ORIGINAL ARTICLE

INTERNATIONAL JOURNAL OF RESEARCH IN PHARMACEUTICAL SCIENCES

Published by JK Welfare & Pharmascope Foundation Journal Home Page: www.ijrps.com

Identification of Bacteria Sample Plaque from Periodontal Disease Among Diabetic Patients

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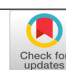
Article History:

Received on: 08 Nov 2020
Revised on: 27 Nov 2020
Accepted on: 14 Dec 2020

Keywords:

Periodontal disease,
Diabetes,
Bacteria

ABSTRACT



Molecular identification of bacteria are very important to develop database gene bank in university. Databases of genes are very important to annotate a collection of nucleotide sequence and protein translation of oral bacteria. This research was conducted to identify bacteria that present in the plaque sample among diabetic patients collected from the Department of Periodontology and Community Oral health Universiti Sains Islam Malaysia (USIM). A total of bacteria was successfully isolated on Blood Agar from periodontal patients' plaques. Colony Forming Unit (CFU/mL) was calculated to estimate the number of viable bacteria in a sample after performed serial dilution. Gram stained was done for all isolated samples to differentiate between Gram positive and negative organisms. About 2.07 x 10^{7} CFU/mL of bacteria was successfully collected from periodontal plaques on diabetes patient. From the numbers, only 17 were selected to identify the species. Seven from the samples were Gram-positive bacteria, meanwhile ten samples were identified as Gram-negative bacteria. PCR products were isolated from the samples by using polymerase chain reaction analysis. *Streptococcus mutans*, *Streptococcus gordonii,Streptococcus oralis,Streptococcus sanguinis,Streptococcus agalactiae,Streptococcus lutetiensis,Streptococcus downei,, Aggregatibacter actinomycetemcomitans, Klebsiella pneumoniae,Enterobacter clocae,Salmonella enterica subs enterica*, *Enterobacter ludwigii*, Enterobacter mori, *Enterobacter roggen kampii*, *Enterobacter cloacae*, *Enterobacter tabaci* and *Klebsiella pneumoniae* were identified from all samples via DNA sequencing.

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ISSN: 0975-7538

DOI: https://doi.org/10.26452/ijrps.v12i1.4147

Production and Hosted by

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INTRODUCTION

Diabetes mellitus cases are expanding globally as more than 300 million people will be affected by year 2025. Diabetic patients who poorly managed their lifestyles are associated with higher susceptibility to oral infection, including periodontitis (Campus *et al.*, 2005; Lalla *et al.*, 2006). Periodontitis is an attachment loss and inflammatory disease mainly caused by bacteria pathogens in subgingival plaque caused by immune-inflammatory response in the liable host (Gajardo *et al.*, 2005). Periodon-

tal disease is common and severe among diabetic patients with underlying complications (Campus *et al.*, 2005). Dental caries and periodontal disease mainly caused by pathogen oral bacteria (Al-Obaida *et al.*, 2020). Five species of periodontal bacteria that are normally found in oral including *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Capnocytophaga ochracea* and *Prevotella intermedia* (Al-Obaida *et al.*, 2020). Molecular biology techniques will be used for specific identification of bacterial species based on exact primer sequence (Paster *et al.*, 2001). PCR analysis has the highest sensitivity for microorganism recognition as we used 16S rRNA sequence primers for PCR component to identify dental pathogens. 16S rRNA gene sequencing is a sensitive, specific and accurate method for bacterial identification (Clarridge, 2004). Comparison of the sequences will be delivered a tool for molecular diversity analysis and phylogenetics evolution by virtue of the well-conserved sequence in 16S rRNA gene of different biological species (Slabbinck *et al.*, 2010). Besides that, 16S rRNA gene is very useful for finding new pathogen of bacteria in patients with antibiotic treatment (Rampini *et al.*, 2011). Identification of oral bacteria can be done by molecular methods (PCR) based on specific primers (Khan, 2012), probes (Salminen *et al.*, 2015) and direct sequencing (Jiang *et al.*, 1978; Riggio *et al.*, 2007). The aim of this study was to identify the diversity of common dental bacteria in diabetic patients, using the 16sRNA universal primers.

