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PCR Identification of Aggregatibacter actinomycetemcomitans isolated from Subgingival Plaque Samples

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ABSTRACT

Aims: This study was performed to confirm the cultural identification of Aggreagatibacter actinomycetemcomitans by PCR identification kit and tocompare detection capability of A. actinomycetemcomitansbyconventional culture with presumptive biochemical tests and culture-enhanced PCR.

Methods: 45 sub-gingival plaque samples were collected from the deep pockets ofpatients with periodontitisby sterile paper points in 5ml BHI broth vials ,each sample divided into two aliquot , 100 µl fromeachaliquot were cultivated on dent-aid selective media,one plate used for biochemical test identification and from the other plate DNA were extracted and used for PCRidentification by amplification of 360 bp.Fragment(amplicon).

Results: By cultural method 21 isolates of A actinomycetyemcomitansfrom 45 samplewere identified as A. actinomycetemcomitans.By culture-enhanced method after cultivation of each sample and extraction of the DNA from the obtained isolates to be identified by PCR identification kitfor A actinomycetyemcomitans,give positive reaction for 31 sample of 45.

Conclusions:PCR confirm the detection and identification of A actinomycetyemcomitans since all the samples that give positive result by culture method produced positive reaction by PCR, combination of the two methods were found to be superior to culturewith presumptive biochemical identification alone and should be the preferred for the detection of A. actinomycetemcomitans in subgingival plaque**.**

Keywords: A.actinomycetemcomitans , PCR, Culture-enhanced PCR.

INTRODUCTION

Aggregatibacter actinomycetemcomitans (A.a., previously Actinobacillus actinomycetemcomitans)described for the first time in 1912by Klinger as coccobacillary bacteria isolatedfrom actinomycotic lesions of man together with Actinomyces[(1)],and had various names over the intervening years.However, DNA homology and 16S rRNA sequencing studies have demonstrated a close relationship to Haemophilus aphrophilus and Haemophilus segnis these 3 species have recently been transferred to the new genusAggregatibacter within the family Pasteurellaceae[(2)].A. Actinomycetemcomitans was recognized as a member of the normal human oral microbiota in the 1950s [(3)].The species have attracted attention because of its association with localized aggressive periodontitis, a severe infection of the gingiva, although it is also associated with non-oral infections. The primary habitat has not been definitively identified, but is most probably dental plaque in the gingival crevice [(4)], as it is not found in edentulous individuals[(5,6)].This organism is a gram-negative, non-spore-forming, non-motile, facultatively anaerobic coccobacillus that grows best in an aerobic environment enriched with $CO_2$5–10%[(7)].Conventional methods used to identify A. actinomycetemcomitans in subgingival plaque samples include culture techniques with biochemical testing [(8)], immunological assays [(9)], and DNA probes [(10)]. These techniques, however, are of limited specificity and sensitivity and/or are time-consuming. Recently, the PCR [(11)] has been described as a technique to identify A. actinomycetemcomitans. Molecular methods for detection and identification of A. actinomycetemcomitans have been described.Genetic studies have shown that the gene for 23S rRNA is split into two smaller forms in A. actinomycetemcomitans while the transcript is continuous in H. aphrophilus H. paraphrophilus H. segnis and H. influenza[(12).]

Materials and Methods

Samples collection &transport : Supra-gingival plaque was removedby using a sterile curette, and the supra-gingival area was isolated with sterile gauze[(13)].45sub-gingival plaque samples were collected by inserting sterile paper point

size 50 into the deep pockets of patients, who attended the Dental Hospital, Department of Periodontics, College of Dentistry at Mosul University asking for diagnosis and treatment, placed in sterile vials containing 5ml brain heart infusion broth(BHI). 100 µl from each5ml sample wasused for bacterial cultivation on two plates ofDentaid-1 agar prepared by using brain, heart infusion agar to which the following compounds were added: 5 g yeast extract, 1.5 g sodium fumarate, and 1 g sodium Formate per liter. The medium was autoclaved for 15 min at 121°C. The final pH was 7.2 ± 0.2. Once the medium was cooled to 50°C, vancomycin was added to a final concentration of 9 µg/ml [(14)]. Incubated at 37C° for 72 hours under anaerobic condition using the anaerobic candle jar. One plate used for Identification of A.actinomycetemcomitans based on colony morphology, gram stains (gram negative coccobacilli) and catalase test (rapid catalase positive) [(15)].Colonies were examined for ability of adherence and difficulty of removal from agar and appearance as a rough or smooth colonyand light microscope (10X).

