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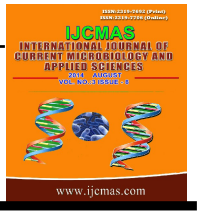

Original Research Article

Study of Microflora in Pulp Tissue of Carious Teeth Using Gram Stain

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Keywords

Microflora, pulp tissue, carious teeth, gram stain, CMPT

A B S T R A C T

Caries is composed of complex and dynamic flora, all of which dental pulp is exposed at one time or the other. Study of individual cultivable bacteria and their cell wall components have been undertaken to have better idea of the pathogenesis of pulpitis. But these culture techniques and molecular biological techniques are time consuming, technique sensitive and expensive as compared to gram staining which is easy, quick, and inexpensive or could be considered as an adjunct to advance techniques. Therefore this study was conducted to study pulp tissue of carious teeth by using gram stain as per the criteria given by various authors. 24 carious extracted teeth were selected and divided into various groups which were then split to expose the pulp tissue which was will be smeared on slide for gram staining. 6 non-carious teeth were selected as control group. Statistical analysis was done It can be concluded that the pulpitis is sequelae of dental caries, it can be selected as a sample to study the caries producing micro flora with the help of gram staining, using various parameters for interpretation which will help the dental professionals to assess the microbial population of carious teeth.

Introduction

The oral cavity is inhabited by an indigenous normal microflora that is composed of more than 500 species, the majority of which still remain uncultivable, of which the number of micro-organisms in an infected root canal may be anywhere between 10^2 -10^8. (Michael et al 2005, John et al 2007, Sjogren et al 1991).

Dental caries and periodontal diseases are the most prevalent infectious diseases of the oral cavity and are responsible for more than 50% cases of tooth mortality (Okell et al 1935, Sunitha et al 2008). Dental caries is a multifactorial disease causing irreversible loss of dental tissue and microorganisms have a critical role in its etiopathogenesis, initiation and progression which has been established by many studies done worldwide (Shafer's, Kakehashi et al 1965, Sundqvist et

al 1976). Although, there is no single species that can be held responsible for growth and progression of caries, (Spratt et al 2003, Schupbach et al 1996, Sansone et al 1993). numerous studies have shown the specificity of microorganisms in the endodontic environment. (Le Goff et al 1997, Fabricius et al 1982).

The morphology of the bacteria may be spherical (cocci), rod shaped (bacilli), filamentous, comma shaped or spiral in shape. Considering the various shapes and sizes the cocci may be arranged singly, in pairs, or in short and long chains. Bacilli may be arranged randomly or in short or long chains also in Chinese letter patterns as palisades or in bundles. (Ananthanarayan et al).

Numerous studies and researches have been carried out to identify the microflora of root canal infections (Brook et al 1991, Dilsha et al 2008, Izabel et al 2011, Saito et al 2006, Tavares et al 2011, Kobayashi et al, Isabela et al 2005) using culturing and microbiologic techniques. But these techniques are difficult to implement on daily basis, as they are expensive and technique sensitive. In our study, we have tried to overcome this shortcoming by employing the gram staining method as a preliminary, primary method of identifying bacteria.

As dental caries is commonly seen in all age groups, without any gender bias and in all socio-economic strata, an easy, quick and effective method should be incorporated to curb the rise in the incidence of these infectious diseases. It should be mandatory to perform gram staining on extirpated pulp to identify the micro-organisms which will help eradicate the infectious process completely and hence increase the success rate of the endodontic treatment. This technique is usually performed on cytological smears (Enver et al 2002, Cigdem et al 2004) but rarely on tissues especially pulpal tissue. Few studies in literature have studied dental pulp tissue , but on decalcified teeth (Pratibha et al 2013, Vytaute et al 2008) and not on freshly extracted teeth. We did not decalcify the teeth as the acid treatment can destroy the bacteria and thus hamper the vitality of micro-organisms.

Therefore this research was carried out to study the pulpal microflora using simple gram staining technique which should be used as an auxiliary technique to provide collaborative evidence in adjunct to diagnosis. The present case–control study was undertaken to identify the microorganism in pulp tissue of carious teeth with and without pulp exposure and to co-relate with clinical type of dental caries using gram stain.

