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COLLECTION AND BIOCHEMICAL EVALUATION OF HUMAN GINGIVAL CREVICULAR FLUID

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1. INTRODUCTION

The mouth contains wide variety of oral bacteria. The tooth surfaces are unique in that they are the only body part not subject to metabolic turnover. Once formed, the teeth are, under the correct conditions, essentially indestructible, as witnessed by their importance in fossils records and forensic medicines. Yet in living individuals, the integrity of the teeth is assaulted constantly by a microbial challenge so great that dental infections rank as the most universal affliction of humankind. The discomfort causes by these infections and their enormous cost (dental infections rank third in medical costs, behind heart and cancer, in the United States) gives dental diseases prominence despite their non-life-threating nature. Dental decay in the late 20th century is a controllable infection and should be preventable in many individuals. Almost 50% of young children are caries-free, and the level of Edentia among individuals over 65, has dropped from 50% to about 20%.[1] The quantification of its constituents is a current method to identify specific biomarkers with reasonable sensitivity for several biological events.[2]

1.1 Gingival Crevicular Fluid

Gingival crevicular fluid (GCF) is an inflammatory exudate derived from the periodontal tissues. It is composed of serum and locally generated materials such as tissue breakdown products, inflammatory mediators, and antibodies directed against dental plaque bacteria. Its constituents are derived from a number of sources, including serum, the connective tissue, and epithelium through which GCF passes on its way to the crevice. GCF plays a special part in maintaining the structure of junctional epithelium and the antimicrobial defence of periodontium.[3]

Definition

"GCF is an exudate that can be harvested from the gingival crevice or periodontal pocket using either filter papers strips or micropipettes." by Manson and Eley.[4]

"GCF is a serum like exudate that bathes the gingival sulcus and follows an osmotic gradient within the local tissues." By Per Axellson.[5]

1.2 Mechanism of production of Gingival crevicular fluid

GCF is produced at the rate of 0.5-2.4 ml/day. At sites in the absence of inflammation and subgingival plaque, the production of GCF is mediated by passive diffusion of the extracellular fluid or by an osmotic gradient. In this situation, the GCF is considered as a transudate. During inflammation, GCF production is mediated by osmotic gradient. When an inflammatory response is provoked by compounds of microbial origin, the permeability of the epithelial barrier and the underlying vasculature increases and the GCF protein concentration is now modulated by extent of plasma protein exudation. Subsequently, the GCF is considered an inflammatory exudate.[6]

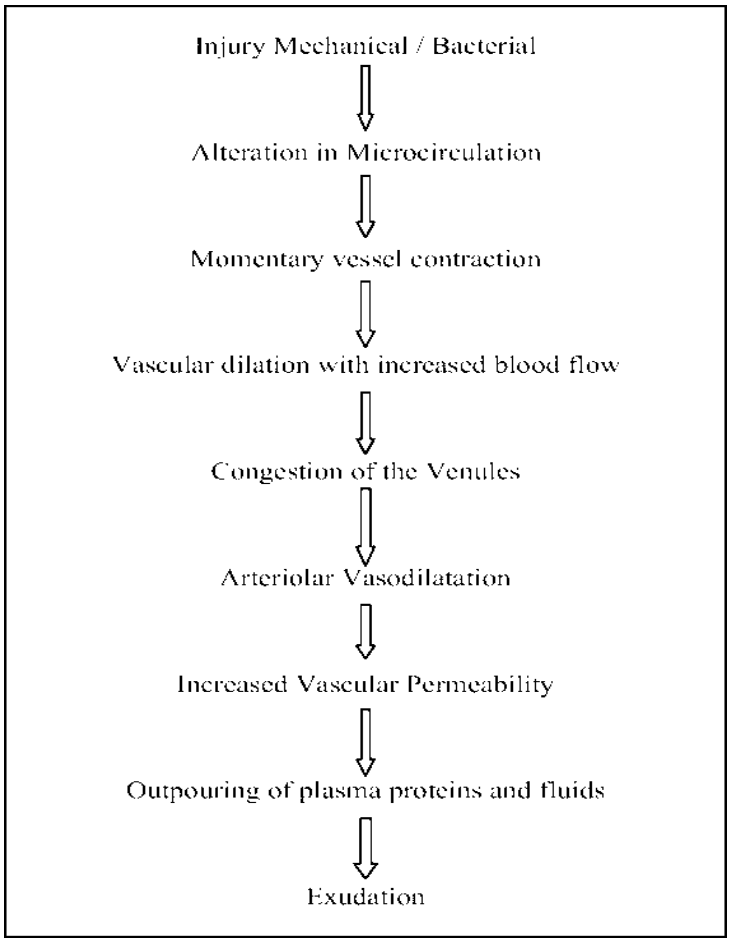

Fig. 1: Mechanism of Gingival Crevicular fluid production.

