with respect to human clinical samples

Buccal samples from 40 subjects (20 each from *A. actinomycetemcomitans*-positive and -negative subjects) were subjected to *A. actinomycetemcomitans* detection by using both cPCR and rPCR. Data for 10 *A. actinomycetemcomitans*-positive samples are shown in Fig. 3. The results showed that 16 of 20 *A. actinomycetemcomitans* culture-positive were *A. actinomycetemcomitans* positive by PCR (sensitivity=80%) while 3 of 20 *A.*

actinomycetemcomitans culture-negative were PCR positive for *A. actinomycetemcomitans* (specificity=85%) (Table 2).

3.3. Identification of *A. actinomycetemcomitans* isolates from monkeys by rPCR

After establishing the efficacy of rPCR for rapid identification of *A. actinomycetemcomitans* from clinical samples, we then utilized the method to study the prevalence of *A. actinomycetemcomitans* among Old World and New World non-human primates. Primers specific for A. actinomycetemcomitans *lktA* sequence (Goncharoff et al., 1993) were used for the identification of *A. actinomycetemcomitans*. Data for 11 strains are shown in Fig. 4 panel A. Twenty seven of 32 rhesus monkeys, 4 of 8 chimpanzees and 1 of 34 marmosets harbored *A. actinomycetemcomitans* as revealed by amplification of *lktA* fragment (Table 3). No *A. actinomycetemcomitans* was detected from cynomolgus, baboon or tamarin group of monkeys.

Identity of *A. actinomycetemcomitans* was further confirmed by *A actinomycetemcomitans*-specific 16S rRNA PCR (Kim et al., 2005). An expected band of 468 bp size was seen in all strains *lktA*-positive for *A actinomycetemcomitans*. *A. aphrophilus* ATCC 33389, a phylogenetic relative of *A. actinomycetemcomitans* was used as a negative control and did not show any band (Fig. 4, panel B).

3.4. Serotyping of monkey A. actinomycetemcomitans isolates

Serotypes of monkey *A. actinomycetemcomitans* were determined by PCR using serotype-specific primers (Table 1) (Suzuki et al., 2001). Among 19 *A. actinomycetemcomitans*-positive rhesus monkeys at NEPRC, 12 had serotype d and four had mixtures of serotypes b, c, d and e but d occurred in 16 of them, while 3 strains were of serotype f (Table 3). In the case of Rutgers rhesus monkeys all four isolates were of serotype f, but two were additionally positive for serotype b or c. From the monkey colony at SNPRC, all three isolates from rhesus monkeys and a single isolate from a marmoset were all serotype f.

4. Discussion

Although microbiological and biochemical tools are an essential part of *A. actinomycetemcomitans* identification, PCR is used as a routine and common technique. In clinical studies involving subjects, rapid identification of *A. actinomycetemcomitans* might be of great advantage since chair-side identification could inform patients of the presence of this pathogenic bacterium. In this study, we demonstrated that using a Super Convection rapid PCR technique, *A. actinomycetemcomitans* could be quickly detected in buccal or plaque samples from humans in 35 min (Fig. 2).

Conventional PCR machines take a longer time for a reaction to complete since heating and cooling are based on diffusion (deMello, 2003; Jia et al., 2007; Kramer and Coen, 2001). In contrast, rapid PCR on AmpXpress is facilitated by the convection heating mechanism, where high velocities of the reaction mixture in rotating tubes impart homogeneous temperature and excellent mixing. This eventually results in less time needed for each cycle (Gidlof et al., 2009; Martensson et al., 2006). Two previous reports have utilized this technology for RT-PCR quantification of non-oral viruses (Gidlof et al., 2009; Martensson et

al., 2006). In those reports, although the technology was the same, thermal cycling and duration of reaction were longer because the experimental setup was RT-PCR. Furthermore, several other rapid PCR systems have been reported in the literature, but the limitations of those systems are either commercial unavailability or non-portability of the equipment (Muddu et al., 2011; Oda et al., 1998; Wheeler et al., 2011). Therefore, this is the first report showing successful utilization of AmpXpress, a portable Super Convection PCR machine for rapid bacterial identification. We first validated the efficacy of the rapid PCR instrument by comparing it against a conventional PCR machine. Amplification of an *lktA* band of similar intensity from both machines suggests that AmpXpress is as efficient as the conventional PCR machine. Furthermore, sensitivity of PCR reaction was also the same on both instruments, i.e., lowest detection limit of 10^3 cfu/ml. This is in agreement with several earlier studies using conventional PCR machines for *A. actinomycetemcomitans* identification (Flemmig et al., 1995; Poulsen et al., 2003).