Materials and Methods

Patient selection

30 cases undergoing extraction were selected randomly from the Department of Oral and Maxillofacial Surgery (fig. 1). A detailed case history was taken and the patient was evaluated clinically. The extracted teeth were immediately preserved in formalin for gram staining. Out of the 30 cases, 24 were carious and 6 were selected for the control group. The 24 carious teeth were further divided into four groups i.e.

-Group A consisted of 6 teeth (cases 1-6) having proximal caries without pulp exposure,
-Group B consisted of 6 teeth (cases 7-12) having proximal caries with pulp exposure,
-Group C consisted of 6 teeth (cases 13-18) having deep occlusal caries without pulp exposure,

-Group D consisted of 6 teeth (cases 19-24) having deep occlusal caries with pulp exposure.

Proximal caries were included in the study sample to evaluate periodontal group of microorganisms affecting pulpal tissue, while the deep occlusal caries which is very commonly seen shows the involvement of the microflora present superficially in the oral cavity.

The control group comprised of teeth that underwent orthodontic extraction as they were non carious and hence no pulpal involvement was present. Non carious deciduous teeth were not selected as control as the root resorption may act as a pathway for microorganisms.

Sampling of Pulp Tissue

Tooth was split into two halves to expose the pulp chamber, which was scraped with a sterile instrument and immediately transferred to a clean slide where it was further immediately heat fixed followed by gram staining. Entire procedure was carried out under sterile environment by using physical mode of sterilisation with the help of two burners close to the sampling procedure. Staining procedure: Pulp tissue was immediately scraped between the two slides and heat fixed followed by gram staining. (Ananthanarayan et al)

All the slides were interpreted using CMPT programs recommended stain reporting criteria, under oil immersion. Detailed observation and results were obtained culture, to confirm the type of strains.

Results and Discussion

The result obtained of the assessed parameter in carious and control group are shown in .Figure.2,3,4,5,6,7,8

Group A) Proximal caries without pulp exposure, shows predominantly gram positive cocci in pairs and very few gram positive & negative bacilli, filamentous group and high neutrophilcount indicative of good host response. P values were ≤ 0.005. (0.005)

Group B) Proximal caries with pulp exposure, showed high gram positive cocci, bacilli and low filamentous and gram negative bacilli, and neutrophil count was moderate. P values were ≤ 0.005. (0.002)

Group C) Deep occlusal caries without pulp exposure shows high gram positive cocci in pairs, chains and also neutrophils were moderate in count. P values were ≤ 0.005. (0.002)

Group D) Deep occlusal caries with pulp exposure, shows gram positive cocci in pairs and chains, gram negative bacilli, filamentous, and Candida showing germ tube formation. P values were ≤ 0.005. (0.001)

Statistical analyses were performed and the following conclusion were deduced. Table-1,2,3,4. Gram stain, originally developed by Christian Gram in 1884, is used to classify bacteria on the basis of their cellular structure, gram reactions, forms and arrangement. (Ananthanarayan et al). It also serves as a critical test for presumptive diagnosis of infectious agents and thus helps in assessing the quality of the clinical specimen. It is commonly performed on smears but in this study, it was performed on extirpated pulp tissue , which has not been done in the past as per the literature review done by all authors of this study.

The initial and most important step for successful endodontic treatment is the

identification of the infectious process. Even though there are recent modalities for endodontic treatment, there are failures as the micro flora has not been extensively studied. Culturing techniques and serial dilution methods were considered as standard for research purposes before the development of newer technology like identification through microscopy, immunological assays and molecular methods. Although techniques like PCR are faster and more sensitive in comparison to the standard techniques, they are expensive to perform and require expertise. (Vytaute et al 2008). Whereas gram staining is easy to perform, cost effective and can give immediate preliminary results. Before any endodontic treatment, the pulp chamber should be scraped and the sample should be provided to the oral pathologists. Thus using gram staining technique the pathologists can provide a rapid preliminary identification of the microorganisms, which can aid in the treatment plan or for the prophylactic treatment of the endodontic infection.

In most of the studies, decalcified sections of teeth have been used to study the microbial flora of pulp tissue, but in this study this technique was not used because after decalcification of teeth, severe reductions of both the number and the Gram-positive stain ability were found. (Wijnbergen et al 1987). Therefore in this study, pulp tissue was directly extirpated from the pulp chamber by using smooth instruments after splitting the tooth.

