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Dental Science

GRAM'S STAINING OF TISSUE BACTERIA: A CASE REPORT AND A COMPARISON OF THREE MODIFICATIONS OF THE TECHNIQUE

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ABSTRACT There are many microorganisms in our environment, but everybody is not susceptible to such organism. Only people who are immunocompromised are usually vulnerable to the infection caused by these microorganism. In most of the cases these organisms are missed in routine H and E section due to lack of experience of the pathologist, hence here comes the importance of special stains. In our study we compared 3 modifications of Gram's staining of tissue-Brown- Hopp's Gram Stain Method, Churukian's modification and Gram Twort stain and concluded that Brown–Hopp's method showed superior staining results. Even though newer technique are now flourished older technique are also used due to lack of infrastructure and cost factor. We did a comparison between 3 stains and our aim was to find out a superior stain among them.

KEYWORDS : Brown- Hopps Gram Stain Method ,churukian's Modification, Gram Twort Stain, Gram Staining.

Case Report

A 48-yr old male patient reported to the outpatient department of Govt. Dental College, Thiruvananthapuram with the complaint of a swelling on palate since 3 weeks. Extra oral examination showed that the patient had a generalized swelling over the right side of face and ptosis of left eye.

Intra-orally a swelling of size 2x 2cm was observed in the anterior mid-palatal region with a yellowish granular slough. Mucosa overlying the lesion was erythematous. The lesion was tender on palpation, firm in consistency with central soft area. Right maxillary central incisor was missing. The remaining teeth were in fairly good condition. Patient was a known diabetic under treatment.

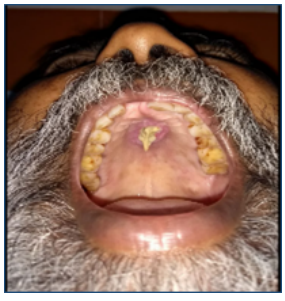


Figure1 Intra-oral view showing swelling on the anterior mid-palatal region with yellowish slough.

Findings in OPG was inconclusive. CT scan reported paranasal sinuses with bony erosion of antero-medial and superior wall of left maxillary sinus with associated pre-maxillay and left pre-orbital soft tissue. Bony erosion of anterior aspect of lamina papyracea on left side with subtle erosion of hard palate on the left side was seen. A differential diagnosis of an infective pathology or carcinoma was made. MRI brain was taken to rule out cavernous sinus involvement. MRI of orbits and PNS also revealed erosion of palate and erosion of medial wall of left maxillary sinus with mild enhancing soft tissue along the floor of orbit.

A punch biopsy was performed after routine blood examination.3 soft tissue masses were obtained which were pearly white in colour with underlying brown areas and were soft to firm in consistency. Bits measured 0.7x0.5x0.3cm,1x0.5x0.1cm and 0.5x0.3x0.1cmeach.

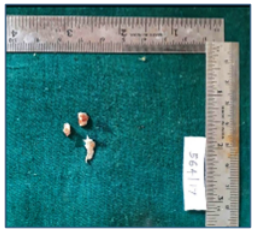
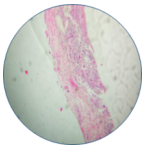


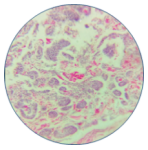
Figure2 : Three formalin fixed soft tissue bits received.

All the bits were processed, 4 µm sections taken and stained with routine haematoxylin & eosin stain. The yellowish granular slough was sent for culture.

Histopathological examination of the tissue revealed a proliferative parakeratotic epithelium overlying a loosely collagenous stroma. Within the stroma there were numerous aggregates of basophilic granules. No carcinomatous features were found. The granules were suspected to be bacterial colonies and a modified grams staining on tissue sections were performed. The staining revealed presence of numerous coccal and bacillary forms. The major population was that of Gram positive cocci. In between the bacterial colonies numerous collections of entangled hyphae were also present.



LOW POWERVIEW



HIGH POWER VIEW

Figure 3 : H& E stained tissue showing aggregates of bacterial colonies in the stroma

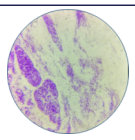


Figure 4 : High power (oil immersion lens) view of Grams stained slide showing aggregates of predominantly gram positive cocci along with gram negative bacteria and numerous entangled fungal hyphae in between.

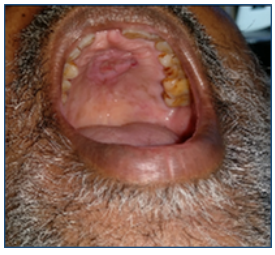
A diagnosis of fungal infection compounded with bacterial infection was made. Culture results revealed a predominance of MRSA strain(Methicillin resistant staphylococcal aureus).