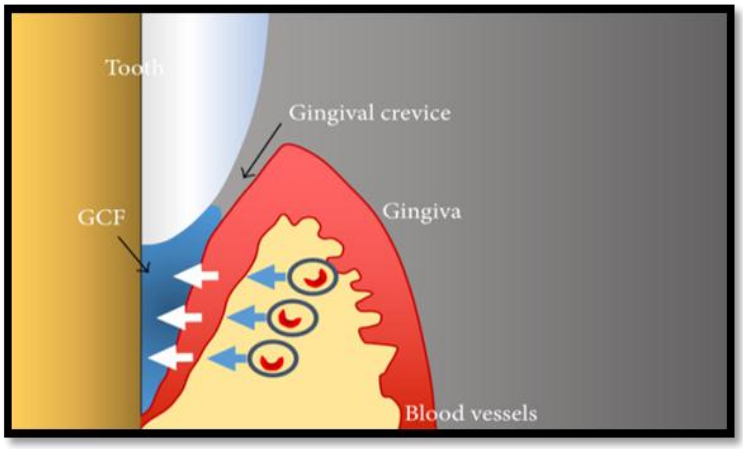

Fig. 2: Gingival Crevicular Fluid formation.

1.3 Composition of Gingival crevicular fluid[7]

The GCF composition is an admixture of molecules originating from blood and host tissues and subgingival plaques including:

- Electrolytes
- Small organic molecules
- Proteins
- Cytokines
- Specific antibodies
- Bacterial antigens
- Enzymes of both host and bacterial origin.

1.4 Functions of Gingival crevicular fluid[8]

- It washes the sulcus, carries out shed epithelial cells, leukocytes and microbes.
- It contains many antimicrobial agents.
- It carries neutrophils and macrophages for phagocytosing bacteria.
- It transports immunoglobins and other immune factors to destroy micro-organisms.
- The monitoring of GCF and quality of its content is used diagnostically to assess the severity of gingival inflammation, effectiveness of oral hygiene, response of tissues to periodontal therapy and effectiveness of chemotherapeuticagents.

1.5 Gingival crevicular fluid as a diagnostic marker[3]

Biomarkers are used as alternative mean of determining the active disease site, prognosis of future site of breakdown and response to therapy. Diagnostic Biomarker facilitates early

detection of a disorder. Gingival crevicular fluid contains elevated levels of a vast array of biochemical factors which offer proper diagnosis of disease activity such as:

- Prostaglandin E2
- β~-glucuronidase
- Neutrophil Elastase
- Aspartate Aminotransferase
- Collagen in gingival crevicular fluid
- Alkaline phosphatase
- Proteoglycans in GCF
- Glycoproteins in GCF

1.6 Factors stimulating the Gingival crevicular fluid flow[6]

- Gingival inflammation, Mastication of coarse food, Pocket depth, Intra-crevicular scraping, Scaling, and Histamine topical application.
- Enzymes and Sex hormones: Female sex hormones increase the gingival fluid flow because they enhance vascular permeability.
- Circadian periodicity: There is gradual increase in gingival fluid amount from 6 am to 10 pm and a decrease afterward.
- Post-periodontal surgery, Restorative procedure, Strip placement, Mobility, Increased body temperature, and Salivary contamination.
- Ovulation, Hormonal contraceptives, and Smoking.

