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Original Study

Detection of endodontic pathogens using Benzoyl-dl-Arginine-Naphthylamide (BANA) in infected root canals

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ABSTRACT

Aim: Culture based identification techniques and molecular techniques are elaborate and a simple quick chairside rapid test will enable quicker detection of endodontic pathogens. The aim of this study was to determine the hydrolysis of BANA with standard strains of common endodontic pathogens in vitro and also the diagnostic accuracy of BANA in detecting microorganisms from infected root canal system in vivo.

Materials and Method: The study was conducted in 2 phases. In the first phase, in vitro hydrolysis of BANA by ATCC strains of common endodontic pathogens were evaluated. After BANA testing, intensity of colour change was noted at different incubation time of 4,6 and 18 hours. In the second phase,intracanal samples from each tooth (Pre-cleaning and shaping, Post- cleaning and shaping) were collected from 85 patients and subjected to both BANA and culture methods simultaneously. The results were tabulated and analyzed using measures of diagnostic validity.

Result: In phase one testing, Porphyromonas gingivalis and Fusobactrium nucleatum showed strong positive reaction at overnight incubation on 1:20 dilution whereas it showed weakly positive reaction at 1:40 and 1:60 dilution. Pseudomonas aeruginosa showed weakly positive reaction at 1:20 dilution at overnight incubation whereas no color change at all other dilutions. Pre-cleaning and shaping diagnostic accuracy of BANA was 69.41% (95%CI, 59%- 79%). Sensitivity and positive predicative value for samples prior to cleaning and shaping was 75% (95%CI) and 80%(95%CI) respectively.

Conclusion: Porphyromonas gingivalis, Fusobacterium nucleatum, Pseudomonas aeruginosa, E. coli responds to BANA except Candida albicans. BANA has 69.41% of clinical diagnostic accuracy in detecting aerobic and facultative anaerobic bacteria present in teeth with necrotic pulps.

***Keywords:** BANA test; Culture method; Endodontic pathogens; Enterococcus faecalis Porphyromonas gingivalis.*

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INTRODUCTION

The main etiological factor for pulpal diseases is microorganisms that invade the dental pulp[i].Endodontic infections are distinct as it is polymicrobial in nature[ii]. The multiplicity of polymicrobial endodontic infections has been proved with 3-12 species of microbes identified in primary endodontic infection[3,4]. The main objective of the endodontic treatment is to lessen the microbial burden from the root canal system for an optimal treatment outcome. Thus, the progress of successful antimicrobial approaches for root canal therapy is reliant upon understanding the makeup of the infective flora of the root canal system. The established methods in microbial detection are culture methods, polymerase chain reaction and immunology-based methods[5]. Recent research has validated the polymicrobial disposition of endodontic infections by employing culture methods, molecular approaches, and biochemical tests. The findings of this study revealed that the bulk of species observed in primary root canal infections belonged to obligate Gram-negative anaerobic bacteria[6].

The association of microbiological outcomes from culture dependent investigations is blighted by certain shortcomings of the culture methods, leading to the underestimation of bacterial variety inside the root canal system[7,8]. This led to the need for a culture independent technique to identify microorganisms without culturing. The introduction of molecular diagnostic methods completely unraveled the bacterial diversity of endodontic microbiota[9]. Thus, molecular diagnostic succeeded over culture methods which has redefined the microbiological profile of different types of endodontic infection. These approaches have positively shown that the root canal flora is more complex than formerly thought. Due to the close relationship between bacteria and endodontic pathosis, bacteriological sampling of the root canal system can be a predictor for assessing the treatment outcome[10]. Cultivation based approaches are considered the gold standard for a dentist to detect the precise targets treatments and to estimate treatment approaches due to their easier accessibility than newer methods[11].