For identification of *A. actinomycetemcomitans* by PCR, different primers specific for *A. actinomycetemcomitans* 16S rDNA have been used (Kim et al., 2005; Tran and Rudney, 1999). In this study, we utilized primers that are specific for the lktA fragment of *A. actinomycetemcomitans* (Goncharoff et al., 1993). These primers are highly specific for *A. actinomycetemcomitans* and do not cross react with any of the 13 other common oral bacteria (Goncharoff et al., 1993). Additionally, we performed PCR for *A. actinomycetemcomitans* identification using species-specific 16S rDNA primers (Kim et al., 2005). That *A. aphrophilus*, a close phylogenetic relative of *A. actinomycetemcomitans*, did not show any band substantiates the specificity of these primers.

Our initial goal was to determine the feasibility of using the AmpXpress method for chair-side identification in clinical samples so that we could inform subjects who had consented to participate in a longitudinal study that they harbored this potentially dangerous microorganism. As a result of both the sensitivity and specificity of the results obtained in this pilot study we decided to include plaque and buccal samples from several primate species to expand the sample size and also to investigate the carriage of *A. actinomycetemcomitans* in different primate species. This allowed us to analyze an additional 86 oral primate samples for *A. actinomycetemcomitans* carriage.

Of interest was the finding that *A. actinomycetemcomitans* was detected in 84% (27 of 32) of rhesus monkeys obtained from three different primate research centers, and only one of 34 marmosets. Four of eight chimpanzees harbored *A. actinomycetemcomitans*; while baboons and tamarins did not show any *A. actinomycetemcomitans*. Admittedly the sample size for these species needs to be expanded in the future. Nevertheless, our preliminary survey of primates shows a significantly higher association of *A. actinomycetemcomitans* with Old World monkeys as opposed to New World primates which is in line with previous studies demonstrating *A. actinomycetemcomitans* bound specifically to buccal epithelial cells from Old World monkeys (Yue et al., 2007). Occurrence of periodontitis in Old World and New World monkeys is ambiguous in the literature. Some studies have reported spontaneous development of the disease in monkeys in wild and in captivity (Dreizen and Levy, 1977; Page et al., 1972). It is possible however, that the form of periodontitis in those animals was chronic periodontitis in older monkeys and may not be aggressive periodontitis.

To gain a greater insight into *A. actinomycetemcomitans* carriage in primates and the effect of *A. actinomycetemcomitans* on disease initiation, we are currently in the process of whole genome sequencing of *A. actinomycetemcomitans* isolates; comparative genomic analysis of *A. actinomycetemcomitans* isolates from monkeys versus humans is also in progress in our laboratory. In conclusion, the rPCR method consisting of Ready-To-Go PCR beads, a quick DNA extraction kit (Epicentre Biotechnologies) and a bufferless fast agarose gel system makes it possible to detect *A. actinomycetemcomitans* in no more than 35 min and thus provides a rapid and accurate method of detecting *A. actinomycetemcomitans* at chair side within a reasonable time period.

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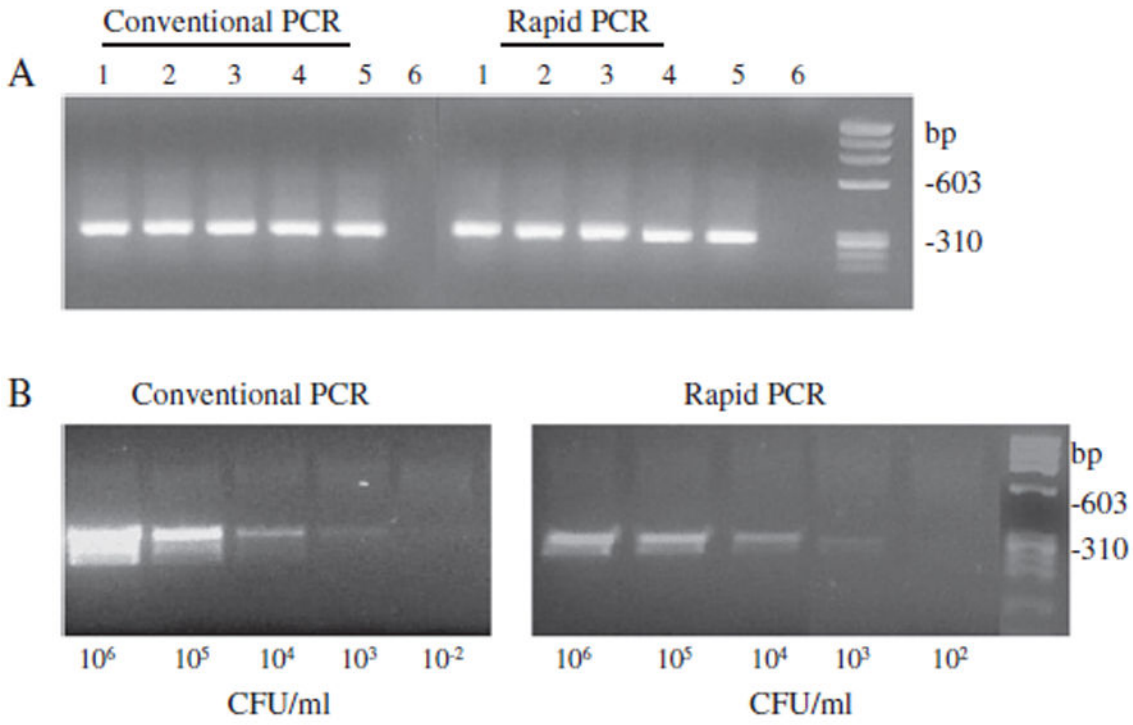