Smooth surface sterile instruments were used for sample collection from the pulp chamber instead of endodontic files that are commonly employed in various studies, whose engaging surface could cause the pulp tissue to get fragmented and thus the quantity of tissue would get minimized. Dental hand piece was not used for pulp extirpation as the heat, water spray and vibrations can disturb the microbial flora.(Bollen et al 1969) Complete sterilization of the working surface and instruments is vital during the extraction of pulp as this will ensure minimum infiltration of microorganisms from the surrounding areas and thus result in more accurate observations.)Ananthanarayan et al)

Even though this technique is applicable to cytological smears, we used intact pulp tissue which was crushed to obtain a smear and heat fixed. After fixation, only microbial population and WBC's were retained on the slide and remaining connective tissue elements were lost. But modifications of gram staining for tissue sections has been done in the literature (Taylor et al 1966).

As per literature different types of caries are associated with specific organisms (Shafer's et al, Rozkiewicz et al 2006) but in this study no such specific association was observed. In the study group of proximal caries with pulp exposure, we found periodontal group of microorganisms which could be due to the infiltration of the microorganisms from the periodontium.

As per documented etiology of dental caries, Candida species does not play an important role in the etiology of dental caries, but in our studies, in the group of deep occlusal caries with pulp exposure Candida species was observed. Candida albicans is usually a normal commensal of the oral cavity, but can become pathogenic in state of immunosupression (Rozkiewicz et al 2006). It is usually a rare entity in untreated root canals but can be seen if the canals are exposed to the oral cavity. They have the ability to survive in root canals as they are resistant to many antibacterials. (Vytaute et al 2008) .

In the study group without clinical pulp

exposure, microorganisms were still observed in varying numbers, which could be due to micro-invasion or invasion through dentinal tubules. Bacteria can also invade the canals from the periodontal pockets, through apical ramifications, accessory canals, isthmuses and other morphological irregularities of the root. (Kobayashi et al)

It has been well studied and documented in various studies that streptococci mutans and lactobacilli are the cariogenic bacteria that play the chief role in etiopathogenesis of dental caries. Numerous lactobacilli were found in our study in the sample of teeth with pulp exposure, although literature says that lactobacilli is usually found in the advancing front of the lesion in dentin and in pulp in limited stage of infection. Almost all the study groups in the current study showed presence of streptococci, thus proving that they are the chief cariogenic agents (Mangala et al 2010, Tomasz et al 2013).

The current study showed presence of gram negative bacilli which could be Prevotella species or Porphyromonas species in the study group of deep occlusal caries with pulp exposure, but culturing or advanced microbiologic techniques are required for confirming the nomenclature.

Control group showed gram positive cocci in one case which could be from the periodontium, anachoresis i.e. bacteria in the blood may be transported to pulp following any operative procedure or trauma, micro-organisms may also migrate from an infected tooth to a healthy pulp via the lateral or principal canals as a consequence of the contiguousness of the tissues. (Lakshmi et al 2010)

There are various documented interpretation criterias for gram stain (Tenover F.C. , J.V.Hirschmann 1990) (Murray P.R., E.J. Barron et al 2003), (Erik Munson, Timothy Block et al) but in this study the interpretation was done using Clinical Microbiology Proficiency Testing (CMPT) as it is a well recognized standardized technique which includes grading of both defense cells (host response cells) and bacteria **(**Tenover,et al 1990, Erik et al 2007, Deidre et al 2000).

Table.1 Group A: - Showing Number of neutrophils and microorganism per field

Group A			Number of microorganism per field				P value
Proximal caries without pulp exposure	Number of Cases	Neutrophils	1	2	3	4	0.005
	1	30	47	45	44	43	
	2	40	46	45	44	49	
	3	50	50	42	43	43	
	4	40	50	46	49	43	
	5	33	44	45	48	50	
	6	40	41	30	42	44	

Table.2 Group B: - Showing number of neutrophils and microorganism per field

Group B			Number of microorganism per field				P value
Proximal caries with pulp exposure	Number of cases	Neutrophils	1	2	3	4	
	1	5	66	55	50	29	0.002
	2	2	77	19	54	55	
	3	10	53	54	51	52	
	4	11	54	55	56	50	
	5	15	59	63	54	64	
	6	12	67	66	72	70	