2. LITERATURE REVIEW

a. **Sultan K., Anik A., (2020):** In this article the author says that, in diabetic children with good metabolic control, type-1 Diabetes mellitus is not associated with GCF level of IL-18 and TNF-α in the presence of gingivitis. However increased level of TNF-α in children with gingivitis is related with inflammation.[9]

b. **Batschtrichkus S., et al., (2018):** In this article the author says that, The Trichloroacetic acid /acetone precipitation now successfully employed for protein precipitation. This precipitation step efficiently reduced the serum albumin content & increased subsequently protein identification by 32%. 317 proteins were identified using this technique.[10]

c. **Chen S., et al., (2016):**In this article the author says that, Hormones (progesterone and estradiol) changes greatly during pregnancy. The prevalence of gingivitis during pregnancy ranged widely from 35-100%. Increased female sex hormones may modulate

the functions of immune cells. Immunological changes during pregnancy are responsible for periodontal conditions. Meanwhile, proinflammatory cytokines play a major role in the progression of gingival inflammation. Interleukin-1β and tumor necrosis factor αregulate the initial inflammation.[11]

d. **Gupta G., (2013):** In this article the author says that, Periodontal disease is a chronic bacterial infection characterized by persistent inflammation, connective tissue breakdown and alveolar bone destruction. In response to bacterial infection, host production of inflammatory mediators, may be the trigger for periodontal disease progression. Major events comprise pro-inflammatory cytokine production and collagenolytic MMPs leading to soft periodontal tissue breakdown. These cytokines along with bone MMPs, will lead ultimately to osteoclastogenesis and alveolar bone resorption.[12]

e. **Verma S., Bhardwaj A., (2012):** In this article the author says that, Steroid sex hormones have a significant effect on different organ systems. In women, during puberty, ovulation, pregnancy, and menopause, there is an increase in the production of sex steroid hormones which results in increased gingival inflammation, characterized by gingival enlargement, increased gingival bleeding, and crevicular fluid flow and microbial changes. Some micro-organisms found in the human mouth synthesize enzymes needed for steroid synthesis and catabolism.[13]

f. **Asikainen S., Turgut Z. et al., (2010):** In this article the author says that, Bacterial colonization and growth on supra- and subgingival tooth surfaces causes chronic inflammation in periodontal tissues. Increased flow of gingival crevicular fluid (GCF) a serum transudate or inflammatory exudate, washes periodontal pockets and thereby provides host-derived substances that shape subgingival bacterial populations. Bacterial species found in GCF reflect the bacterial populations detached from subgingival biofilms. GCF bacteria may provide new prospects for studying dynamic properties of subgingival biofilms.[14]

g. **Van Winkelhoff AJ., Winkel E G., (2007):** In this article the author says that, Smoking has been identified as one major risk factor for destructive periodontal diseases scaling and root planning have been shown to be less effective in smokers with periodontitis. Smoking is a determining factor for the composition of the subgingival microflora in adult patient with periodontitis. Microbiological characteristics of untreated smokers were a higher prevalence of Peptostreptococcus microbes and Fusobacterium nucleatum.[15]

h. **Andrew J. D., Van Dyke T. E. et al., (2003):** In this article the author says that, GCF is an admixture of molecules originating from blood, host tissues and subgingival plaques,

including: electrolytes, small organic molecules, proteins, cytokines, specific anti-bodies, bacterial antigens, and enzymes ofboth host and bacterial origin. GCF also contain diverse populations of cells, which include: bacteria from the adjacent plaques mass; desquamated epithelial cells which are passively washed out of the sulcus into the oral cavity by the outward flow of GCF; and transmigrating leucocytes, [including polymorphonuclear cell (PMNs), monocytes/macrophages, and lymphocytes.[16]

i. **Giuseppe P., Paolantonio M., (2002):** In this article the author saysthat, Alkaline phosphatase activity in Gingival crevicular fluid reflects the biologic activity and hence, used as a diagnostic tool for monitoring orthodontic tooth movement in clinical practices.[17]

j. **Emberry G., Waddington R., et al., (1994):** In this article the author says that, Periodontal diseases are associated with presence of bacteria which are recognized as the principle etiological agents, the factors derived from these bacteria may be useful indicators of their presence and metabolic activity**.** Lipopolysaccharide (Endotoxin) found in outer membrane of the cell wall of the Gram- negative bacteria. The level of endotoxin is related to the number of gram-negative bacteria.[18]

3. AIM AND OBJECTIVE

AIM

The aim of this research work is to collect and perform biochemical evaluation of the unknown sample of Human Gingival Crevicular Fluid.

OBJECTIVE

- To perform the microbiological evaluation of Gingival Crevicular Fluid.
- To determine the class of bacteria present in Gingival Crevicular Fluid**.**

4. MATERIAL AND METHODS

A. Sample collectionmethods of Gingival crevicular fluid

For study, the sample was collected by using microcapillary pipette.