Till now, there is no quick chairside test to perform the microbial sampling of root canal system. Meanwhile, Losche et al demonstrated the presence of trypsin like enzyme is capable of hydrolyzing the synthetic substrate benzoyl-dl-arginine-naphthylamide (BANA) produced by P.Gingivalis, T.forsythesis and T.denticola could be used as diagnostic marker[12]. The BANA test reveals the presence of this enzyme through a chromogenic reaction obtained by adding a drop of fast garnet dye to suspension[13]. This indicates that the existence of trypsin enzyme in the root canal sample shows the presence of BANA positive pathogens. Taxonomic studies of the microorganisms found in subgingival plaque revealed 6 other types of Porphyromonas gingivalis, Treponema Denticola showing positive response to BANA. Subsequent research in this direction has resulted in the development of a chairside kit to detect periodontal pathogens[14]. The plausible use of BANA in detecting microorganisms inside the root canal is worth exploring, since they are like periodontal pathogens. Quick chairside evaluation will be convenient in formulating root canal disinfection strategies. The evaluation of the response of ATCC strains of common endodontic pathogens to BANA will enable the identification of BANA positive and negative endodontic organisms.

The study was conducted in 2 parts. In the first part, an *in-vitro* determination of hydrolysis of BANA by common endodontic pathogens in different concentration and proportion was done. This was done to identify the BANA positive and BANA negative endodontic pathogens. In the second part, diagnostic accuracy of BANA in detecting microorganisms from infected root canal system was assessed.

MATERIALS AND METHODS

Protocol Registration

The research methodology was approved by the ethical committee at Meenakshi Academy of Higher Education and research institute (MAHER) Ref-MAHER/COE-113/2013.

In-vitro detection of bacterial strains by BANA

Pure cultures

ATCC strains of Porphyromonas gingivalis, Fusobacterium nucleatum, Candida albicans, Pseudomonas aeruginosa, Enterococcus faecalis was sub-cultured in BHI agar medium and incubated at 37°C for 18 hours. The solitary pure colonies were incubated in BHI broth and incubated at 37°C for 4 hours and the turbidness was altered to 0.5% McFarland standard. This standardized broth culture was used for further testing. The standard strains were serially diluted into 1:20, 1:40, 1:60. BANA solution was prepared as described by Bretz et al. Briefly, the BANA stock solution was formulated by adding 44mg to 1mg of dimethyl sulfoxide and diluted to 1:100 in Sorensen phosphate buffer. 1ml of BANA solution was added to all standardized broth culture. After 2nd hour of incubation, 100µm was taken from 1:20 dilution of all strain to which 10µl of fast garnet dye was combined. The amount of colour change was noted. This procedure was repeated for rest of the dilutions at different incubation times of 6, 12 and 18 hours. The intensity of colour change was noted. The BANA test findings were read visually and recorded as negative (Yellow), weakly positive (yellowish orange), positive (orangish red), strongly positive (red).

In-vivo detection of microorganisms by BANA

Samples for this clinical study were selected from the teeth among the outpatients attending the department of conservative dentistry and endodontics, Meenakshi Ammal Dental College, and hospital, Maduravoyal, Chennai based on the following criteria. Teeth with non-vital pulp, apical periodontitis and periapical abscess were included in the study. Root canal treated teeth, teeth which were vital after access opening, teeth with calcified canals, gross destruction of crown and teeth with root resorption were eliminated from the study. Written informed consent was taken from all the selected patients.

Sampling from root canals was done under stringent aseptic conditions. For each tooth isolation was done with rubber dam. Any restorations present or caries was removed with sterile high-speed and low-speed burs after which the tooth and the adjacent field were cleaned with 30% hydrogen peroxide for 30 seconds followed by 2.5% sodium hypochlorite solution for an extra 30 seconds. Later, the disinfectant was deactivated with sterile 5% sodium thiosulphate for 2 minutes.

Sterile endo access burs (Dentsply Maillefer, Ballaigues, Switzerland) and instruments helped to gain access to the root canal system. Prior to entering the pulp chamber, the access cavity was disinfected with the same procedure as above and the sterility was again examined by taking a swab sample of the cavity exterior and streaking on blood agar plates. Canal patency was checked with #10 ISO K file (Mani, Inc., Tochigi, Japan). The canals were instrumented with K files up to ISO #20. Two #20/02 sterile paper points were positioned in the canal and left there for 30 seconds. Paper points were removed to a sterilised tube containing1 ml Viability Medium Göteborg Agar (VMGA) III transport medium. The samples were transported to a microbiological laboratory within 15 minutes. One paper point was now transferred in 2ml of BHI broth vial

and another one placed in 1 ml of BANA solution vial. Working length was found using apex locator and established with a radiograph.