METHODOLOGY

Processing of subgingival plaque

A total of 900 μL Thioglycollate broth (TB) was pipette into 1.5 mL microcentrifuge tube. Then, the broth was incubated at 37°C under anaerobic condition for 24 hours. After incubation, the subgingival plaque (100 μL) of diabetes patient (Collected from the Department of Periodontal and Public health of USIM) were added into the TB. The samples were mixed properly and keep in -80 °C refrigerated until bacteria growth steps. The same method was repeated for sample plaque collection from different diabetic patients.

Bacteria growth and morphology observation

An amount of 100 μL of sample plaque (from -80°C refrigerated) were pipette into 2.5 mL TB and the sample was incubated at 37°C under anaerobic condition for 24 hours. Then, the bacteria culture from the plaque sample in the TB was observed. After that, a serial dilution method was performed from 10^{-1}until 10^{-4} to estimate the concentration (number of colonies of bacteria) of the TB. Then, an amount of 10 μL of each diluted culture was pipette and spread onto Blood agar (BA) for microbial enumeration and incubate at 37°C under anaerobic condition for 24 hours. The morphology and the number of colony growth on the BA were observed. The colonies were counted by using colony counter and the colony forming unit calculation (CFU/μL) were performed and recorded for each BA plate. The gram staining method was performed for each sample from BA plate 10^{-4} and observed under the microscope. The microscopic data were recorded. After that, a single colony from each BA plate were picked and subcultured onto prepared brain heart infusion agar (BHIA) and incubated at 37°C in anaerobic chamber for a day. Then, the growth of subcultured colonies on BHIA was observed, and the gram staining method was performed and observed under the microscope. Next, a single colony from BHIA was subcultured and inoculated into the prepared brain heart infusion broth (BHIB). The inoculated BHIB was incubated at 37°C in the anaerobic chamber. After a day, the subcultured BHIB was observed. Then, the culture that is grown in BHIB was preserved in glycerol broth by freezing method. An amount of 900 μL of culture was preserved in 100 μL glycerol solution in a micro centrifuge tube and stored in -80°C refrigerated.

DNA extraction

DNA extraction was done by using heat shock technique following the manufacturer's manual (Instagene matrix, Biorad). A total of 1000 μL of bacteria broth was put into 1.5 mL eppendorf tube. The sample was centrifuge for 12000 rpm at 1 minute. The supernatant was discarded. Then, about 200 μL of Instagene matrix solutions was added to the pellet and incubated at 56°C for 15-30 minutes. The samples had been vortex at high speed for 10 seconds. The samples had been placed in boiling water bath (100°C) for 8 minutes. The sample was centrifuge for 1 minute at 12000 rpm. Quality of DNA was measured by A260/280 ratio.

Bacteria identification

Product PCR was isolated from samples by using 16sRNA primers (Mohammadi *et al.*, 2003). The primers for the analysis were selected and purchased from Apical Scientific Oligo company. The primer pairs used for the PCR amplification were Forward: TCCTACGGGAGGCAGCAGT and Reverse: GACTACCAGGGTATCTAATCCTGTT. The predictable of product PCR size was 300 bp. The PCR was carried out in a total volume of 25μl containing 20 μl of reaction mixture and 5 μl of the DNA samples.