There are three methods to collect the gingival crevicular fluid.

a. Micropipette Method

Micropipette method is inserting capillary tubes, with specific diameter, into the entrance of the gingival crevice and the fluid migrates into the tube by capillary action. The use of

micropipettes permits the absorption of fluid by capillarity. Capillary tubes were placed in the pocket and their content was later centrifuged and analyzed.[20]

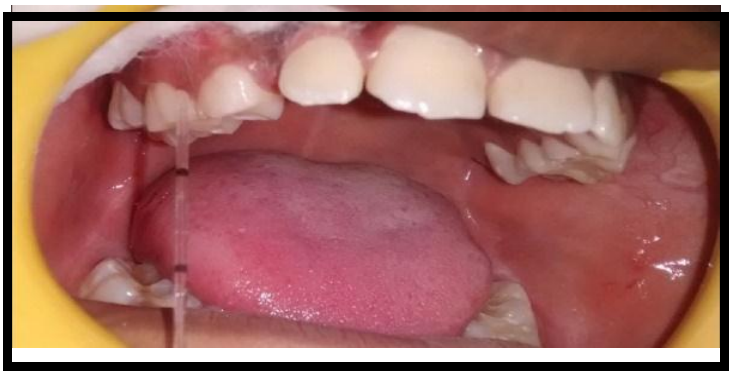

Fig. 3: Micropipette method.

Advantages of Micropipette methods[21]

- It provides an undiluted sample of GCF whose volume can be accurately assessed.
- Larger volume can be collected.

Disadvantages of Micropipette method[21]

- It has long collection period.
- Fluid collection is difficult because viscosity of fluid makes aspiration difficult.
- It is difficult to remove complete sample from tube.

b. Absorbent Filter Paper Strips

The most used method for GCF collection is made with specifically designed absorbent filter paper as endodontic paper points or perio-papers. The paper strips are inserted into the gingival crevice and left in situ for 5 to 60 seconds to allow the GCF to be adsorbed by the paper.[22]

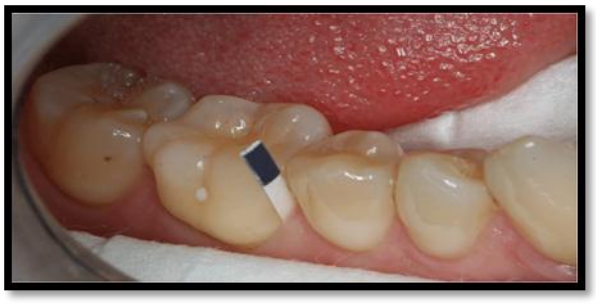

Fig. 4: Absorbent Filter Paper Strip method.

Advantages of Absorbing paper strips[23]

- Easiest method for collection of GCF sample.
- More precised method.

Disadvantages of Absorbing paper strips[23]

- Method introduces a degree of irritation of sulcular epithelium than can, by itself triggering oozing of fluid.

c. Preweighed Twisted Threads

The threads were placed in the gingival crevice around the tooth, and the amount of fluid collected was estimated by weighing the sample threads.[22]

d. Crevicular washing

The appliance designed by Oppenheim, permits collection of gingival fluid without disturbing the integrity of the marginal gingiva and this is a modification of that described by Takamori and the acrylic splint. It consists of a hardacrylic plate covering the maxilla with soft borders and a groove along the gingival margin, which is connected to four plastic cubes. Gingival washings are obtained by rinsing the sulcular area for a fixed period from one side to another through the palatine and buccal channels with 4–6 ml of solution using a peristaltic pump.[24]

Advantages of Crevicular washing method[25]

- Useful for studying the number and state of cells & bacteria from the crevicular area.
- Useful for clinically normal gingival.
- Useful for longitudinal studies.
- Permits collection without disturbing the marginal tissues.
- Contamination is least.

Disadvantages of Crevicular washing method[25]

- Complex procedure.
- Represents a dilution of crevicular fluid.

B. Equipments

Table 1: List of equipments.

Sr. No.	Instruments	Company Name
1.	Incubator	Aarson Scientific Works
2.	Autoclave	Aarson Scientific Works
3.	Hot air oven	Aarson Scientific Works

C. Composition of Media

a. Brain Heart Infusion Agar media.