Detection of A.actinomycetemcomitans by culture-enhanced PCR: The other plate used for PCR analysis,isolates were randomly transferredinto400 µl PBS (8g/l Nacl,1.21 g/lK_2HPO_4,0.3g/l KH_2PO_4 (Ph7.3)sterile eppendorff tubes. After centrifugation at 6000g for 5 min, the supernatant was discarded and the pellet stored at –20 °C for DNA extraction'DNA extraction using genekam universal DNA isolation kit(Germany) by spin silica membrane, concentration ofextracting DNA was measured using biodropmost ,of samples had DNA concentration above 25 ng/µl, DNA samples was kept at -20°C.

PCR detection of A.actinomycetemcomitans: PCR identification kit (Genekam biotechnology,Germany)is one step system by amplification of the 360 bp fragment which was detected by gel electrophoresis.

PCR analytical procedure:mark the micro tubes with a sample number and with control +ve and control –ve,add 8 µl of tube A to each tube,add 10 µl of tube B to each tube **,**add 2 µl of extracting DNA template,add 2 µl of solution "positive control" to control +ve tube**,**add 2 µl of the solution "negative control" to control -ve tube**,** Putting the tubes in the Eppeddorf personal thermo cyclers and run the program in table 1:

Table 1: Thermo cycler running program

Segments	No of Cycles	Denaturation	Annealing/ extention
1	1	95 °C 600 seconds	
2	40	95 °C 60 seconds	61 °C 60 seconds 72° C 300 seconds
3	1		72° 600 seconds

Gel preparation & Electrophoresis Electrophoresis is carried out using TBE (Tris- Borate-EDTA buffer)Good quality agarose gel were prepared 2% TBE (1X),added (0.5µg/ml) ethidium bromide **.**After PCR step was finishedtest tubes were moved from thermocycler(UK) **,** 8µl from each amplicon specimen or control(+ve,-ve) to new empty test tube **,** 2µl of dye was added to each test tube ,mix and added the content of each tube to the lane carry the same name of the test tube **,**added 10 µl of marker to the first lane of agarose gel **,**run the gel for 35 min. at 120 Volt. ,400 Amp,view the gel under UV transilluminator360 bp band of amplicone appear in control +ve and +ve samples A.actinomycetemcomitans , no band in control negative or negative samples .

Results

The frequency of detection of A.actinomycetemcomitans as identified by culture and culture enhanced PCR are reported in Table(2).from 45 sample detection of A.actinomycetemcomitans by culture and conventional biochemical tests only 21(46%) isolates were capsulated gram negative coccobacilli , all were catalase positive ,16 were strongly adherent on agar had rough surface star shape inner structure colony when examined under 10X light microscope while 5 isolate were non adherent and easily removed from agar surface had smooth surface colony. Detection of A.actinomycetemcomitansby culture enhanced PCR figure(1):by detection of amplicon (360bp), increase the ability of detection 31(68.8%) and at the same time confirm the identification of A.actinomycetemcomitansby conventional culture-biochemical tests since all the positive isolates by cultural method appear positive by culture enhanced PCR.

Table 2 - Presence of A.actinomycetemcomitansdetermined by two separate detection methods in subgingival plaques taken from diseased sites

Detection method	No.of samples	No,of (+ve) isolates of A.actinomycetencomitans	%
Culture	45	21	46%
Cultur –enhanced PCR	45	31	68%

