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ORIGINAL ARTICLE

Effective anaerobic culture method for identifying the organisms in deep dentinal caries and testing the antimicrobial efficacy of the pulp capping agents- comparison of 2 techniques

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Abstract

Background: Effective pulp capping cements with good antimicrobial properties is a requirement to prevent propagation of deep dentinal caries to healthy pulp. Previously, the studies on antimicrobial efficacy were performed using commercially available strains. The present study was carried out using the samples collected directly from deep dentinal caries. Two different methods direct contact test (DCT) and agar diffusion method (AGM) of anaerobic incubation were assessed to predict the antimicrobial efficacy of the dental cements. **Aims**: To test, compare and gauge 2 anaerobic methods- DCT and ADM to detect the antimicrobial efficacy of the cements. **Materials and Methods:** Carious material (n=25) from deep dentinal caries. Glass ionomer (GIC Fuji II), CaOH2 in form of Dycal, mineral trioxide aggregate (MTA) and Zinc oxide eugenol (ZnOE) were tested for antimicrobial efficacy using DCT and ADM. **Results and statistical analysis**: The anaerobes could be grown with both the techniques (100%). Contamination was detected in 12% of cases on DCT; MTA plates were contaminated the most. Lack of diffusion of cements across the agar was seen in 12% of cases of ADM. Both the techniques showed ZnOE to be more effective followed by MTA, Dycal and GIC with a p value of >0.001 and was statistically significant. **Conclusion**: DCT is an extensive procedure and selective colonies could be grown. ADM is easy to perform and allowed the growth of mixed culture. The disadvantages of DCT were contamination and prolonged procedure, whereas; of ADM were poor diffusion of the cements across the agar.

Keywords: Anaerobic culture, Mixed microflora, Deep dental caries, Pulp capping agents, Anaerobic culture technique, Direct contact test, Agar diffusion method

1 | INTRODUCTION

Deep dentinal caries if treated appropriately can save the vitality of the tooth and prevent an extensive procedure such as the root canal treatment. Often during the preparation of such cavities, total removal of carious dentin is not advised for the fear of exposure of healthy pulp which has shown no signs of degeneration. In such an event, pulp capping procedures give an opportunity to revive the tooth and maintain the vitality. A layer of affected dentin which may still contain pioneer microorganisms in the dentinal tubules can be temporarily left behind with the placement of good dental cement to allow time for pulp to heal and help reparative dentin formation.[1,2,3] In such a case, use of dental cements with good antimicrobial properties becomes essential to prevent progression of caries. Clinically, based on a survey, practitioners commonly use calcium hydroxide cement (CaOH2), that is commercially available as Dycal, glass ionomer cement (GIC), mineral trioxide aggregate (MTA) and zinc oxide eugenol (ZnOE) as pulp capping agents.[1,2,4,5]

Currently, the preferred cement for pulp capping is CaOH2 in the form of Dycal cement, since it can induce remineralization due to high alkalinity. The antimicrobial action of the cement is due to release of hydroxide ions which enzymatically inhibit the growth of microorganisms.[5,6,7]

MTA gets converted to CaOH2 after setting and has a high pH of 12.5. Calcium oxide reacts with tissue fluids to form CaOH2 which is antibacterial and biocompatible.[3,4,8]

ZnoE releases eugenol which is antibacterial.[4]

GIC provides excellent bacterial seal and in turn inhibits the growth of microorganisms.[4]

Various researches have been carried out to study the antibacterial effect of the cements using the available commercial strains of microorganisms found in the deep dentinal caries. The organism that have been tested include lactobacillus species and streptococcal species.[1,2,5] The other major cariogenic organism that can be found is bifidobacterium species.[9]

The present study was done using the caries sample obtained directly from deep dentinal caries as it would normally contain polymicrobes.[10] Two different culture methods were tested- Direct contact test (DCT) and Agar Diffusion Method (ADM) to verify the antimicrobial efficacy of 4 commonly used cements that include Dycal, MTA, GIC and ZnOE. The aims and objectives of the study were to identify the anaerobic method that is easy to perform, would allow growth of the mixed anaerobic microorganisms with minimal contamination and allow quantification of antimicrobial efficacy of the cements.