Table 2: Formulation table for BHI Media.

Sr. No.	Ingredients	Quantity given
1.	BHI Media	37.0g
2.	Distilled water	1000ml

b. Muller's Hinton media

Table 3: Formulation table for Muller's Hinton Media.

Sr. No.	Ingredients	Quantity given
1.	Muller's Media	132.5g
2.	Distilled water	1000ml

c. SIM broth Media

Table 4: Formulation table for SIM broth Media.

Sr. No.	Ingredients	Quantity given
1.	Sim Broth Media	36.23g
2.	Distilled water	1000ml

d. MR-VP broth Media

Table 5: Formulation table for MR-VP Media.

Sr. No.	Ingredients	Quantity given
1.	MR-VP Media	17g
2.	Distilled water	1000ml

e. Starch Agar Media

Table 6: Formulation table for Starch Agar Media.

Sr. No.	Ingredients	Quantity given
1.	Starch Agar Media	30g
2.	Distilled water	1000ml

f. Simmons' Citrate Agar Media

Table 7: Formulation table for Simmons' Citrate Agar Media.

Sr. No.	Ingredients	Quantity given
1.	Simmons' Citrate Agar Media	24.28g
2.	Distilled water	1000ml

g. MacConkey Agar Media

Table 8: Formulation table for MacConkey Agar Media.

Sr. No.	Ingredients	Quantity given
1.	MacConkey Agar Media	49.53g
2.	Distilled water	1000ml

5. EXPERIMENTAL WORK DONE

A. Sample Collection

The sample of Gingival Crevicular fluid was collected from the 30 year old, female patient having chronic periodontitis oral infection from Dr. Chavhan Karanja Dental Clinic. The sample was collected from gums using micropipette**.**

B. Isolation of Microbes

The sample containing bacteria was inoculated on plates of both, the Brain Heart Infusion agar media & Muller's Hinton Agar Media by streak plate technique, incubated at 37°C for 24 hrs. After 24 hrs, the petri plates were observed for bacterial colonies.

C. Biochemical Test

a. Indole Test

Principle: Indole test is a biochemical test which differentiates the coliform from other members of Enterobacteriaceae by detecting their ability to produce the enzyme tryptophanase. This enzyme hydrolyses the amino acid tryptophan into indole, pyruvic acid and ammonia. It is the intracellular enzyme (endoenzyme). When indole is combined with Kovac's Reagent, the solution turns from yellow to cherry red. Because amyl alcohol is not water soluble, the red colouration will form in an oily layer of the top of the broth.[26]

Procedure

1. Weigh the accurate quantity of SIM broth media as per given in formulation table.
2. Dissolve the media in 20ml of Distilled water. Heat, if required.
3. Sterilized media by autoclaving at 121°C and 15 lbs for 30min.
4. In sterilized test tube, add 4ml of SIM broth media.

5. Inoculate the test tube aseptically by taking the loopful growth from 18-24 hrs culture of Gingival Crevicular fluid sample.
6. Incubate the test tube at 37°C for 48 hrs.
7. After the incubation period, add 0.5ml of Kovac's reagent to the broth culture.
8. Observe the colour change in test tube immediately.[27]

Inference: Positive test: Formation of pink-red colour on addition of reagent.

Examples: *Aeromonas hydrophila, Aeromonas punctata, Bacillus alvei, Edwardsiella sp., Escherichia coli, Flavobacterium sp., Haemophilus influenzae, Klebsiella oxytoca, Proteus sp. (not P. mirabilis and P. penneri), Plesiomonas shigelloides, Pasteurella multocida, Pasteurella pneumotropica, Enterococcus faecalis, and Vibrio sp.*

Negative test: No colour change was observed.

Examples: most *Bacillus sp., Bordetella sp., Enterobacter sp., Lactobacillus spp., most Haemophilus sp., most Klebsiella sp., Neisseria sp., Pasteurella haemolytica, Proteus mirabilis, Pasteurella ureae, P. penneri, Pseudomonas sp., Salmonella sp., Yersinia sp, Serratia sp.*[26]

b. Starch Hydrolysis Test

Principle: In the starch hydrolysis test, the test bacteria are grown on agar plates containing starch. If the bacteria have the ability to hydrolyse starch, it does so in the Media, particularly in the areas surrounding their growth while the rest of the area of the plate still contain non-hydrolysed starch. Since no colour change occurs in the Media when organisms hydrolyse starch, iodine solution is added as an indicator to the plate after incubation. While the non-hydrolysed starch forms dark blue colour with iodine, its hydrolyse end products do not acquire such dark blue colour with iodine.[28]

