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Research Article

COLLECTION, ISOLATION, CHARACTERIZATION OF HELICOBACTER PYLORI FROM DENTAL PLAQUE AND ANTIBACTERIAL ACTIVITY OF NOVEL DRUG 1-PHENYL-3-(4'-AMINOPHENYL)-5-(2", 4"-DICHLOROPHENYL)-2-PYRAZOLINE

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ABSTRACT

Collection of dental plaque from 194 patients with a history of smoking, drinking, tobacco chewers, oral hygiene status and stress conditions. Isolation of H.pylori from plaque by using laboratory diagnostic tests such as Urease, Catalase, Motility, Gram Staining. Culturing of H.pylori on Campylobacter agar medium, Brucella Broth to increase the detection rate of H.pylori. To determine Anti bacterial susceptibility test of phenyl compound against H.pylori.

Keywords: Helicobacter Pylori, Pyrazolines, Gram Staining, Antibacterial activity.

INTRODUCTION

Plaque is defined clinically as a structured, resilient, yellow-greyish substance that adheres tenaciously to the intraoral hard surfaces, including remove and fixed restorations. Plaque is primarily composed of bacteria in a matrix of salivary glycoprotein's and extracellular polysaccharides. This matrix makes it impossible to removable the plaque by rinsing or the use of sprays. Plaque can thus be differentiated from other deposits that may be found on the tooth surface, such as material Alba and calculus. Material Alba refers to soft accumulations of bacteria and tissue cells that lack the organized structure of dental plaque, and it is easily displaced with a water spray. Calculus is a hard deposit that forms by mineralization of dental plaque, and it is generally covered by a layer of unmineralized plaque.(Madinier IM, Fosse TM, Monteil RA 1997)

Dental plaque is composed primarily of microorganisms. One gram of plaque (wet weight) contains approximately 10^{11} bacteria. The number of bacteria in supragingival plaque on a single tooth surface can exceed $10^{9.}$ More than 500 distinct microbial species are found in dental plaque. One individual may harbor 150 or more different species. Non bacterial microorganisms that are found in plaque include Mycoplasma species, yeasts, protozoa, and viruses. The microorganisms exist within an intercellular matrix that also contains a few host cells, such as epithelial cells, macrophages, and leukocytes.

Dental plaque is broadly classified as supragingival or sub gingival based on its position on the tooth surface toward the gingival margin as follows:

- Supragingival plaque is found at or above the gingival margin, when in direct contact with the gingival margin, it is referred to as marginal plaque.
- Sub gingival plaque is found below the gingival margin, between the tooth and the gingival pocket epithelium.

Supragingival plaque typically demonstrates a stratified organization of a multilayered accumulation of bacterial morphotypes. Gram positive cocci and short

rods predominate at the tooth surface, whereas gram negative rods and filaments, as well as spirochetes predominate in the outer surface of the mature plaque mass. In general, the sub gingival microbial differs in composition from the supragingival plaque, primarily because of the local availability of blood products and a low oxidation-reduction potential, which characterizes the anaerobic environment.

The environmental parameters of the sub gingival region differ from those of the supragingival region; the gingival pocket is bathed by the flow of crevicular fluid, which contains many substances that bacteria may use as nutrients. Host inflammatory cells and mediators are likely to have considerable influence on the establishment and growth of bacteria in the sub gingival region. Both morphologic and microbiologic studies of sub gingival plaque reveal distinctions between the tooth associated and tissue associated regions of sub gingival plaque. The tooth associated cervical plaque, adhering to the root cemented, does not greatly differ from that observed in gingivitis. At this location filamentous microorganisms dominate but cocci and rods also occur. This plaque is dominated by gram positive rods and cocci. In the deeper parts of the pocket, however the filamentous organisms become fewer in numbers, and in the apical portion, they seem to be virtually absent. The apical border of the plaque mass is separated from the junction epithelium by a layer of host leukocytes, and the bacteria of this apical tooth- associated region shown an increased concentration of gram-negative rods.

The layers of microorganisms facing the soft tissue lack a definite intermicrobial matrix and contain primarily gram-negative rods and cocci, as well as large number of filaments, flagellated, Rods and spirochetes. (Micheal et al., 2001).

Plaque as a biofilm

Biofilm in general have an organized structure. They are composed of micro colonies of bacterial cells none randomly distributed in a shaped matrix or glycocalyx. In the lower plaque layers, which are dense, microbes are bound together in a polysaccharide matrix with other organic and inorganic materials. On top of the lower layer, a loose layer appears that is often irregular in appearance; it can extend into the surrounding medium. The fluid layer bordering the Biofilm has a rather stationary sub layer and a fluid layer in motion. Nutrient components penetrate this fluid medium by molecular diffusion. Steep diffusion gradients, especially for oxygen, exist in the more compact lower regions of Biofilm, which further explains changes in microbial composition.