2 | MATERIALS AND METHODS

Institutional Ethical Clearance for the study was obtained on 13/07/2016 and an informed consent for each case was obtained before collecting the carious sample. 25 samples of last carious material removed before the placement of the restoration was collected under aseptic conditions and stored in reduced transport medium (RTF). A spoon excavator of 1 mm diameter was used to collect the sample and was placed in 1 ml of RTF. The samples were stored at 4 degree Celsius. At the time of the experiment, the sample in RTF was vortexed to disperse the microorganisms into the RTF. The techniques followed are as described below:

a] Direct contact test: 10 microliters of each of this sample was placed in 1 ml of thyioglycolate broth with hemin and vitamin K, an anaerobic liquid media. Five such vials were made. The first vial without cement was taken as positive control. The second, third, fourth and fifth contained 1 mm^3 cement block of GIC (GIC Fuji II restorative material), Dycal (Dentsply International Ltd), MTA (Prevest DenPro Ltd) and ZnoE (Prime Dental Products Pvt

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Ltd) respectively in thioglycolate broth [Figure 1]. After 24 hours of anaerobic incubation [Figure 2], 10 microliters from each of the 5 vials were streaked on to 3 selective media designated for lactobacillus (lactobacillus agar), streptococcus mutans (Mitis salivarius agar with bacitracin and potassium tellurite) and bifidobacterium species (Bifidobacterium agar) which are commercially (Himedia, India Pvt Ltd) available. The growth of the organisms was compared in each of the medium through colony counting [Figure 3]. The liquid broth allowed the growth of mixed culture, and by sub-culturing we could quantify the antimicrobial efficacy by comparing the colony forming units (CFUs)

B] Agar diffusion test: Anaerobic blood agar plates were used. Four wells of 1 mm^3 dimensions were created to match with the $1mm^3$ block of cement that was used for direct contact test. GIC, Dycal, MTA and ZnOE cements were freshly mixed and placed into the wells to completely fill and not extend beyond the dimension of the wells prepared. 10 microliters of the sample in RTF was streaked on to these cement wells containing agar plates and incubated under anaerobic conditions for 24 hours. The then produced zone of inhibition including the diameter of the well was measured [Figure 4A]. An anaerobic blood agar plate without the cement wells was used as control and it showed the growth of mixed culture [Figure 4B]

In both the techniques, the samples were incubated anaerobically in the anaerobic jar and commercially available anaerobic Gaspak. Methylene blue chemical indicator was used to check the anaerobic conditions.

3 | OBSERVATIONS AND RESULTS

The techniques were compared for 1] Ease of handling and working. 2] The method that would best allow growth of the culture. 3] Easy detection and quantification of the anti microbial efficacy.

Ease of handling and working: Direct contact test was an extensive procedure as the thiglycolate broth containing the cements had to be first incubated anaerobically for 24 hours and then streaked onto the selective plates, incubated again for 24 hours to compare the colonies formed in each of the selective media with cements and without cement. Initially, an attempt was made to study the turbidity obtained after growth with optical spectrophotometer, but MTA and ZnOE themselves produced turbidity as they have the feature of dissolving in fluids, thus accurate measurements would not be received. Thus the samples after 24-hour anaerobic incubation were streaked on to the selective plates and the colonies formed compared.

Agar diffusion method was easily followed as commercially available anaerobic blood agar plates that would allow mixed culture growth after 24 hour anaerobic incubation showed zones of inhibition around the cement wells.

The method that would best allow growth of mixed culture: Both the methods allowed the growth of anaerobic organisms (100%). In case of direct contact test, selective media were employed for each of the commonly found bacteria in deep dential caries. All the 3 selective media showed the growth of the organism reinstating that mixed colonies could be grown in the liquid thioglycolate broth. The most growth was of bifidobacterium with a mean log 10 growth of 3.10 (table 1) followed by lactobacillus with a mean log growth of 2.31 (table 2) and then streptococcus species with a mean log 10 of 1.99 (table 3). On Krusal Wallis analysis, the results on difference of mean CFUs obtained were statistically significant. The sub-culturing offered a better analysis as the species could be isolated and the most grown organism was identified. Few cases showed contamination.

In blood agar, mixed colonies of various morphologies were easily cultures and detected.

Easy detection and quantification of the anti microbial efficacy: In direct contact test, the colonies formed with cement treatment and without cement treatment were easily compared and the number of colonies formed was quantified. Multiple comparison of mean differences in CFUs of Bifidobacterium Species between groups showed that ZnOE group showed significantly lesser CFUs as compared to Broth [$P<0.001$] and other cements followed by MTA, Dycal and then GIC (table 4). Multiple

comparison of mean differences in CFUs of Lactobacillus Species between groups showed that MTA group showed significantly lesser CFUs as compared to Broth [P<0.001] and other cements followed by ZnoE, Dycal and then GIC (table 5). Multiple comparison of mean differences in CFUs of Streptococcus Species between groups showed that ZnOE group showed significantly lesser CFUs as compared to Broth [P=0.009] and other cements followed by MTA, Dycal and then GIC (table 6). This showed that ZnoE had better antimicrobial properties.