Table.3 Group C: - Showing number of microorganisms per field

Group C			Number of microorganism per field				P value
Deep occlusal caries without pulp exposure	Number of cases	Neutrophils	1	2	3	4	
	1	22	11	22	52	55	0.002
	2	24	20	33	45	55	
	3	25	16	25	30	55	
	4	30	21	24	66	67	
	5	21	23	44	55	70	
	6	22	30	50	44	34	

Table.4 Group D: - showing number of microorganism per field

Groups D			Number of microorganism per field				P value
	Number of cases	Neutrophils	1	2	3	4	
Deep occlusal caries with pulp exposure							
	1	5	42	43	42	43	0.0018
	2	4	40	45	43	42	
	3	9	39	46	35	40	
	4	7	44	43	41	42	
	5	5	14	9	57	40	
	6	8	10	59	50	51	

Figure.1 A shows proximal caries without pulp exposure. B shows deep occlusal caries without pulp exposure. C shows proximal caries with pulp exposure. D shows deep occlusal caries with pulp exposure

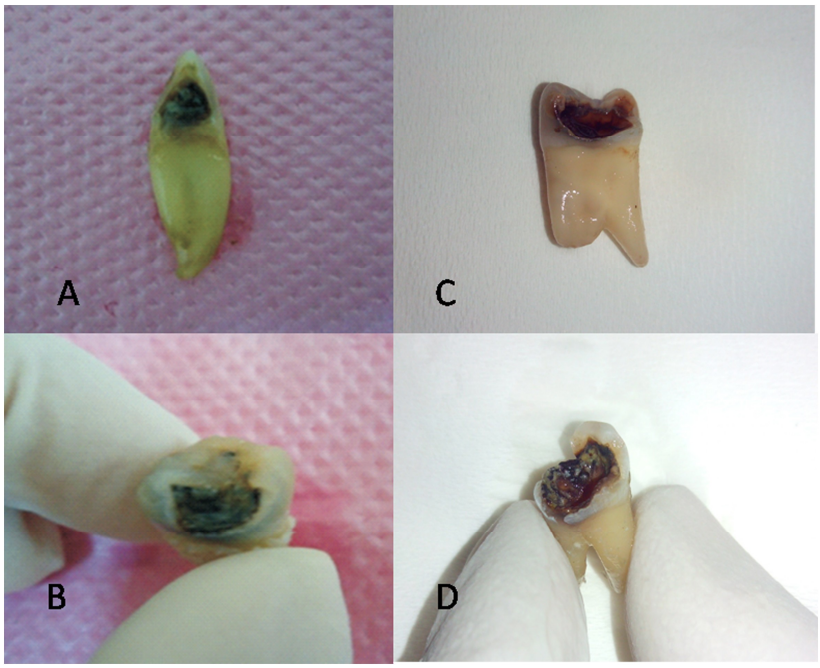


Fig.2 A-shows gram positive cocci and bacilli, B-shows gram negative cocci in pairs and chains, gram negative bacilli, filamentous organism and Candida showing germ tube. C-shows gram positive cocci in pairs and chains and neutrophils. D-shows gram positive cocci in pairs and neutrophils.