The dental plaque Biofilm has a similar structure. It is heterogeneous in structure, with clear evidence of open fluid-filled channels running through the plaque mass. These water channels permit the passage of nutrients and other agents throughout the Biofilm, acting as a primitive “circulatory” system. Nutrients make contact with sessile micro colonies by diffusion from the water channels to the micro colony, rather than from the matrix. The bacteria exist and proliferate within the intracellular matrix through which the channels run. The matrix confers a specialized environment, which distinguishes bacteria that exist within the Biofilm from those that are free floating, the so called plank tonic state in solutions such as saliva or crevicular fluid. The Biofilm matrix functions as a barrier. Substances produced by bacteria within the Biofilm are retained and essentially concentrated, which fosters metabolic interactions among the different bacteria.

The intercellular matrix consists of organic and inorganic materials derived from saliva, gingival crevicular fluid and bacterial products. Organic constituents of the matrix include polysaccharides, proteins, Glycoprotein’s, and lipid material. Albumin, probably originating from crevicular fluid, has been identified as a component of the plaque matrix. The lipid material consists of debris from the membranes of disrupted bacterial and host cells and possibly food debris. Glycoprotein’s from saliva are an important component of the pellicle, which initially coats a clean tooth surface, but they also become incorporated into the developing plaque Biofilm. Polysaccharides produced by bacteria, of which dextran is the predominant form, also contribute to

the organic portion of the matrix. (Vanderende A et al., 1996).

The inorganic components of plaque are predominantly calcium and phosphorus, with trace amounts of other minerals, including sodium, potassium, and fluoride. The source of inorganic constituents of supragingival plaque is primarily saliva. As the mineral content increases, the plaque mass becomes calcified to form calculus. Calculus is frequently found in areas of the dentition adjacent to salivary ducts. The inorganic components of sub gingival plaque are derived from crevicular fluid. Calcification of sub gingival plaque also results in calculus formation. The fluoride component of plaque is largely derived from external sources such as fluoridated toothpastes, rinses and fluoridated drinking water. Fluoride is used therapeutically to aid in remineralization of tooth structures, prevention of demineralization of tooth structures, and inhibition of the growth of many plaque microorganisms (Uma Sudhakar et al., 2008).

Initial adhesion and attachment of bacteria

This diagram of events can also clarify the importance of hard-surface characteristics in plaque formation.

PHASE 1

Transport to the surface

The first stage involves the initial transport of the bacterium to the tooth surface, random contacts may occur, for example through Brownian motion (average displacement of 40 µm/hour), through sedimentation of microorganisms, through liquid flow or through active bacterial movement.

PHASE 2

Initial adhesion

The second stage results in an initial, reversible adhesion of the bacterium, initiated by the interaction between the bacterium and the surface, from a certain distance (50nm), through long-range and short range forces, including vanderwaals attractive forces and electrostatic repulsive forces. Derjaguin, landau, verwey and overbeek (DLVO) have postulated that above a separation distance of 1 nm, the summation of the previous two forces describes the total long-range interaction, shows the total interaction energy also called the total Gibbs energy. The result of this summation is a function of the separation distance between a negatively charged particle and a negatively charged surface in a medium ionic strength suspension medium (eg. saliva) for most bacteria. For bacteria in the mouth, the secondary minimum does not reach large negative values, which means a “weak” reversible adhesion. If a particle reaches the primary minimum (<1nm from the surface), a group of short-range forces (e.g., hydrogen bonding), ion pair formation, steric interaction dominates the adhesive interaction and determines the strength of adhesion.

PHASE 3

Attachment

After initial adhesion, a firm anchorage between bacterium and surface will be established by specific interactions (covalent, ionic, or hydrogen bonding). This follows direct contact or bridging true extracellular filamentous appendages (with length up to 10nm). On a rough surface, bacteria are better protected against shear forces so that a change from reversible to irreversible bonding occurs more easily and more frequently. The film between the interacting surfaces has to be removed before short-range forces can be involved. The bonding between bacteria and pellicle is mediated by specific extra cellular proteinaceous components of the organism and complementary receptors on the surface.

PHASE 4

Colonization of the surface and biofilm formation

When the firmly attached microorganisms start growing and the newly formed bacterial clusters remain attached, micro colonies or a Biofilm can develop. From this stage forward, new mechanisms are involved because now intra bacterial connection may occur. Essentially all oral bacteria possess surface molecules that foster some type of cell to cell interaction.