In case of agar diffusion method, measurement of zones of inhibition around the cements helped to quantify the anti-microbial effect of the cements. On comparison, ZnOE showed highest mean zone of inhibition of 4, followed by MTA, GIC and Dycal (table 7).

4 | DISCUSSION

Research is on to save the vitality of the tooth; in this regard pulp capping procedures and materials play a major role in controlling the progression of carious process and avoiding extensive measures like root canal treatment. One of the etiological factors causing dental caries is microbial biofilm. Deep dentinal caries has varied microbial colonies which are predominantly anaerobic.

As per the study done by S. Saini et al [10], polymicrobes both aerobic and anaerobic, are found in deep seated carious lesions. The most common anaerobic species found are Lactobacillus, Veillonella and Actinomyces whereas the most common facultative aerobic organisms found was streptococcus species. S. Saini et al performed an antibiotic sensitivity test using various antibiotics.

The present study also showed growth of polymicrobes from samples obtained from deep dentinal caries and on direct contact test, the commonly found organism was bifidobacterium. Anaerobic incubation was followed as most of the organisms found in the deep dentinal caries would be anaerobes. Here the antimicrobial efficacy of cements was tested.[10]

The present study followed 2 different antimicrobial efficacy tests with an aim to identify an affable technique for growth of mixed culture that would allow detection of antimicrobial effect of the cements and its quantification.

Yalcin et al[5] studied the antimicrobial efficacy of CaOH2, Calcimol LC and BioAggregate on 10 microliter lactobacillus commercial strain suspension using the technique of direct contact test where a 96-well microliter plate coated with the 3 cements was used. The growth pattern in each well was recorded non-stop at 650 nm every 30 min, using a temperature controlled spectrophotometer. The results showed that none of the pulp capping agents had antimicrobial effect as a logarithmic growth increase of the test strain was observed as against the control.

In the present study, initially a optical spectrophotometer was used to quantify the anaerobic growth based on the turbidity obtained in the liquid thioglycolate broth, but some of the cements like MTA and ZnOE produced turbidity themselves which gave false results on spectrophotometer, hence, the samples after incubation in liquid thioglycolate broth were sub-cultured to detect the presence of different microbes using media specific for 3 main microorganisms ie lactobacillus, streptococcus and bifidobacterium species. Few of the samples were contaminated (12%) while following this method.

Cepowicz et al [1]studied the antimicrobial efficacy of different forms of GIC (Fuji triage, Fuji IX, Ketac Molar and Ketac Silver) using agar diffusion method using commercially available streptococcus and lactobacillus species where the samples were swabbed on solid tryptic-soy agar medium (TSA) and wells were made into which the freshly mixed cements were placed. The culture plates were left at room temperature for 30 min and incubated at 37o C for 7 days. The inhibition zones around the wells were measured in millimetres. Their study showed that the different forms of GIC had an inhibitory effect on streptococcal species but none had an effect on lactobacillus species.[1]

Agar diffusion method in the present study allowed the growth of mixed culture and showed zones of inhibition around the cement wells. Since the antimicrobial efficacy was tested on mixed culture and inhibitory zones were obtained, it could be stated that cements would be effective against polymicrobial

organisms.

Both the techniques compared in the present study allowed the growth of mixed culture and also detection and quantification of the antimicrobial efficacy.

Direct contact test was an extensive procedure as the liquid broth had to be sub-cultured to detect and quantify the antimicrobial efficacy and few cases presented with contamination. The advantages of this technique was that the sub-culturing allowed detection of common microbes involved and quantification was better detected by comparing the colony counts.

Agar diffusion method was easy to perform, allowed the growth of mixed culture and antimicrobial efficacy was detected by comparing the zones of inhibition obtained. The main disadvantage of this method was inconsistency in diffusion of the cements across the agar medium.

Both the methods showed that ZnOE has better antimicrobial properties followed by MTA, Dycal and GIC.

In one of the clinical studies, when various cements were used to assess the antimicrobial efficacy, sterility was 61.4% of all the tooth cavities filled with calcium hydroxide as against 81.8% of cavities filled with zinc oxide-eugenol cement.[10]

The present study utilized the carious material directly from deep carious lesions before placing the capping cement which is has been followed in very few studies. On comparison of the two techniques, DCT was an extensive procedure whereas AGM was simple and easy to perform. Detection and quantification of antimicrobial efficacy could be performed in both the techniques.

5 | CONCLUSION

The right choice of the technique depends on the parameters on which the antimicrobial efficacy is tested. In case, the effect of cements on different species has to be detected then DCT is the method of choice and care should be taken that the procedure is performed under complete aseptic conditions to prevent contamination. In case, the aim is to just to identify the cement that has good antimicrobial properties against mixed culture, then AGM is simple and effective method to perform.