Procedure

1. Weigh the accurate quantity of Starch Agar Media as per given in formulation table.
2. Dissolve the weighed media in 20ml of water. Heat, if required.
3. Sterilized media by autoclaving at 121°C and 15 lbs for 30min.
4. Pour the media in petri plate and allow to solidify undisturbed.
5. Using sterile technique, inoculate the culture of Gingival Crevicular fluid sample using streak plate technique.
6. Incubate the inoculated petri plate for 48hrs at 37°C.

7. After incubation, flood the surface of the plates with Iodine solution for 30 sec. and pour off excess of Iodine.
8. Examine the bacterial growth around the line of inoculation.[28]

Inference: Positive test: A clear zone around the line of growth after addition of Iodine solution indicating that, organism has hydrolysed the starch.

Examples: *Bacillus subtilis, Bacillus cereus, Bacillus megaterium.*

Negative test: A blue, purple or black colouration of Media.

Example: *Streptococcus agalactiae, Staphylococcus epidermidis, Escherichia coli.*[29]

c. Catalase Test

Principle: The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into Hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. The culture should not be more than 24 hours old. Bacteria thereby protect themselves from the lethal effect of Hydrogen peroxide which is accumulated as an end product of aerobic carbohydrate metabolism.[29]

Procedure

1. Take 3ml of Hydrogen peroxide solution in test tubes.
2. Add growth colonies of 18-24 hrs Gingival Crevicular fluid sample culture in test tube containing Hydrogen peroxide, using sterile loop.
3. Observe immediately the oxygen bubbles produced in test tube.[29]

Inference: Positive test: Copious bubbles produced, active bubbling.

Examples: *Staphylococci, Micrococci, Listeria, Corynebacterium diphtheriae, Burkholderia cepacia, Nocardia,* the family Enterobacteriaceae (*Citrobacter, E. coli, Enterobacter, Klebsiella, Shigella, Yersinia, Proteus, Salmonella, Serratia*), *Pseudomonas, Mycobacterium tuberculosis, Aspergillus, Cryptococcus, and Rhodococcus equi.*

Negative test: No bubbles formation.

Examples: *Streptococcus mutans and Enterococcus* spp.[30]

d. Motility Test

Principle: Motility is the ability of an organism to move by itself by means of propeller-like flagella unique to bacteria or by special fibrils that produce a gliding form of motility. Motility by bacterium is demonstrated in semi solid agar media. The Media mainly used for this purpose is SIM Media (Sulphide Indole Motility Media) which is a combination of differential Media that tests three different parameters, Sulfur Reduction, Indole Production and Motility. The media has a very soft consistency that allows motile bacteria to migrate readily through them causing cloudiness. Bacterial motility is evident by a diffuse zone of growth extending out from the line of inoculation. Some organisms grow throughout the entire Media, whereas others show small areas or nodules that grow out from the line of inoculation. The non-motile bacteria will only grow in the soft agar tube and only the area where they are inoculated.[31]

Procedure

1. Weigh the accurate quantity of SIM broth media as per the given in formulation table.
2. Dissolved the media in 20 ml of distilled water. Heat the media if required.
3. Sterilized the media by autoclaving at 121°C at 15 min for 30min.
4. Sterilize the loop and inoculate the Gingival Crevicular fluid sample culture by stabbing in the middle of the sterilized test tube containing SIM broth media.
5. Incubate the test tube at 35-37°C and examine daily for up to 7 days.
6. Observe a diffuse zone of growth around the line of inoculation.[32]

Inference: Positive test: Diffuse, hazy growth, that spread throughout the media rendering it slightly opaque.

Example: *Escherichia coli.*

Negative test: Growth that is confined to the stab line, with sharply defined margins and leaving the surrounding media clearly transparent.