Patient was administered antibiotics and antifungals as treatment, to which he responded positively

Gram's staining of tissue

Grams staining of smear is different from that of tissue. A smear is heat fixed before grams whereas histological tissue is not. One of the pioneer methods of histological Grams staining was proposed by Brown and Brenn in 1931.Although this established the stepping stone, it was not free of flaws. The major drawback of this method was that the staining of Gram negative organisms were faint and establishing enough contrast from the background was difficult. Hence this method was later modified in different ways.

Figure 5 : Intra oral view showing healing of lesion after a course of antibacterials and antifungals



BROWN & BRENN METHOD		
PROCEDURE	**PREPARATION OF REAGENTS**	**RESULT**
1.Deparaffinise and hydrate to distilled water 2.Crystal violet-sodium bicarbonate solution x 1 min. 3.Rinse in distilled water. 4.Flood with Gram's Iodine x min. 5.Rinse with water and carefully blot with filter paper to complete dryness. 6.Decolorize with Acetone-Alcohol,by dropping onto the slide until no more color runs off. 7.Stain in the Basic Fuchsin solution x 1 minute 8.Wash in water 9.Blot carefully but not to complete dryness 10.Differentiate in Acetone, one quick dip 11.Transfer immediately to the Picric Acid – Acetone Solution, 0.1% until sections show yellowish pink. 12.Rinse quickly in Acetone; 13.Then rinse in Acetone-Xylene 14.Clear in 3-4 changes Xylene, 15.Mount with Permount	Ÿ Crystal violet-sodium bicarbonate solution Ÿ Mix 1.0ml (20 drops) Crystal Violet, 1% Aq. with 5 drops Sodium Bicarbonate, 5% Aq Ÿ Acetone –alcohol Ÿ Equal proportion of acetone and alcohol Ÿ Picric a cid-acetone Ÿ Picric acid 0.1 g Ÿ Acetone 100 ml	Gram+ Bacteria, -blue Gram- Bacteria, Nuclei- red Additional tissue elements -yellow

COMPARISON OF 3 MODIFICATIONS OF GRAM'S STAINING

For this study we performed 3 modifications of the Brown and Brenn method in the biopsy of the same patient

method in the biopsy of the same patient
1.Brown and Hopp's modification
2.Churukian's modification
3.Gram Twort stain

Table 2 :Cherukian's modification method

CHURUKIAN'S MODIFICATION		
PROCEDURE	**PREPARATION OF REAGENTS**	**RESULT**
1.Deparaffinize and rehydrate through graded alcohols to distilled water. 2.Stain with crystal violet solution, 1 minute. 3. Rinse well in distilled water. 4. Iodine solution, 1 minute. 5.Rinse in distilled water, blot slide but NOT the tissue section. 6.Decolorize by dipping in alcohol-acetone solution until the blue color stops running. (One to two dips only!) Counterstain in working basic fuchsin for 1 minute 7.Rinse in distilled water and blot slide but not section. 8.Dip in acetone, one dip. 9.Dip in picric acid-acetone until the sections have a yellowish-pink color. 10.Dip several times in acetone-xylene solution. Keep checking for proper differentiation. 11.Clear in xylene and mount.	Ÿ Ethyl alcohol-acetone solution Ÿ Ethyl alcohol, absolute 50 ml Ÿ Acetone 50 ml Ÿ Picric acid-acetone Ÿ Picric acid 0.1 g Ÿ Acetone 100 ml Ÿ Acetone-xylene Ÿ Acetone—50 ml. Ÿ Xylene—50 ml	Ÿ Gram-positive organisms, fibrin, some fungi,Paneth cell granules, keratohyalin, and keratin-blue Ÿ Gram-negative organisms- red Ÿ Nuclei - red Ÿ Other tissue elements -yellow

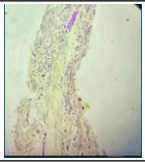
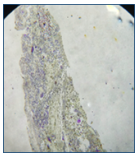
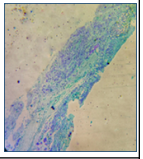
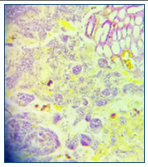
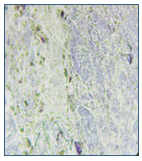
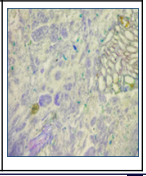
Table 3: Grams –twort method