Fig. 1.

Validation of the efficacy of the AmpXpress rPCR. Panel A: Efficacy of AmpXpress rapid PCR machine was compared to that of a regular PCR machine. *A. actinomycetemcomitans*-specific *lktA* primers (Goncharoff et al., 1993), were used to amplify a 262-bp fragment of the *lktA* gene. Purified genomic DNA from *A. actinomycetemcomitans* strains was used for PCR at 50 ng per reaction. Lanes: *A. actinomycetemcomitans* strains IDH781 (lane 1), HK1651 (lane 2), JP2 (lane 3), CU1000 (lane 4), NJ4500 (lane 5) and negative control (lane 6). Panel B: Detection limit of cPCR and rPCR. Purified genomic DNA from a serial 10-fold dilution of *A. actinomycetemcomitans* IDH781 was used. Equal volume of DNA sample from each dilution was used in PCR reactions.

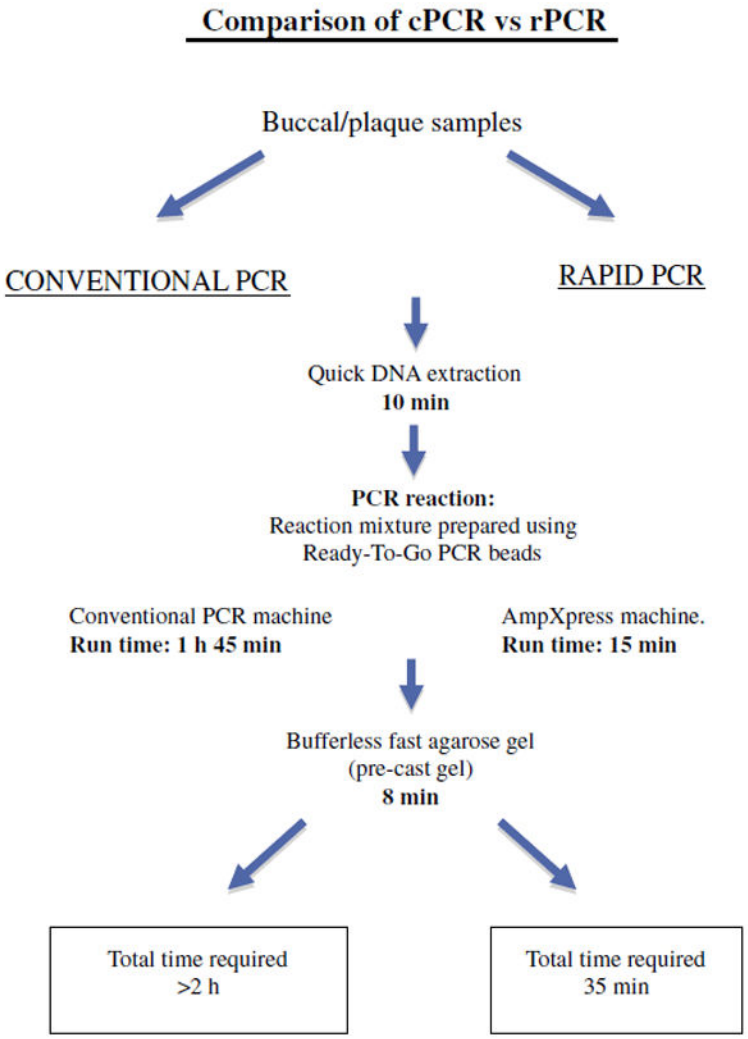

Fig. 2.