The reactions of mixture PCR were contained 1x MIX

Table 1: The total of bacteria successfully isolated from subgingival plaque diabetes patient

Sample	Age	Gender	Number of isolated bacteria
P1	38	Male	1
P2	44	Male	2
P3	48	Male	3
P4	46	Female	2
P5	55	Male	1
P6	55	Female	2
P7	58	Female	2
P8	61	Female	2
P9	63	Male	2

PCR buffer (Promega), 100 pmol of each primer and 50 ng/μl DNA samples. The setting programmed for PCR was set up. The step for denaturation was 95°C for 5 min, followed by amplification step for 30 cycles including denaturation at 95°C for 1 min, primers annealing at 55°C for 1 min and primer extension at 72°C for 2 min. After completing the cycles, final extension step had been done at 72°C for 10 min. PCR analysis was done by using thermal cycler from Biorad, USA.

Sequencing analysis

A total of 17 product PCR was successfully isolated from all samples. All the products were purified using PCR Clean Up Kit (Promega). All PCR products were sent to Apical Scientific sequencing for sequencing process. Results of sequencing were analysed by using bio-informatics tools Basic Local Alignment Search Tool (BLASTn).

RESULTS AND DISCUSSION

Sampling and bacteria isolation

In this study, about nine subgingival periodontal plaque samples were successfully collected from diabetes patients. A total of 17 bacteria have been successfully isolated from that patients (Table 1). The subgingival plaque samples were obtained from patient representing both genders. Among 9 patients, there were four females and five males. The mean age of the patient was 50, ranging in age from 30 to 70 (Table 1). Figure 1 show the age ranges in this study by gender. Some researchers reported that females were found to be more affected than males, particularly in the younger age group (21-25 years). This finding was in agreement with Khorasani and Samiezadeh (2008); Bataineh and Al (1985); Piironen and Ylipaavalniemi (1981). Previous study from Anjum *et al.* (2014), reported that more males were affected than female in a ratio of 1.3:1. A few previous studies also reported more incidents of male compared to female (Tegginamani and Prasad, 2013; Singh *et al.*, 2018). The difference of findings on gender among a periodontal patient suggests that there is no correlation between genders that may affect the inflammation to occur.

Bacterial identification using standard microbiological test

The subgingival plaque samples were cultured on blood agar media to enhanced growth of strictly anaerobic bacteria. The morphologies of bacteria from most samples were small and greyish white. However, some of the bacteria were large, circular, white to grey and translucent. The subculture is carried to obtain pure culture for each colony.

Table 2: Predominant bacteria of periodontal samples among diabetes patient

Predominant bacteria	Number of bacteria
Gram positive, cocci	7
Gram negative, rod	10
Total	17

A total of seven Gram-positive and ten Gram-negative bacteria was found in samples plaque. Based on bacterial identification using standard microbiological test, gram-negative were found to be the predominant group identified in this study as summarized in Table 2.

The microbiological data confirmed that bacteria pathogen observed in the study was noticed common in biofilms formation.

This observation supports the statement that the oral cavity can be as reservoir and a cause by these microorganisms. Previous report mention that that different structures and tissues of oral are colonized by dissimilar microbial colony (Mager *et al.*, 2003; Hanafiah *et al.*, 2015).

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Table 3: Morphology of all isolated bacteria from periodontal plaque among diabetes patients

Bacteria	Microscopic morphology	Morphology culture
1	Gram positive, coccus and chain	small and greyish white
2	Gram positive, coccus and chain	small and greyish white
3	Gram positive, coccus and chain	small and greyish white
4	Gram positive, coccus and chain	small and greyish white
5	Gram positive, coccus and chain	small and greyish white
6	Gram positive, coccus and chain	small and greyish white
7	Gram positive, coccus and chain	small and greyish white
8	Gram negative, rod and cluster	large, circular, white to grey and translucent
9	Gram negative, rod and cluster	large, circular, white to grey and translucent
10	Gram negative, rod and cluster	large, circular, white to grey and translucent
11	Gram negative, rod and cluster	large, circular, white to grey and translucent
12	Gram negative, rod and cluster	large, circular, white to grey and translucent
13	Gram negative, rod and cluster	large, circular, white to grey and translucent
14	Gram negative, rod and cluster	large, circular, white to grey and translucent
15	Gram negative, rod and cluster	large, circular, white to grey and translucent
16	Gram negative, rod and cluster	large, circular, white to grey and translucent
17	Gram negative, rod and cluster	large, circular, white to grey and translucent