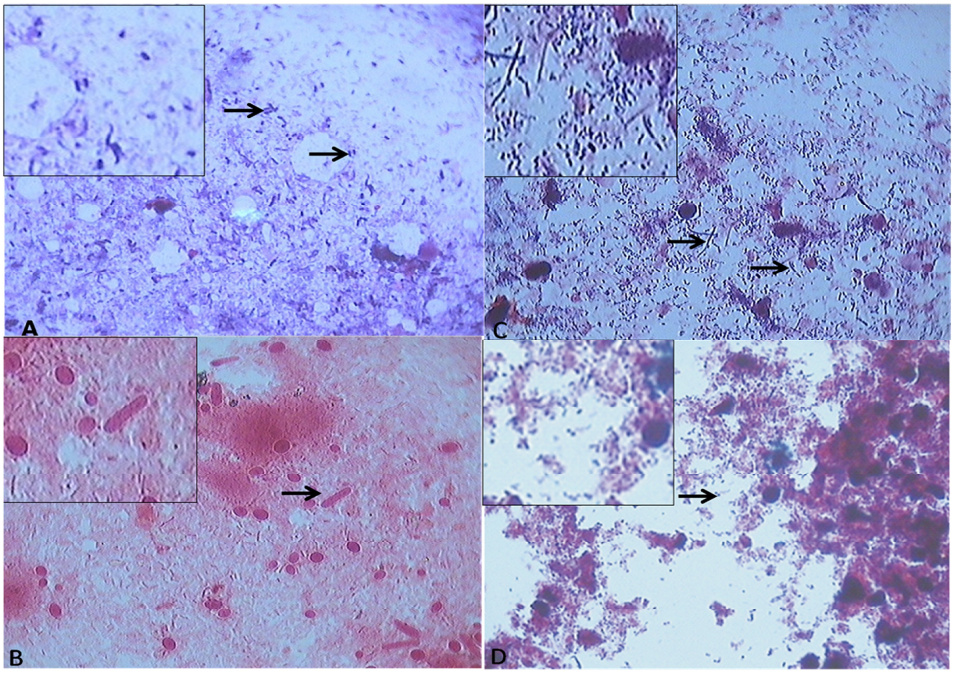


Figure.3 Shows the number of various bacteria that were seen among the different groups which were selected for the study. It shows that the number of bacteria are more in the group with pulp exposure as compared to the group without pulp exposure.

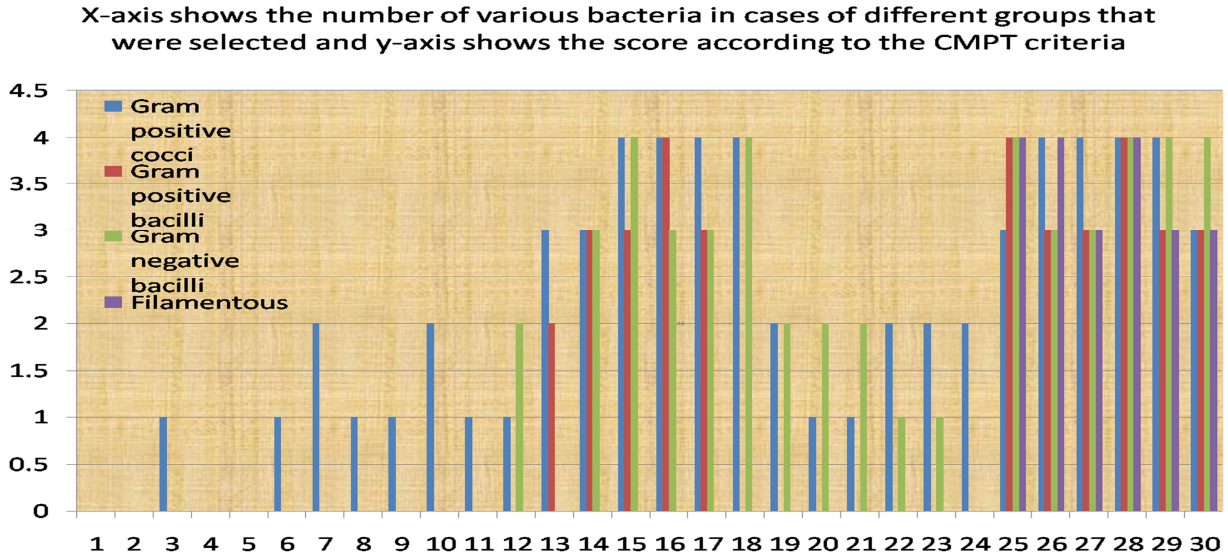


Figure.4 Show that gram positive cocci can be seen in all the four study groups, it is also seen in one of the case of control group.