GRAMS-TWORT STAINING		
PROCEDURE	**PREPARATION OF REAGENTS**	**RESULT**
1.Deparaffinize and rehydrate through graded alcohols to distilled water. 2.Stain in crystal violet solution x 3 minutes. 3.Rinse in gently running tap water. 4.Treat with Gram's iodine x 3 minutes. 5.Rinse in tap water, blot dry, and completely dry in a warm place. 6.Differentiate in preheated acetic alcohol (preheated to 56°C) until no more color washes out.The section should be light brown or straw colored. 7.Rinse briefly in distilled water. 8.Stain in Twort's x 5 minutes. 9.Wash in distilled water.	Ÿ Acetic alcohol Ÿ 2% acetic acid in absolute alcohol(ethanol) Ÿ · Twort's stain Ÿ 1% neutral red in ethanol 9 ml Ÿ 0.2% fast green in ethanol 1 ml Ÿ Distilled water 30 ml Ÿ Mix immediately before use or not more than 30 minutes before staining	Ÿ Gram-positive organisms blue-black Ÿ Gram-negative organisms -pink-red Ÿ Nuclei -red Ÿ Red blood cells and most cytoplasmic structures-green Ÿ Elastic fibres-black

Table 4:Brown and Hopp's modification

BROWN AND HOPP'S MODIFICATION		
PROCEDURE	**PREPARATION OF REAGENTS**	**RESULT**
1.Deparaffinize and hydrate to distilled water. 2. 1% crystal violet x 2 minutes. 3.Wash in tap water to remove excess crystal violet. 4. Gram's iodine for 5 minutes. 5. Wash in tap water to remove excess iodine. 6. Blot, but not to dryness. 7. Differentiate in acetone until blue color ceases to run from the slide-two dips per second for a few seconds 8. Quickly rinse in tap water and wash thoroughly to remove acetone. 9. Working basic fuchsin solution x 5 minutes. 10. Wash briefly in tap water. 11. Gallego's solution x 5 minutes (blowing on solution occasionally to agitate) 12. Wash thoroughly in tap water and blot, but not to dryness. 13. Acetone, three quick dips. 14. Picric acid-acetone, three quick dips. 15. 16. Acetone, three quick dips. 17. Acetone-xylene, five quick dips. 18. Xylene, ten quick dips. 19. Xylene, two times. 20. Mount in Permount.	Ÿ Gallego's solution: Ÿ Distilled water—50 ml. Ÿ Formalin (37 to 40% solution)—1 ml. Ÿ Glacial acetic acid—0.5 ml. Ÿ Picric acid-acetone solution Ÿ Picric acid—0.5 Gm. Ÿ Acetone—1,000 ml Ÿ Acetone-xylene Ÿ Acetone—50 ml. Ÿ Xylene—50 ml	Ÿ Gram-positive bacteria—stain blue Ÿ Gram-negative bacteria—stain red Ÿ Background tissue-yellow Ÿ ·Nuclei and epithelium—stain light red

Table 5 : Comparison of the three stain

	BROWN AND HOPP'S MODIFICATION	CHURUKIAN'S MODIFICATION	GRAM TWORT STAIN
LOW POWER VIEW			
HIGH POWER VIEW			

RESULT

All 3 modifications of Grams method of tissue staining could demonstrate gram positive organisms well. Among the methods that were compared, the contrast between gram positive and gram negative bacteria was highest in the Brown and Hopp's method. Fungal hyphae were also demonstrated well in this technique. We concluded that Brown and Hopp's method was superior to Churukian's modification and Gram Twort method not only because of better contrast and detailing, but also because clearing in xylene after staining led to some degree of decolourisation in the latter two methods while the colour was preserved in the former

DISCUSSION

Most of the times infectious organisms and their cytopathic effects may be clearly identified by routine H & E examination, additional histochemical stains are often needed for their complete characterization. Nowadays highly specific molecular techniques, such as immunohis tochemistry, in situ hybridization and nucleic acid amplification, may be needed in certain instances to establish the diagnosis of infection[4].

Conventional method is the gold standard for isolation of the bacteria where culture followed by its identification is the best way to identify any pathogen to establish infectious etiology in any disease. Improper specimen collection, transportation and processing may lead to poor isolation rate of microorganisms for bacterial culture from tissue biopsies. The histopathology of infectious diseases, i.e., direct microscopic visualization of tissue samples for identification of the infectious agent, is particularly useful when cultures cannot be made or the infectious agent is slow growing or fastidious [5]. The cytological identification of microorganisms, no matter how specific, is not intended to replace microbiologic techniques [6].