This process occurs primarily through the highly specific stereo chemical interaction of protein and carbohydrate molecules located on the bacterial cell surfaces, in addition to the less specific interactions resulting from hydrophobic, electrostatic and vanderwaals forces. Most co aggregations among strains of different genera are mediated by lectin like adhesions and can be inhibited by lactose and other galactosides. (Pytko-polonczyk et al., 1996).

Growth dynamics of dental plaque

Important changes in the plaque growth rate can be detected within the first 24 hours. During the first 2 to 8 hours, the adherent streptococci saturate the salivary pellicle binding sites and thus cover 3% to 30% of the enamel surface. Instead of the expected steady growth during the next 20 hours, a short period of rapid growth is observed. After 1 day, the term Biofilm is fully deserved because organization takes place within it. Microorganisms packed closely together, form a palisade, whereas others start to develop a pleomorphism. Each crack is filled with one type of microorganism. As the bacterial densities approach approximately 2-6 million bacteria/mm^2 on the enamel surface, a marked increase in growth rate can be observed to 32 million bacteria/mm^2. This further growth of the plaque mass occurs preferably by the multiplication of already-adhering microorganisms rather than by new colonizers. This growth period is appears to be dependent on cell density. The thickness of the plaque increases slowly with time increasing to 20 to 30µm after 3 days. (Desai HG et al., 1991).

Special bacterial behavior in biofilms

Bacteria growing in microbial communities' adherent to a surface do not "behave" the same as bacteria growing suspended in a liquid environment. Organisms in a Biofilm are 1000 to 1500 times more resistant to antibiotics. The mechanisms of this increased resistance differ from species to species, from antibiotic to antibiotic, and for Biofilm growing in different habitats. The resistance of bacteria to antibiotics is affected by their nutritional status, growth rate, temperature, Ph, and prior exposure to sub-effective concentrations of antimicrobial agents. Another important mechanism of resistance appears to be the slower rate of growth of bacterial species in a Biofilm, which makes them less susceptible to many, but not all antibiotics (Hardo PG et al.,1995).

Helicobacter Pylori

Robert warren, a pathologist and Barry Marshall a medical student at the time of the discovery received the 2005 Nobel Prize in medicine for their identification in 1982 of Helicobacter pylori. H. pylori are a gram negative, microaerophillic, rod-shaped bacterium that colonizes the human stomach. It resides beneath the gastric mucous layer, adjacent to the gastric epithelial cells; it causes inflammation of the gastric mucosa. Infection with this organism is now recognized as a serious, transmissible infectious disease, linked to duodenal and gastric ulcers and gastric carcinoma. (Lee A, 1994).

It contains a hydrogenase which can be used to obtain energy by oxidizing molecular hydrogen that is produced by intestinal bacteria; it produces oxidase, catalase and urease. It is capable of forming Biofilms and converts from spiral to a possibly viable but non culturable coccid form, both likely to favor its survival and be factors in the epidemiology of the bacterium**.**

Prevalence of H.Pyroli

The prevalence of H.pylori infection varies widely by geographic area, age, race, and socioeconomic status. The acquisition rate of H.pylori appears to be more rapid in developing than developed countries. The differences in H.pylori prevalence by race, ethnicity and nationality may reflect differences in social and or hygiene factors or the widespread use of antimicrobials for treatment of other common infections, especially during childhood. (Goodman KJ et al., 1996).

Pyrazolines

Pyrazolines are heterocyclic organic compound containing two nitrogen atoms next to each others in a five-membered ring,

called 1,2-diazol. Pyrazole is a colourless solid, melting point 70^0 C and crystallizes in long needles. Pyrazoles exhibit aromatic character with properties resembling both pyrrole and pyridine.

Pyrazolines are reduced form of nitrogen-containing five membered heterocyclic compounds . Pyrazolines possess important biological activity and therefore useful materials in drug research. Moreover Pyrazolines have played crucial role in the development of theory in heterocyclic chemistry and also used extensively as useful synthons in organic chemistry. The Pyrazolines function is quite stable and has inspired to utilize this stable fragment in bioactive materials to synthesis new compounds. 1-pyrazoline, 2-pyrazoline and 3-pyrazoline are the three important partially reduced forms of pyrazole structure with the different positions of the double bonds. Among these 2-Pyrazoliens are the most popular and frequently studied Pyrazolines isomers.2-pyrazoline exhibits monoimino character and hence more stable than the rest even though all the three types have been synthesized. (Anjani S and Smruti L., 2009).