Time requirements by cPCR and rPCR—from sample collection to data acquisition. Schematic presentation of time required for each step in the detection of *A. actinomycetemcomitans* by rPCR as compared to cPCR. In the case of rPCR, it is necessary that all equipments and reagents be ready before beginning in order to complete quickly in 35 min. Notwithstanding the use of a quick DNA extraction method and a fast agarose gel electrophoresis system, cPCR takes approximately 2 h, which is still outside a “chairside” timeframe.

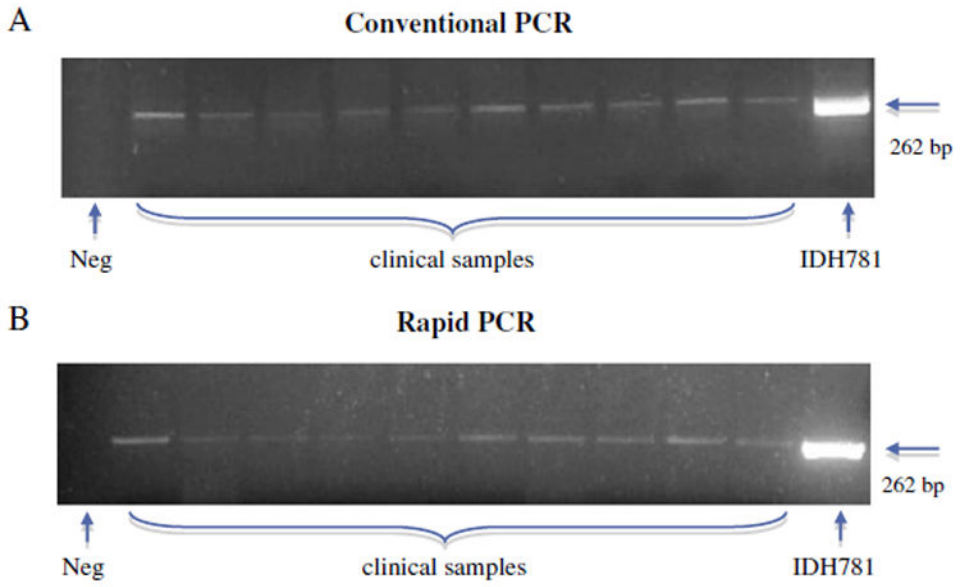

Fig. 3.

Detection of *A. actinomycetemcomitans* from human clinical samples by cPCR and rPCR. DNA purified from buccal samples from 40 subjects (20 each from A. actinomycetemcomitans-positive and *A. actinomycetemcomitans*-negative subjects) were used for PCR detection of *A. actinomycetemcomitans*. Panel A: cPCR; DNA was purified using the Qiagen kit, PCR performed on a regular PCR machine and PCR products were run on a conventional agarose gel. Panel B: rPCR; DNA was purified using QuickExtract™ solution, PCR performed on the AmpXpress rapid PCR machine, and PCR products were run on a 2% agarose gel in a bufferless agarose gel system for 6 min. Lanes: Data from 10 random *A. actinomycetemcomitans*-positive samples is shown. Lanes 1–10: LAP samples; lane 11: *A. actinomycetemcomitans* IDH781, used as positive control. Neg = negative control.