Table 4: DNA concentration and analysis using UV spectrophotometer on bacteria

Bacteria	DNA concentration (ng/μL)	A260/280	A260/230
1	54	1.803	2.001
2	67.0	1.801	1.998
3	75.0	1.809	2.000
4	89.5	1.798	1.999
5	66.3	1.800	2.000
6	88.8	1.805	1.998
7	65.0	1.799	2.000
8	130.0	1.800	2.001
9	322.0	1.801	2.000
10	118.2	1.802	1.998
11	210.5	1.802	2.001
12	320.06	1.809	2.002
13	218.26	1.798	2.000
14	135.43	1.803	2.002
15	210.68	1.801	2.002
16	190.49	1.799	1.999
17	210.45	1.800	1.999

Table 5: Bacterial identification and percentage (%) of alignment in database National Centre Biotechnology Information (NCBI)

Bacteria	Name of Bacteria	Percentage of similarity (%)
1	*Streptococcus mutans*	98
2	*Streptococcus gordonii*	100
3	*Streptococcus oralis*	99
4	*Streptococcus sanguinis*	99
5	*Streptococcus agalactiae*	100
6	*Streptococcus lutetiensis*	98
7	*Streptococcus downei*	99
8	*Aggregatibacter actinomycetemcomitans*	99
9	*Klebsiella pneumoniae*	98
10	*Enterobacter clocae*	98
11	*Salmonella enterica subs enterica*	98
12	*Enterobacter ludwigii*	99
13	*Enterobacter mori*	98
14	*Enterobacter roggen kampii*	98
15	*Enterobacter cloacae*	99
16	*Enterobacter tabaci*	98
17	*Klebsiella pneumoniae*	99

Mostly, Gram-positive bacteria will be growth depend on medium from dental plaque. Early stage of biofilm will be predominantly by Gram positive, aerobic, non-motile and anaerobic bacteria, however for late stage, biofilm will be predominantly colonized by Gram-negative and mobile bacteria (Komori *et al.*, 2012).

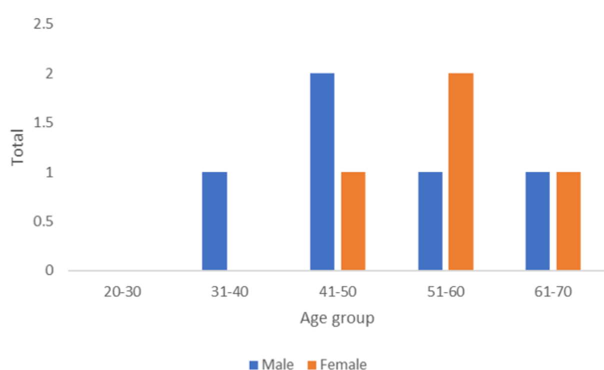

Figure 1: The age range of subjects in this study

Staining technique was done after the single colony of the pure culture obtained to distinguish the colony, whether it is Gram-positive or Gram-negative bacteria for further identification. Gram staining shows the sizes, shapes, and arrangement of the cells. Table 3 showed morphology microscopic of the isolated bacteria. Morphology cultures of the gram-positive bacteria were small and greyish white, meanwhile gram-negative bacteria were large, circular white to grey and translucent. The morphologies of isolated bacteria were coccus and rod (Figure 2). Meanwhile, the arrangements were chain and cluster (Figure 2). The gram staining is critical in this study as it well defined the method at DNA extraction part. There is a slightly additional step for DNA extraction of Gram-positive bacteria because peptidoglycan in the cell wall is thicker than Gram-negative bacteria.