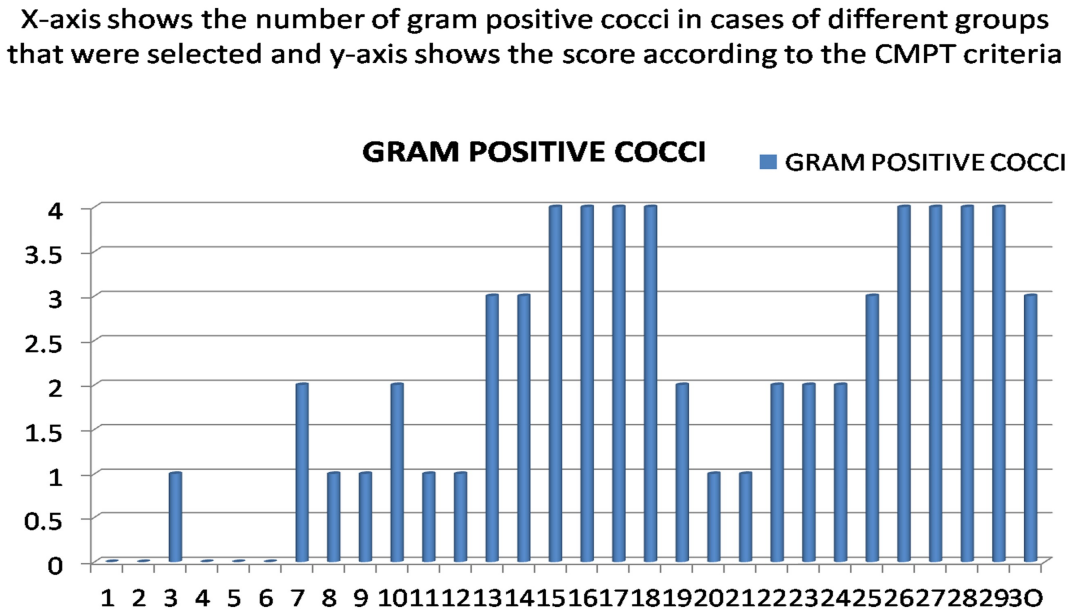


Figure.5 shows- the number of gram positive bacilli are more in group with pulp exposure and proximal caries without pulp exposure, while group with occlusal caries without pulp exposure and control group shows no gram positive bacilli.

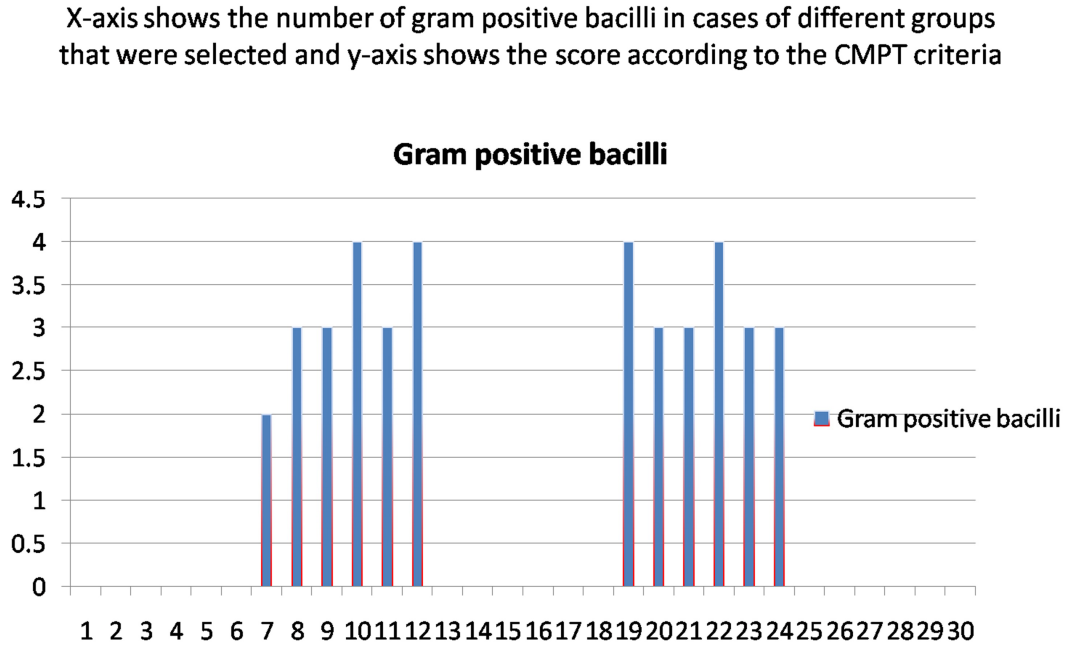


Figure.6 shows- the number of gram negative bacilli is more in the group with pulp exposure, while control group shows no gram negative bacilli