The root canals were shaped using Protaper universal Ni-Ti rotary instrumentation (Dentsply Maillefer, Ballaigues, Switzerland) using a crown-down technique using profuse irrigation with saline, 5% NaOCl (Prime Dental product Pvt Ltd, Mumbai, India) and 17% ethylene diamine tetra acetic acid (EDTA) (Prime Dental product Pvt Ltd, Mumbai, India). Final irrigation was done with 2% chlorhexidine (Septodont Ind Pvt Ltd, New Delhi, India). Following this two sterile paper points were again placed inside the canal and left inside for 30 seconds. Paper points were shifted to a disinfected tube containing 1 mL VMGA III transport medium. The samples were transported to a microbiological laboratory inside 15 minutes. One paper point was transferred to BHI broth vial (1ml) and another one placed in BANA solution vial (1ml).

Later the vials were marked appropriately for the ease of identification. All the vials were incubated at 37°C overnight. Samples from BHI broth was streaked on BHI agar plates and then incubated at 37°C during the night. Microbial colonies were tallied on plates with positive growth under microscope (Allied surgicals, New Delhi, India). The culture plates were counted twice, and an average was considered. A mean colony count of more than 5000 colonies were considered as culture positive. Subsequently, the type of organism was identified centred on the colony characteristics (size, shape, color, haemolysis consistency, surface, and brightness) identified using Gram's staining test.

Meanwhile, BANA solution was prepared as described by above. Samples vials with BANA solution were taken from the incubator and one drop of 0.1ml of fast garnet dye was included and the strength of chromogenic reaction was noted visually and recorded as described previously. An independent examiner scored the BANA test results. Both examiners (culture and BANA) were masked to the results of the other test and to the clinical information pertaining to the samples.

The results for the samples were noted and recorded. Then they were subjected to statistical analysis (SPSS software version 16). The validity of BANA was ascertained by calculating sensitivity, specificity, positive predictor valve and diagnostic accuracy values.

RESULTS

Part I

Porphyromonas gingivalis (ATCC 33277) and Fusobactrium nucleatum (ATCC 25586) showed strong positive reaction at overnight incubation on 1:20 dilution whereas it showed weakly positive reaction at 1:40 and 1:60 dilution. P.Aeruginosa showed weakly positive reaction at 1:20 dilution at overnight incubation whereas no color change at all other dilutions. Candida albicans, Enterococcus faecalis showed negative reactions at all incubation times (Table 1).

Table 1: Hydrolysis of BANA with standard strains of primary endodontic pathogens

INCUBATION	Fusobacterium nucleatum	Porphyromonas gingivalis	Pseudomonas aeruginosa	E. Faecalis	Candida albicans
			DILUTION		

	1:20	1:40	1:60	1:20	1:40	1:60	1:20	1:40	1:60	1:20	1:40	1:60	1:20	1:40	1:60
2 hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4 hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6 hours	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18 hours	++	+	+	++	+	+	+	-	-	-	-	-	-	-	-

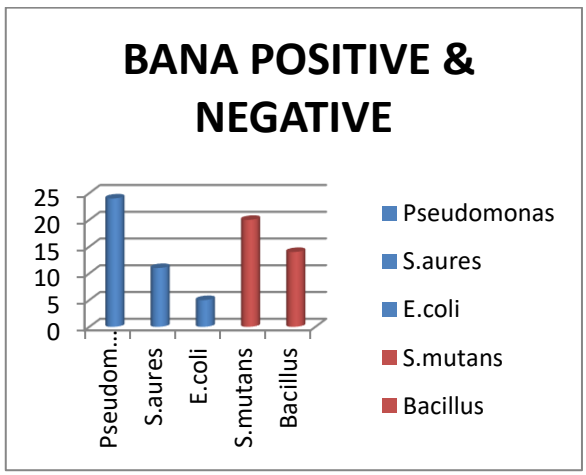
- Negative, + weakly positive, ++ strongly positive

Part II

Culturing procedure:

Measurement of validity of BANA test for pre and post cleaning and shaping samples is depicted in table 2. The proportions of organisms detected and undetected before cleaning and shaping using BANA is presented in graph 1.