Example: *Staphylococcus aureus.*[32]

e. Methyl Red Test

Principe: Some bacteria have ability to utilize the glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product. These bacteria initially metabolise glucose to pyruvic acid which is further metabolized through the mixed acid pathway to produce the stable acid. The type of acid produced differs from species to species and depends on the specific enzymatic pathways present in the bacteria. The acid so produced

decreases the pH to 4.5 or below, which is indicated by a change in the colour of methyl red from yellow to red.[33]

Procedure

1. Weigh the MR-VP broth media as per given in formulation table.
2. Dissolve the media in 20ml of Distilled water. Heat media if required.
3. Sterilized media by autoclaving at 121°C and 15 lbs for 30min. Cool the media.
4. Inoculate the media lightly using 24 hrs young culture of Gingival Crevicular fluid sample.
5. Incubate at 37°C for 24hrs.
6. After incubation, transfer 2ml broth in separate sterilized test tube.
7. Add 2-3 drops of Methyl red indicator to test tube.
8. Observe for red colour produced immediately.[34]

Inference: Positive test: Formation of distinct red colour on addition of Methyl red indicator.

Examples: *Escherichia coli*, *Shigella* species, *Salmonella* species, *Citrobacter* species, *Proteus* species, *Yersinia* species.

Negative test: Formation of yellow colour or lack of colour change on addition of Methyl red indicator.

Examples: *Enterobacter* species, *Hafnia* species, *Serratia marcescens*, *Klebsiella pneumoniae*.[34]

f. Voges Proskauer Test

Principle: The Voges-Proskauer (VP) test is used to determine if an organism produces acetyl methyl carbinolfrom glucose fermentation. If present, acetyl methyl carbinol is converted to diacetyl in the presence of α-naphthol**,** strong alkali (40%KOH), and atmospheric oxygen. The α-naphthol was not part of the original procedure but was found to act as a colour intensifier by Barritt's and must be added first. The diacetylandquinidine-containing compounds found in the peptones of the broth then condense to form a pinkish red polymer.[35]

Procedure

1. Weigh the MR-VP broth media as per given in formulation table.
2. Dissolve the media in 20 ml of Distilled water. Heat media if required.

3. Sterilized media by autoclaving at 121°C and 15 lbs for 30min. Allow to cool.
4. Inoculate the media lightly using 24 hrs young culture of Gingival Crevicular fluid sample.
5. Incubate at 37°C for 24hrs.
6. After incubation, transfer 2ml broth in separate sterilized test tube.
7. Add 6 drops of Barritt's reagent A and mix well to aerate. Add 3 drops of 40% KOH & mix well to aerate.
8. Observe the pink-red colour at the surface within 30 min.
9. Shake the tube vigorously during the 30 min period.[36]

Inference: Positive Test: Formation of pink-red colour at the surface.
Examples: *Klebsiella* species, *Enterobacter* species, *Hafnia* species, *Serratia* species.
Negative test: Lack of formation of pink red colour at surface.
Example: *Escherichia coli*.[36]

g. Citrate Test

Principle: Simmons Citrate Agar is used for differentiating Gram negative bacteria on the basis of citrate utilization. The media contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen.

Bacteria that can grow on this Media produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle. When the bacteria metabolize citrate, the ammonium salts are broken down to ammonia, which increases alkalinity. The shift in pH turns the Bromothymol blue indicator in the media from Green to Blue above pH 7.6.[37]

Procedure

1. Weigh the accurate quantity of Simmons' Citrate Agar media as per given in formulation table.
2. Dissolve the media in 20 ml of Distilled water. Heat media if required.
3. Sterilized media by autoclaving at 121°C and 15 lbs for 30min.
4. Prepare slant by keeping test tube on one side.
5. After solidifying the agar, inoculate a loopful culture of Gingival Crevicular fluid sample slightly on the slant by touching the tip of the loop.

6. Incubate the test tube 37°C for 24hrs. Some organisms may require up to 7 days of incubation due to their limited rate of growth on Citrate media**.**
7. Observe the bacterial growth and colour change in media.[37]

Inference: Positive Test: Growth will be visible on slant surface and Media will be intense Prussian blue. The colour changes from the original green colour to intense blue colour. **Examples:** *Salmonella, Edwardsiella, Citrobacter, Klebsiella, Enterobacter, Serratia, Providencia,* etc.

Negative test: Traces or no growth on slant surface and no colour change observed. The Media will remain deep forest green coloured.