Pathologists are well versed with histopathology for infectious diseases. Microbiologists often lack knowledge regarding the direct microscopic visualization of infectious agents in tissue biopsies[1] .Bacteria are the most difficult microorganisms to detect in routine H and E-stained histologic sections. Several modifications of Gram stains can be used for the detection of bacteria in tissue sections such as Brown-Hopp's Gram Stain Method, Churukian's modification and Gram Twort stain [7]. Tissue diagnosis of a bacterial infection begins with the recognition of a consistent pattern of inflammation in H&E-stained sections, although it is important to remember that the inflammatory response varies depending on the immune status of the host [8] and in our case the inflammatory component was less.

Gram Staining is the common, important, and most used differential staining techniques in microbiology, which was introduced by Danish Bacteriologist Hans Christian Gram in 1884[9]. The staining procedure as originally presented by Gram used Ehrlich's aniline gentian violet, an aqueous solution of iodine-potassium iodide, absolute alcohol as a decolourizer, and sometimes Bismarck brown as a counter stain. The method is now fundamentally the same; however, a long series of important modifications has resulted in procedures which produce more reliable results, and which are much more convenient than the original [10].

This test differentiates the bacteria into Gram positive and Gram negative bacteria, which helps in the classification and differentiations of microorganisms. In addition this stain also allows determination of cell morphology, size, and arrangement of the organism. It is typically the first differential test run on a specimen brought into the laboratory for identification. In some cases, a rapid, presumptive identification of the organism or elimination of a particular organism is possible. It can be used especially in emergency situation [11].

- The differences in cell wall composition of Gram positive and Gram negative bacteria accounts for the Gram staining differences. Gram positive cell wall contain thick layer of peptidoglycan with numerous teichoic acid cross linking which resists the decolourization.
- Crystal violet is the primary stain used.
- When added, iodine interacts with crystal violet to form large crystal violet iodine complexes within the cytoplasm and outer layers of the cell.
- The decolorizing agent interacts with the lipids of the membranes of both gram-positive and gram negative bacteria.
- The outer cell membrane made of lipopolysaccharide layer in gram negative bacteria is lost from the cell wall leaving the

peptidoglycan layer exposed. Gram-negative cells have thin layers of peptidoglycan, one to three layers deep with a slightly different structure than the peptidoglycan of gram-positive cells. With ethanol treatment, gram-negative cell walls become leaky and allow the large crystal violet iodide complexes to be washed from the cell [11,12].

Table 6: Gram staining procedure

PROCEDURE OF GRAM STAINING OF SMEAR		
Steps in staining	**Gram positive organism**	**Gram negative organism**
Ÿ Heat fix and air dry Ÿ Stain with crystal violet / methyl violet / gentian violet for 1 min	Stains purple/violet	Stains purple/violet
Wash in running water	Purple /violet	Purple/violet
Flood the slide with Grams iodine for 1min (Mordant)	Purple/violet	Purple/violet
Wash in running water	Purple/violet	Purple/violet
Flood slide with acetone (10-15 sec)	Retains the colour	Decolorizes
Flood Carbolfuchsin/ safranine/ neutral red for 30sec- 1min (Counter stain)	Purple/violet	Red
Wash with tap water blot dry	Purple/violet	Red

In H & E staining, bacteria in tissue appear as blue-gray granular masses which are often invisible or obscured by cellular debris. Hence the method of Grams staining for bacteriae in tissue is different from that of smear so as to differentiate them from the background of stromal tissue.

Few of these methods are
1. Brown-Brenn Gram Stain Method
2. Taylor's method
3. Brown-Hopps Gram Stain Method: 4.Modification of original method
5. Churukian's modification
6. Gram Twort staining
7. MacCallum-Goodpasture Gram Stain Method:
8. Humberstone Gram Stain Method
9. Modified Humberstone Gram Stain Methods

CONCLUSION

Although newer methods of microbial detection like immunohi stochemistry and Polymerase chain reaction are far superior to special staining, the traditional methods are still widely used mainly due to cost factor and lack of infrastructure and summary results. They can be used in empirical diagnosis before opting for more expensive investigation. Gram staining is one of the oldest staining method which has still not lost its glory. In our study we compared 3 modifications of Grams staining of tissue-Brown-Hopps Gram Stain Method, Churukian's modification and Gram Twort stain and concluded that Brown–Hopp's method showed superior staining results. Further improvisation of techniques including stringent methods of standardization should be adopted to substantiate the present finding.

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