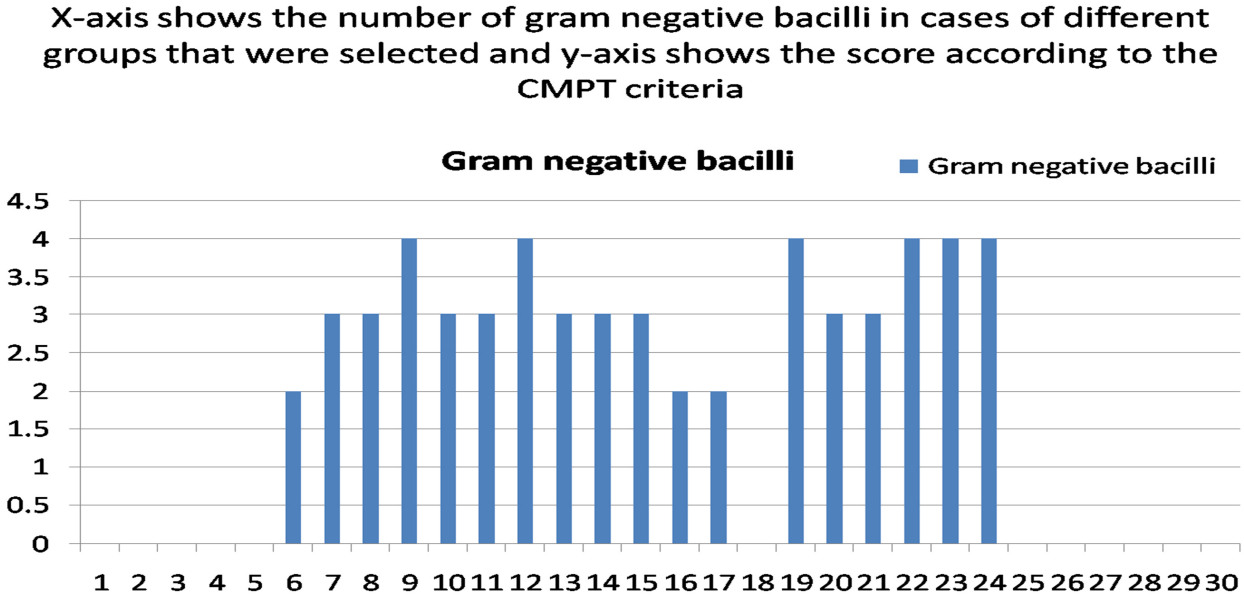


Figure.7 shows- filamentous bacteria were present only in the groups with proximal caries with and without pulp exposure, and also with the group of deep occlusal caries with pulp exposure.