Figure 1 - Proportions of organisms detected and undetected in Pre-cleaning and shaping using BANA



The pre-cleaning and shaping diagnostic sensitivity ranged from 63% to 84.22% with mean of 75% and the positive predictor value of 80.4%, which shows that BANA had the ability to detect culture positive. The pre-cleaning and shaping diagnostic specificity ranged from 37% to 73.33% with mean of 56% and the negative predictor value was 48.3%. Hence, diagnostic interpretation has to be exercised with caution when BANA is negative. The diagnostic accuracy for the pre-cleaning and shaping samples was 69.41% (59%-79% at 95% CI) indication BANA is highly reliable in detecting initial load of microorganism (Table 2).

Table 2: Measurement of validity of BANA test for Pre and Post –cleaning and shaping samples respectively

Parameter	Estimate (95% CIs)	
	Pre –cleaning and shaping	Post–cleaning and shaping
Sensitivity	75% (63-84.22)	1.69% (0.30-9.00)
Specificity	56% (37- 73.33)	69.23% (50.01-84)
Positive Predictive Value	80.4% (68.2- 89)	11.11% (2- 44)
Negative Predictive Value	48.3% (31.4-66)	23.7% (15.5-34.4)
Diagnostic Accuracy	69.41% (59-79)	22.4% (1-32.3)

The post-cleaning and shaping diagnostic sensitivity is 1.69% with the positive predictive value of 11.11% showing that BANA does not have the ability to detect culture positive. The post-cleaning and shaping specificity was 69.23% with the negative predictor value of 23.7%, indicating that diagnostic interpretation must be exercised with caution. The diagnostic accuracy range for the post clinical samples was 22.4% (1%-32.3% at 95% CI) indicating that BANA is not reliable in detecting microorganisms in post cleaning and shaping samples (Table 2).

DISCUSSION

Infected root canal complicates the treatment outcomes in endodontics[15]. Several studies reported that the assessment of lingering bacteria during obturation may be a good predictor of long-term outcomes[16,17,18.]

The diagnostic accuracy of BANA was evaluated to develop a less invasive method to assess the microbial load of infected root canal. This can improve the disinfection approaches and aftermath of the endodontic treatment. The BANA assay was compared with culture method as it is traditionally considered as primary standard.

This study was done to assess the capability of BANA to identify microorganisms from the infected root canals which are compared with traditional culture methods. In addition, ATCC strains of Porphyromonas gingivalis, Fusobacterium nucleatum, Candida albicans, Pseudomonas Aeruginosa, Enterococcus faecalis response to BANA was also evaluated. Although the structure of microbial community differs in different types of endodontic infection, certain key species play a major role in pathogenicity of that community. Thus, invitro BANA testing was done only in common endodontic pathogens. BANA is available in a colourless powder form. It has a powerful affinity to proteolytic enzymes such as trypsin. For the analysis Sorensen buffer, monopotassium phosphate, disodium phosphate at pH 7.2 was employed because only in this pH enzymatic activity of the microorganism is well preserved. BANA though colourless, upon hydrolysis releases napthylamide that reacts with fast garnet dye to form-colored products which can be viewed visually and can be interpreted as negative, weakly negative, weakly positive and positive based on the color produced.

The use of BANA for the identification of microorganisms from clinical samples showed 69.41% diagnostic accuracy when tested prior to cleaning and shaping. This indicates that BANA has the potential to clearly identify the true positives and true negatives to a considerable extent. Among the 85 samples tested prior to cleaning and shaping, 11 samples showed false positive, and 15 samples showed false negative. The reason for false positive reaction could be attributed to the culture method. In our study, culture methods promote growth of aerobes and facultative anaerobes. The 11 samples that showed BANA positive and culture negative could be result on anaerobic that could not be cultured. The reason for this false negative could be attributed to the lack of trypsinase activity among the microorganisms detected by culture. Among the 15 samples which was negative to BANA predominantly constituted streptococcus mutans and Bacillus sp. It is known from literature

that these two organisms lack trypsinase enzymes. The clinical relevance of this study is that BANA test when developed as a chair side kit will facilitate the detection of initial microbial load before commencement of treatment which will enable establishment of treatment planning. BANA test performed post cleaning and shaping will be helpful in evaluating the antimicrobial efficacy of treatment protocols or effectiveness of treatment employed. The samples showing positive reaction to BANA indicates treatment strategies were not adequate to reduce the microbial load. Thus, these cases will receive intracanal medicament to enhance the antimicrobial activity and multivisit endodontic treatment will be recommended for these patients.