EXPERIMENTAL

MATERIALS AND METHODS

Patients

The study group consisted of 194 patients, age ranging from 20-60 years, in and around the Bhimavaram who visited Shri Vishnu dental college. Subjects with a history of smoking, drinking, tobacco chewers, drinking water, ineffective brushing, stress, gastritis and duodenal ulcers.

Sampling of the dental plaque

Dental plaque was removed from the tooth surfaces with a sterile curette. Plaque was collected by an upward scrape against the tooth surface. The sample was dispersed separately in 1ml urea broth with phenyl red indicator to detect the urease activity and in 1ml normal saline which is used for culturing on selective media. (Honda K et al., 2001).

Isolation procedure

The sample collected in urea broth was monitored for a change in color from orange to pink which is due to the production of urease that converts the urea to ammonia. The broth is monitored for 24 hours. The change occurring within 15-30 minutes denotes a positive reaction.The sample in saline was transformed to the microbiology lab within 20 minutes and stored at 2-8°c. (Malaty HM et al., 1992).

Urease test

Urease test have been widely used because they are simple, cheap and easy to carry out.

Purpose

To assess the presence of H.pylori in dental plaque samples.

MATERIALS

Equipments required: Sterile test tubes, conical flask, Membrane filters (0.45µ), Laminar air flow, Micropipette, Cotton.

Urea broth composition (I.P)

Potassium dihydrogen orthophosphate	9.1g
Anhydrous disodium hydrogen phosphate	9.5g
Urea	20.0g
Yeast extract	0.1g
Phenol red	0.01g
Water	1000ml

Method

Mix, all the ingredients in 1000ml water, sterilize through membrane filtration (pore size 0.45µm), under laminar air flow and distribute aseptically in sterile container, cotton should be plugged. Transfer broth into test tubes by using membrane filters (pore size 0.45 µm). plaque samples containing H.pylori is introduced into test tubes which contains urea broth, the urease breaks the urea down into carbon dioxide and ammonia. The ammonium ion increases the PH, and a PH indicator, so phenol red changes color from yellow to red.

Transport media

Purpose

The success in culturing H.pylori from dental plaque is influenced by the transport conditions from periodontal lab to microbiology lab. Specimens taken to microbiology should be processed for culture as quickly as possible because the viability of the organism is reduced by exposure to atmospheric oxygen. Transport media such as 0.9% physiological saline as the selective enrichment medium. Samples should be processed within 2 to 3 hours of receipt. They should be stored at 4°c and processed as soon as possible thereafter.

Requirements

0.9% saline solution, sterile test tubes, Cotton, Conical flask, Autoclave, Hot air oven.

0.9% Physiological saline solution

Ingredients	Grams
Nacl	0.9g/100ml

Method**:** Dissolve 9 grams of Nacl in 100ml of water, sterilize by autoclaving at 15lbs square inch pressure (121ºc) for 15 min. cool transfer aseptically into sterile test tubes. Test tubes are sterilized by using Hot air oven at 121°c. Samples which are collected are stored at 4°c. (Parsonett JK *et al*1988).

Membrane filtration method

Membrane filtration method requires skill as it is a complicated method and based on the porosity of the membrane filter.

Apparatus:

The sterility test apparatus consists of a closed reservoir and a container to collect the filtrate, between which a properly supported membrane of appropriate porosity is placed. Membrane generally suitable for sterility testing has normal porosity of 0.45µm, diameter about to (Gaud RS *et al*., 2006).

Culture media

Purpose

There are notable differences in media recipes used for isolating and culturing H.pylori and the ideal medium remains to be developed. Culturing on solid media is the standard technique used in most laboratories for the isolation of H.pylori, selective media such as Skirrow Campylobacter medium. Although, success in H.pylori isolation and growth depends on many factors, such as the method of specimen collection time, procedure for tissue processing, composition of culture media, including growth supplements and environment (co_2 and humidity). (Hu W et al., 2002)

Requirements: Sterile Petri plates, sterile Macney bottle, Skirrow Campylobacter Agar, Laminar air flow, Sterile cotton swab.