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TABLE 1: Growth of Bifidobacterium species

Table 1: Comparison of mean CFUs (in Log10) of Bifidobacterium Species between different

Groups		Mean	SD	Min	Max	P-Value
Broth	25	3.10	0.99	1.0	4.3	
GIC	25	2.77	0.92	0.6	4.2	
Dycal	25	2.50	1.06	0.5	4.3	
MTA	25	1.99	0.99	0.0	4.0	
ZnOE	25	1.80	1.10	0.0	4.0	

*Statistically significant

TABLE 2: Growth of Lactobacillus species

Table 2:Comparison of mean CFUs (in Log10) of Lactobacillus Species between different study

Groups		Mean	SD	Min	Max	P-Value
Broth	22	2.31	1.16	0.0	4.3	
GIC	22	2.06	1.19	0.0	4.1	
Dycal	22	1.86	1.09	0.0	3.5	
MTA	22	0.64	0.77	0.0	3.7	
ZnOE	22	1.03	1.02	0.0	3.8	

TABLE 3: Growth of Streptococcus species

Table 3: Comparison of mean CFUs (in Log10) of Streptococcus Species between different

Groups		Mean	SD	Min	Max	P-Value
Broth	22	1.99	0.95	0.0	4.5	
GIC	22	1.60	1.01	0.0	4.2	
Dycal	22	1.59	0.83	0.0	2.6	
MTA	22	1.33	0.84	0.0	2.5	
ZnOE	22	1.24	0.85	0.0	3.1	

TABLE 4: Comparison of mean CFUs of Bifidobacterium species

Table 4: Multiple comparison of mean diff. in CFUs of Bifidobacterium Species b/w

(I) Groups	(J) Groups	Mean	95% CI for the Diff.		P-Value
			Lower	Upper	
Broth	GIC	0.33	-0.47	1.12	0.08
	Dycal	0.59	-0.20	1.39	0.02*
	MTA	1.10	0.31	1.90	0.001*
	ZnOE	1.29	0.50	2.09	<0.001*
Dycal	Dycal	0.27	-0.53	1.06	0.34
	MTA	0.78	-0.02	1.57	0.003*
	ZnOE	0.97	0.17	1.76	0.001*
MTA	MTA	0.51	-0.29	1.31	0.04*
	ZnOE	0.70	-0.10	1.50	0.02*
ZnOE	ZnOE	0.19	-0.61	0.99	0.42

TABLE 5: Comparison of mean CFUs of Lactobacillus species

Table 5: Multiple comparison of mean diff. in CFUs of Lactobacillus Species b/w groups using Mann

(I) Groups	(J) Groups	Mean	95% CI for the Diff.		P-Value
			Lower	Upper	
Broth	GIC	0.25	-0.64	1.13	0.22
	Dycal	0.45	-0.43	1.34	0.23
	MTA	1.67	0.78	2.55	<0.001*
	ZnOE	1.28	0.39	2.16	0.001*
Dycal	Dycal	0.21	-0.68	1.09	0.87
	MTA	1.42	0.54	2.31	<0.001*
	ZnOE	1.03	0.15	1.92	0.001*
MTA	MTA	1.21	0.33	2.10	0.002*
	ZnOE	0.83	-0.06	1.71	0.01*
ZnOE	ZnOE	-0.39	-1.27	0.50	0.04*

TABLE 6: Comparison of mean CFUs of Streptococcus species

Table 6: Multiple comparison of mean diff. in CFUs of Streptococcus Species b/w

(I) Groups	(J) Groups	Mean	95% CI for the Diff.		P-Value
			Lower	Upper	
Broth	GIC	0.40	-0.36	1.15	0.10
	Dycal	0.40	-0.35	1.15	0.09
	MTA	0.67	-0.09	1.42	0.02*
	ZnOE	0.75	0.00	1.50	0.009*
Dycal	Dycal	0.00	-0.75	0.76	0.62
	MTA	0.27	-0.48	1.02	0.31
	ZnOE	0.36	-0.40	1.11	0.16
MTA	MTA	0.27	-0.49	1.02	0.19
	ZnOE	0.35	-0.40	1.10	0.09
ZnOE	ZnOE	0.09	-0.67	0.84	0.43

TABLE 7: Comparison of zones of inhibition on agar diffusion method of various cements

Table 7: Comparison of mean Zone of Inhibition (in mm) between different study

Cements		Mean	SD	Min	Max	P-Value
Dycal	25	0.96	0.36	0.0	2.0	
GIC	25	1.94	0.95	0.0	3.0	<0.001*
MTA	25	2.91	0.70	0.0	3.6	
ZnOE	25	4.00	0.91	0.0	5.0	

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