The limitations of this study are that prolonged (18 hours) incubation time for BANA analysis and detection of microorganisms by aerobic culture method. This protocol was followed to check the efficacy of BANA using primary chemical ingredients. This original BANA liquid assay has to be modified to a chair side kit with less incubation time similar to solid state format (Perioscan) that was developed in periodontics for assessment of periodontal

infection. In this study, the authors intended to check the response of BANA to aerobes and facultative anaerobes as detected by aerobic culture methods. The encouraging finding of this study paves way for future studies that could test BANA against bacteria isolated with anaerobic culture methods and/or PCR methods. This will provide further information of facultative anaerobic bacteria that could be detected by BANA.

CONCLUSION

Within the constraints of this clinical study, it can be determined that Porphyromonas gingivalis, Fusobacterium nucleatum, Pseudomonas aeruginosa respond to BANA except Candida albicans and enterococcus faecalis. The BANA test has a diagnostic accuracy of 69.41% in detecting aerobic and anaerobic bacteria.

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Conflicts of interest - There are no conflicts of interest.

REFERENCES

1. Chavez de Paz LC. Redefining the persistent infection in root canals: possible role of biofilm communities. J Endod 2007;33:652–62
2. Siqueira JF, Rocas IN. Community as the unit of pathogenicity: an emerging concept as to the microbial pathogenesis of apical periodontitis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;107:870–8.
3. Sundqvist G, Johansson E, Sjogren U. Prevalence of black-pigmented Bacteroides species in root canal infections. J Endodon 1989;15:13-9.
4. Baumgartner JC, Watkins BJ, Bae KS, Xia T. Association of black-pigmented bacteria with endodontic infections. J Endod 1999;25:413-415.
5. Lazcka O, Del Campo FJ, Munoz FX. Pathogen detection: a perspective of traditional methods and biosensors. Biosens Bioelectron. 2007 15;22:1205-17.
6. Pourhajibagher M, Ghorbanzadeh R, Bahador A. culture dependent approaches to explore the prevalence of root canal pathogens from endodontic infections. Braz Oral Res. 2017, 18;31:e108.Hugenholtz P. Exploring prokaryotic diversity in the genomic era. Genome Biol 2002,3:Reviews0003.1.
7. Relman DA. New technologies, human-microbe interactions, and the search for previously unrecognized pathogens. J Infect Dis 2002, 186(Suppl 2):254-258.

8. Siqueira JF Jr, Rôças IN. Exploiting molecular methods to explore endodontic infections: Part 2—Redefining the endodontic microbiota. J Endod 2005;31:488-498.Sathorn C, Parashos P, Messer HH. How useful is root canal culturing in predicting treatment outcome? J Endod 2007;33:220-5.
9. Fouad AF. Endodontic Microbiology and Pathobiology: Current State of Knowledge. Dent Clin North Am. 2017;61:1-15
10. Loesche, W. J. 1986. The identification of bacteria with periodontal disease and dental caries by enzymatic methods. Oral Microbiol.Immunol.1:65-70
11. Laughon BE, Syed SA, Loesche WJ. API ZYM system for identification of Bacteroides spp., Capnocytophaga spp., and spirochetes of oral origin. J Clin Microbiol. 1982;15:97-102.
12. Loesche WJ, Bretz WA, Kerschensteiner D, Stoll J, Socransky SS, Hujoel P, Lopatin DE. Development of a diagnostic test for anaerobic periodontal infections based on plaque hydrolysis of benzoyl-DL-arginine-naphthylamide. J Clin Microbiol. 1990;28:1551-9.
13. Ng YL, Mann V, Rahbaran S, Lewsey J, Gulabivala K. Outcome of primary root canal treatment: systematic review of the literature -- Part 2. Influence of clinical factors. Int Endod J. 2008;41:6-31.
14. Sundqvist G, Figdor D, Persson S, et al. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998;85(1):86–93.
15. Sjogren U, Figdor D, Persson S, et al. Influence of infection at the time of root filling on the outcome of endodontic treatment of teeth with apical periodontitis. Int Endod J 1997;30:297–306.
16. Waltimo T, Trope M, Haapasalo M, et al. Clinical efficacy of treatment procedures in endodontic infection control and one year follow-up of periapical healing. J Endod 2005;31:863–6.

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