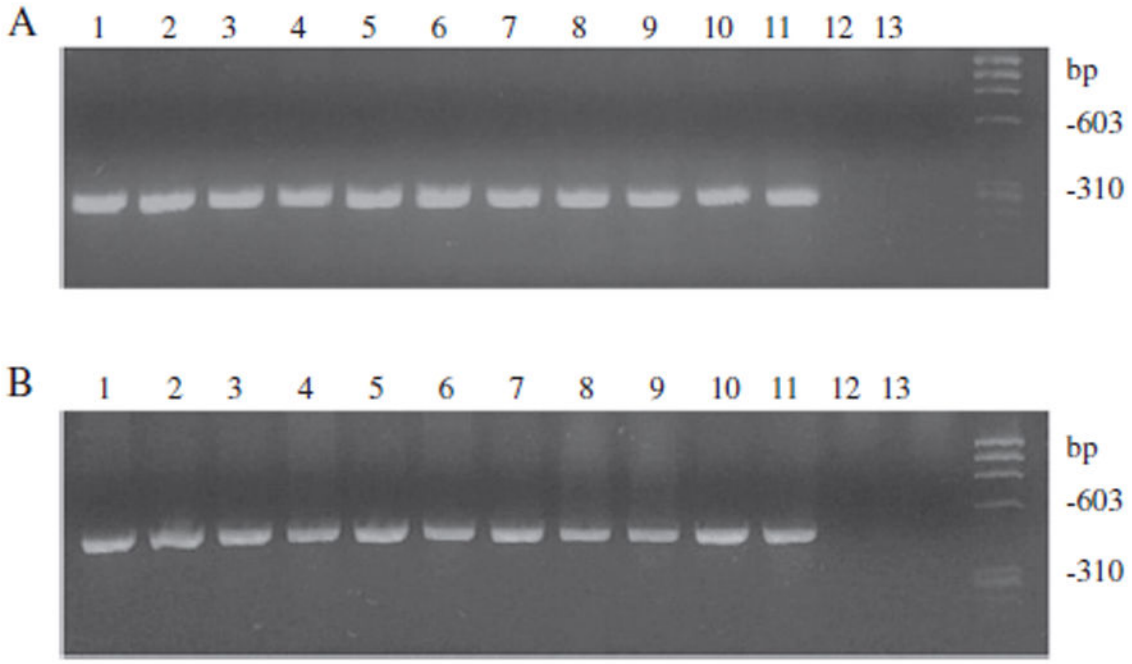

Fig. 4.

Identification of *A. actinomycetemcomitans* isolates from monkeys by rPCR. Panel A: *A. actinomycetemcomitans*-specific *lktA* primers were used for the identification of *A. actinomycetemcomitans* by rPCR. Panel B: *A. actinomycetemcomitans*-specific 16S rDNA primers were used to confirm the results from l*ktA* PCR. Lanes: Eleven randomly selected monkey *A. actinomycetemcomitans* isolates (lanes 1–11), *A. aphrophilus* ATCC 33389 (lane 12) and negative control (lane 13).

Table 1

PCR primers.

Primer	Sequence (5′–3′)	Amplicon	Reference
lktA	GGAATTCCTAGGTATTGCGAAACAATTTGATC	262	Goncharoff et al. (1993)
	GGAATTCCTGAAATTAAGCTGGTAATC		
Aa 16S rDNA	TAGCCCTGGTGCCCGAAGC	428	Kim et al. (2005)
	CATCGCTGGTTGGTTACCCTCTG		
Serotype b	TCTCCACCATTTTTGAGTGG	333	Kaplan et al. (2001)
Serotype c	GAAACCACTTCTATTTCTCC	268	Kaplan et al. (2001)
Serotype f	CCTTTATCAATCCAGACAGC	232	Kaplan et al. (2001)
Universal Fwd for sero b, c and f	ARAAYTTYTCWTCGGGAATG		
Serotype a	TGGGTCATGGGAGGTACTCC	293	Kaplan et al. (2001)
	GCTAGGAACAAAGCAGCATC		
Serotype d	GGAACGGGTATGGGAACGG	411	Kaplan et al. (2001)
	GGATGCTCATCTAGCCATGC		
Serotype e	ATTCCAGCCTTTTGGTTCTC	311	Kaplan et al. (2001)
	TGGTCTGCGTTGTAGGTTGG		

Table 2

Specificity and sensitivity of cPCR and rPCR in detecting *A. actinomycetemcomitans* from buccal samples from subjects.

	Culture results	Conventional PCR	Rapid PCR
Aa[a] positive	20	16	16
Aa negative	20	17	17

	Culture vs PCR	cPCR vs rPCR
Sensitivity	80%	100%
Specificity	85%	100%

[a]Aa: A. actinomycetemcomitans.

Table 3

Monkeys used in this study.

Monkey group	Age (years) Mean (range)	Sex	*A. actinomycetemcomitans*-positive	Serotype
Rhesus (n=32)	8.5 (2–15)	Male=16 Female=16	27 (84%)	d=12 b+c+d+e=4 c+f=1 f=10
Chimpanzee (n=8)	28.2 (15–33)	Male=2 Female=6	4 (50%)	b=3 c=1 f=4
Cynomolgus (n=4)	5.7 (5–6)	Male=4 Female=0	0	
Baboon (n=4)	15.7 (14–19)	Male=1 Female=3	0	
Tamarin (n=4)	2 (2–2)	Male=2 Female=2	0	
Marmoset (n=34)	5 (2–8)	Male=20 Female=14	1 (2.9%)	f=1