Bacterial identification by molecular analysis

DNA Extraction

Bacterial DNA of was successfully extracted by using Instagene matrix. The DNA concentration and the purity of the DNA are recorded in Table 4. The ratio of A260/280 is commonly used to investigate protein contamination in DNA samples.

The approximate purities of double-stranded DNA preparations are valued by determination of the absorbance ratio at 260 and 280 nm (A_{260}/A_{280}). The purity ratio of DNA (A_{260}/A_{280}) is 1.8. The purity of RNA has been reading of around 2.0. The 260/280 ratio for protein is 0.5 until 1. The DNA sample has been reading above than 1.8 suggests that there is RNA contamination. If the reading obtained is less than 1.8, this suggests there is a protein in the samples. The ratio of A260/230 will indicate the presence of organic contaminants, including TRIzol, phenol, and other compounds. If the ratio (260/230) below than 1.8, the samples considered to contain contaminants and will be interfered with downstream applications.

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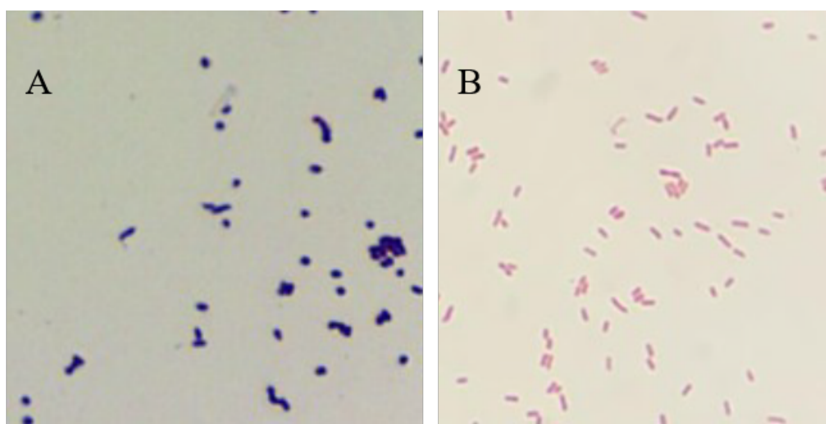

Figure 2: Microscopic morphology of bacteria from plaque samples. A: coccus and chain (Gram positive), B: Rod and cluster (Gram negative)

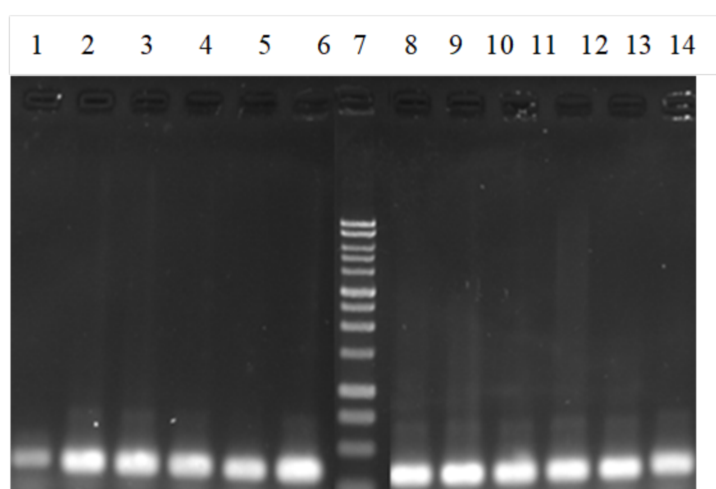

Figure 3: Gel stain polymerase chain reaction (PCR)amplicons separated in 1.5% TAE agarose gel (100V, 40 minutes) at 300bp. Lane1-6: Product PCR samples of 1-6. Lane 7: DNA ladder containing DNA fragments of defined length (1kb) (Promega). Lane 8-14: Product PCR samples of 8-13

The purity ratio of DNA (A_{260}/A_{230}) is 2.0. In these results, all samples are good according to the value of 260/280 and 260/230 ratio. The absorbance spectrum of DNA samples shows a quality purity with excellent reading in A260/280 and A260/230 ratios. However, the spectrophotometer reading is might not be reliable because there have many factors of contamination. If the DNA is low, the purity difficult to assess with A260/280 and A260/230 ratios. The DNA may be actually well, but it cannot be measured by Nanodrop.