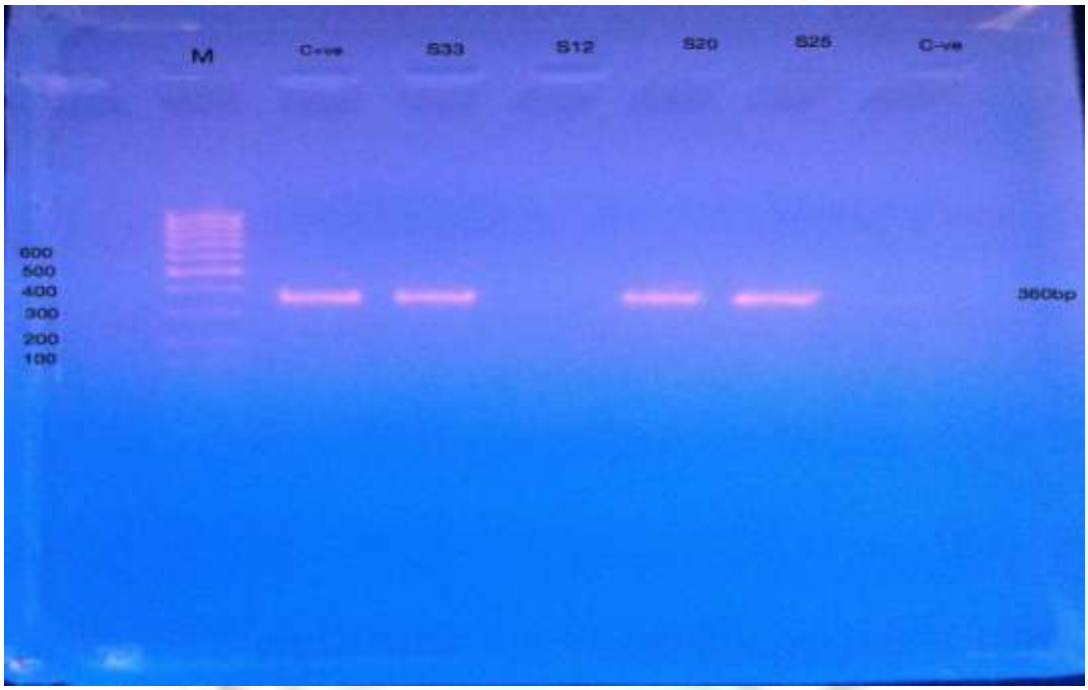

Figure (1): PCR method for identification of A.actinomycetemcomitans by detection of 360 bp. Fragment (amplicon)

This study compared a molecular method-culture enhanced PCR and theconventional culture method used for a long time in our oral microbiology laboratory statistical analysis was done by using **Mann-Whitney TestTable (3)**which show significant difference between the two methods (0.034).

Table(3): Mann-Whitney Test

Test Statistics[a]

	1
Mann-Whitney U	787.500
Wilcoxon W	1822.500
Z	-2.122-
Asymp. Sig.(2-tailed)	.034

a. Grouping Variable: method

Ranks

	Method	N	Mean Rank	Sum of Ranks
1	Culture	45	40.50	1822.50
	culture-enhanced pcr	45	50.50	2272.50
	Total	90		

Discussion

In determining the principal pathogens in the samples of subgingival plaque of patients with periodontal diseases. These two techniques were compared because their detection limit was similar (10^3-10^4)cells for anaerobic cultures, 10^2-10^3 cells for PCR)[16].From the analysis of our results and from thosein literatures it is possible to observe that no onewants to block the use of the culture method forthe search of oral microorganisms,but it is suggested that thenew molecular method can determine these microorganismsmore accurately ,despite the passing of the years and the largenumber of studies, much confusion persists onwhich method is more appropriate for the searchof periodontopathogenic bacteria, considering alsothat recent works by different authors continueto consider the great utility that the culturemethod continues to offer despite the advent ofreal time PCR and more sensitive DNA

probes[17].In conclusion, the ideal technique for accurate detection of pathogens in subgingival plaque samples has yet to be developed. The high sensitivity and specificity of multiplex PCR justifies its use in epidemiological studies of periodontal diseases. Both these techniques can detect multiple bacterial species coincidentally, but the bacterial cultures can detect unexpected bacteria and also allow the determination of antibiotic resistance ,colony morphology and biochemical charecterstics[18].

Conclusions

PCR confirm the detection and identification of A actinomycetyemcomitans since all the samples that give positive result by culture method produced positive reaction by PCR, combination of the two methods were found to be superior to culture with presumptive biochemical identification alone and should be the preferred for the detection of A. actinomycetemcomitans in subgingival plaque**.**

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