Example: *Escherichia, Shigella, Morganella, Yersinia* etc.[37]

6. RESULTS AND DISCUSSION

Gingival crevicular fluid

The biochemical microbial examination was carried out of Gingival crevicular fluid sample. The sample was first inoculated on the Brain Heart Infusion agar & Muller's Hinton agar plate. The subculture was used for various biochemical tests**.**

a. Indole Test

Test	Observation	Inference
Test tube containing SIM media + culture + incubate for 48 hrs at 37°C + add 0.5 ml of Kovac's reagent.	No colour change was observed after the addition of reagent	Negative test

From the above test, it was confirmed that the test sample contain *Lactobacillus* spp., *Klebsiella* sp.

b. Starch Hydrolysis Test

Test	Observation	Inference
Test tube containing starch agar media + culture + incubate for 48 hrs at 37°C + Flood the surface of petri plate with Iodine solution.	A clear zone around the line of growth after addition of iodine solution was observed**.**	Positive test

From the above test, it was confirmed that the test sample contain *Bacillus subtilis.*

Fig 4: Starch Hydrolysis test.

c. Catalase Test

Test	Observation	Inference
Add 3ml of Hydrogen peroxide solution in test tube + add colonies of 24 hrs old culture in test tube + observe immediately the oxygen bubbles produced in test tube.	No bubble formation, was observed.	Negative test

From the above test, it was confirmed that the test sample contain *Streptococcus mutans* species.

d. Motility Test

Test	Observation	Inference
Test tube containing SIM media + stab the loop containing culture in middle of test tube + incubate at 37°C for 7 days and examine daily + observe the growth around the line of inoculation.	Growth was observed around the inoculated line with clearly transparent medium.	Negative test

From the above test, it was confirmed that the test sample contain *Staphylococcus aureus.*

e. Methyl Red Test

Test	Observation	Inference
Test tube containing MR-VP Broth media + culture + incubate at 37°C for 24 hrs.+ take 1 ml broth in test tube + add 3 drops of Methyl red indicator + observe the red colour.	A pink-red colour was observed at the surface of test tube**.**	Positive test

From the above test, it was confirmedthat the test sample contain*Salmonella* species, *Escherichia coli*, *Citrobacter species.*

Fig. 5: Methyl red test.

f. Voges Proskauer Test

Test	Observation	Inference
Test tube containing MR-VP Broth media + culture + incubate at 37°C for24 hrs + take 2ml of cultured media in test tube + add 6 drops of 5% α-naphthol + 3 drops of 40% KOH + mix well to aerate + observe pink colour at the surface on addition of reagent.	A Pink -red colour was observed at the surface	Positive Test

From the above test, it was confirmed that the test sample *Klebsiella species.*

Fig. 6: Voges Proskauer test.

g. Citrate Test

Test	Observation	Inference
Prepare Slant containing Simmons' Citrate Agar media + inoculate the culture + incubate at 37°C for 24 hrs – 7 days + observe the growth with colour change.	Growth with colour change from Green to intense blue observed along the test tube.	Positive test

From the above test, it was confirmed that the test sample*Citrobacter, Salmonella* Spp.*Klebsiella* species

Fig. 7: Citrate test.

7. CONCLUSION

From this present study, I have concluded that, the micropipette method for sample collection was time consuming whereas, absorbing paper strips were easy to handle and faster.

The sample of human gingival crevicular fluid was collected using microcapillary tube and evaluated using various biochemical tests. The sample was found to be contained anaerobic gram positive and gram negative bacteria such as Streptococcus *mutan*, *Klebsiella*, *Salmonella* spp., *Lactobacillus* spp., *Citrobacter freundii.*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, etc. belonging to class Bacilli and Gamma proteobacteria, belonging to family of Enterobacteriaece and Streptococcus. All these bacteria were found during the study, streptococcus *mutans* causes dental carries where above other bacteria triggers the periodontal diseases and stimulates the production of gingival crevicular fluid in healthy as well as in non-healthy patients.

The Crevicular fluid based tests are recently attracting interest in not only the clinical but also in academics and industrial levels. Using these techniques, the diagnosis will be faster and much easier.

8. FUTURE SCOPE

The study of GCF is must needed as it provides media for measuring variety of molecules and bacteria present in the oral cavity. Increased coverage of GCF proteome will help in understanding the pathophysiology of various periodontal diseases as well as microflora present in the oral cavity. GCF as biomarker will help to detect root resorption study and will

help to diagnose the various periodontal diseases. Use of GCF based diagnostic test will be cost effective due to rapid detection of diseases.

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