PCR Analysis

The DNA extracts of bacterial have been amplified through PCR. The product PCR was analysed by running electrophoresis gel to observe the quality of PCR products. The primers used in this study produced a 300bp PCR products from the 16S rRNA for all bacteria samples identified. Figure 3 showed the representative PCR products separated in an agarose gel. DNA bands observed in lane 1 to 14 showed the successful amplification of the target sequence of product PCR DNA samples (1-13). The PCR analysis has detected several 17 bacterial species associated with periodontal disease and diabetes.

The PCR method is simple and provides rapid results as compared to the standard microbiological method. In PCR, the DNA will copy from the target area based on the levels of stringency and the high temperature of PCR reaction. The stringency is the matching between primers and DNA samples. The matching will be controlled by level of salts and temperature. There is a good match between primers and DNA targets at a high temperature. This process will be ensured the primer does not attach elsewhere and cause non-target DNA copy. Hence, the PCR results will be achieved high level of accuracy technique. This is important in eliminating any

false-positives or false-negatives in disease diagnosis (Lorenz, 2012).

Sequencing Analysis

Further investigation was carried out to identify the bacterial species of the amplified DNA gene. Sequencing analysis successfully identified 17 bacterial strains from samples. Percentage of similarity (%) was listed in Table 5. Most of the bacteria were Gram-negative, rod and the remain of bacteria were Gram-positive cocci. The nucleotide sequences were compared using Basic Local Alignment Search Tool (BLAST) to another nucleotide in the database of GenBank from National Centre for Biotechnology Information (NCBI). The identification ranges of similarity bacteria are between 98% to 100% for all samples. In this research, identification of bacteria was chosen by 98-100% similarity with other species in NCBI data. Sequence analyses have been applied to determine phylogeny tree of microorganism families. It also can be used to identify horizontally transferred genes and recombined sequences. The sequencing method is very useful to further analysis such as next-generation sequencing and whole genome sequencing. Furthermore, alignment of DNA is the important analysis to annotate the conserved protein domains and tracking the phenotype-related in sequence of polymorphism. It also can be used to re-construct the ancestral DNA sequences and determine the rate of sequence evolution. DNA matching will produce three-dimensional protein structures based on homology-modeling (Zielezinski *et al.*, 2017).

CONCLUSIONS

In conclusion, there are several types of bacteria present in plaque samples collected from diabetic patients in the dental clinic USIM. Microscopic morphology of bacteria showed that 10 bacteria were gram negative and rod while 7 bacteria were gram positive and coccus. Sequencing analysis showed that the percentages of alignment in the database were 98-100%. *Streptococcus mutans*, *Streptococcus gordonii,Streptococcus oralis,Streptococcus sanguinis,Streptococcus agalactiae,Streptococcus lutetiensis,Streptococcus downei,*, *Aggregatibacter actinomycetemcomitans, Klebsiella pneumoniae,Enterobacter clocae, Salmonella enterica subs enterica*, *Enterobacter ludwigii*, Enterobacter mori, *Enterobacter roggen kampii*, *Enterobacter cloacae*, *Enterobacter tabaci* and *Klebsiella pneumoniae* were identified from all samples via DNA sequencing.

ACKNOWLEDGEMENTS

This study had been acknowledged to the Department of Periodontol and Public Health (USIM) for providing plaque samples.

Funding Support

This study was financially supported by Ministry of Higher Education Malaysia under MRUN-RAKAN RU-2019-002/2.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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