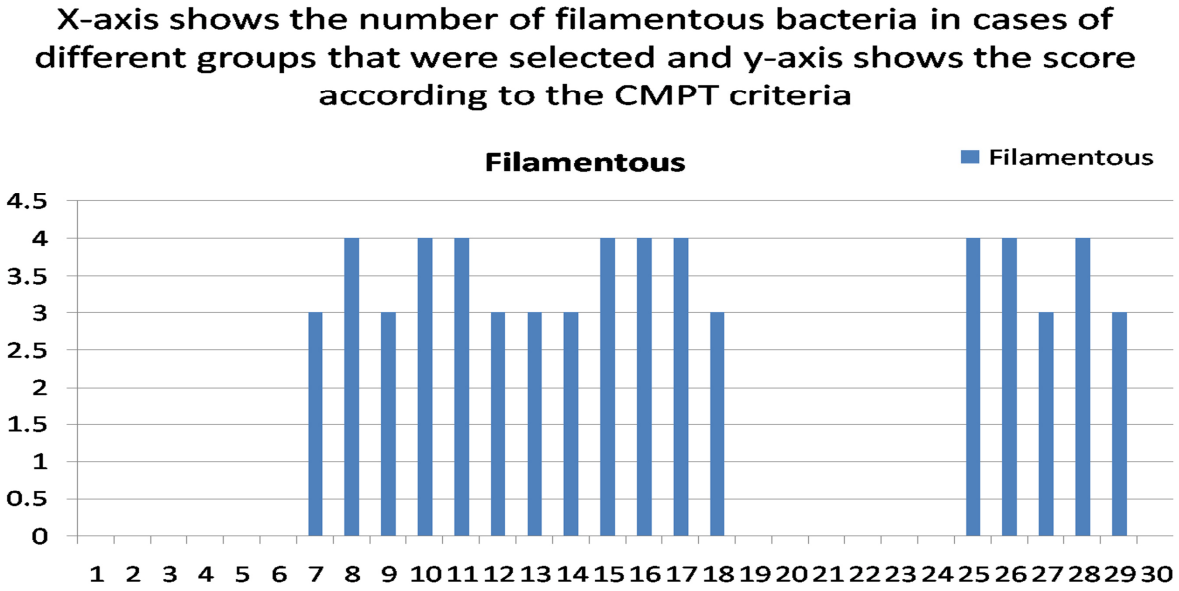
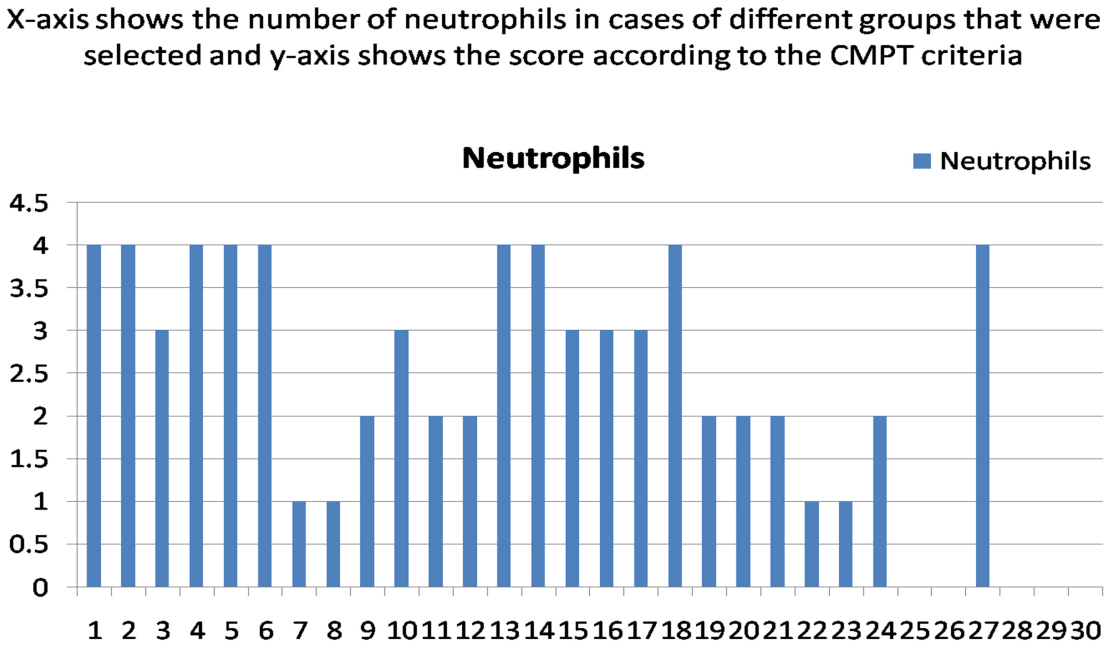


Figure.8 shows- large number of neutrophils were seen in the group without pulp exposure.



In conclusion, data presented showed that the number of microorganisms were less in the pulp of non carious teeth. Few samples in control group showed numerous bacteria in the form of cocci and bacilli possibly due to periodontal infection. In the study group, with and without pulp exposure more number of neutrophils were seen which could be due to host response against the carious process. This study also suggested different levels of concentration of microorganism in both carious and non carious teeth. Streptococci were seen in almost all groups that were studied, but their concentration was altered. Few other bacteria like Lactobacilli, Prevotella, Actinomyces and fungi like Candida were also found.

Endodontic infections and its sequelae place a substantial burden on individuals, communities and the general healthcare system; hence there should be prompt diagnosis and appropriate intervention. With the help of trained pathologists or microbiologists, the gram reaction, arrangement and structure of the pulpal microflora can be studied and a preliminary identification can be given in the initial stages of endodontic treatment to help determine the prophylactic and supportive treatment. Gram staining of the pulp tissue of carious teeth is a preliminary diagnostic tool in addition to advance molecular biologic techniques which can be performed with a simple set up in all laboratories. There is a lack of standardization for the interpretation of gram stain. There is a need for good quality clinical trials of sufficient size to develop software that helps for the analysis and interpretation of gram stain in pulp tissue.

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