Campylobacter agar base

INGREDIENTS	Grams/Liter
Protease peptone	15.00
Liver digest	2.00
Yeast extract	5.00
Sodium chloride	5.00
Agar	12.00
Final pH (at 25°c)	7.4 + 0.2

Method

Suspend 19.75gm in 500ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15lbs pressure (160ºc) for 15minutes. Cool to 45-50ºc and aseptically add 5-7% sterile lysed horse blood or sterile defibrinated sheep blood and rehydrated contents of one vial of campylobacter supplement-I (Blaser-wang) or campylobacter supplement-III (skirrow). Mix well and pour into sterile Petri plates. Wait for 15 minutes to solidify the agar base. Take organism from saline solution with a sterile loop, gently streak on the surface of the Campylobacter agar base. Wait for 45 minutes for complete absorbance of organism by the agar. (Jyostna V et al., 2009)

Incubation conditions

There are marked differences in the compositions of the microaerobic atmospheres used for culturing H.pylori. Concentrations ranging from 5-6% O_2, 7-12% CO_2, 10% H_2 and 0-85% N_2 have been reported. These can be achieved by using a gas generation kit, a variable atmospheric incubator or an anaerobic jar evacuated to 200mm Hg and refilled with a 10% CO_2, 10% H_2 and 80% N_2 gas mixtures. Although cultures should be incubated under microaerobic conditions at 37°c for at least four to five days, colony formation reported

on campylobacter agar plates after long term anaerobic incubation has been reported. Failure to detect the organism may be due to insufficient duration of incubation. It is important to maintain a humid atmosphere in the jars by placing moist filter paper or moist cotton wool at the bottom of the jar. However, this allows growth of fungi and other contaminant. The jars should be wiped with 70% alcohol between each use. Identification should be done on the basis of colony morphology, cell morphology, Gram's stain reaction, and positive biochemical reactions such as Catalase, Urease and Motility tests. (Kjoller M et al., 1991).

Catalase test

Purpose

Catalase is a common enzyme found in nearly all living organisms which are exposed to oxygen where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen.

Action of catalase

The reaction of catalase in the decomposition of hydrogen peroxide is:

$$2H_2O_2 \longrightarrow 2H_20 + O_2$$

Materials

Sterile test tube, Hydrogen peroxide, Microscope Slide.

Method

The catalase test is also one of the main three tests used by microbiologists to identify species of bacteria. The presence of catalase enzyme in the test isolate is detected using hydrogen peroxide. If the bacteria posses catalase (i.e. are catalase positive), when a small amount of bacterial isolate is added to hydrogen peroxide, bubbles of oxygen are observed.In microbiology, the catalase test is used to differentiate between bacterialspecies in the lab. The test is done by placing a drop of hydrogen peroxide on a microscope slide.

Result

- If bubbles or froth forms, the organism is said to be catalase positive.
- If not, the organism is catalase negative. (Jones DM et al., 1984).

Motility test

Purpose

This test is used to determine if an organism is motile or non motile. Motile Organisms are generally bacilli although a few motile cocci do exist.

Reagents and Equipment

Hanging drop method

- Liquid bacterial culture.
- Microscope slide with a central depression.
- Cover slip.
- Petroleum jelly or plasticine.

Methodology

- Place a small drop of bacterial culture in urea broth in the centre of a cover slip.
- Keep petroleum jelly on four sides of cover slip.
- Invert a slide with a central depression over the cover slip.
- The cover slip will stick to the slide and when the slide is inverted the drop of bacterial culture will be suspended in the well.
- Examine microscopically for motile organisms

Result

Positive result: spiral movement is observed.

Negative result: No movement is observed. (Thijs JC et al., 1996) .

Gram Staining

To study the plaque sample of H.pylori by Gram staining.

Procedure:

- Bacterial smear are prepared on a glass slide and thereafter heat fixed.
- A drop or two of crystal violet is put on the smear.
- After 1-2 minutes the slide is washed with tap water to remove excess of stains.
- The washed slide is subjected to few drops of grams iodine and waited for 1-2 minutes and thereafter washed with tap water for decolorization.
- The slide is put in 95% ethanol.
- Safranin is added to the slide after 1-2 minutes.
- The smear mounted in glycerin and observed under the microscope. Violet

and iodine (CV–I) within the inner and outer layers of the cell. Iodine is often referred to as a mordant, but is a trapping agent that prevents the removal of the CV-I complex and therefore color the cell.

When a decolorizer such as alcohol or acetone is added, it interacts with the lipids of A Gram stain of mixed Staphylococcus aureus (Gram positive cocci) and Helicobacter pylori (Gram negative bacilli).

Antibacterial activity

The Pyrazolines compound was screened for antibacterial activity, as the literature survey reveals that Pyrazolopyrimidines possess the same. For determination of bacterial susceptibility test, gram negative organism H.pylori is used. A stock solution of amoxicillin was prepared and the dilutions are prepared. All the strains were maintained by weekly sub culturing on nutrient agar slant, stored at 4 ^{0}C after previous 24 h incubation at 37 ^{0}C. Before each experiment, the organism was activated by successive sub culturing and incubation. The activity is studied by using agar diffusion method.

Standardization of test organism

A 10 mL volume of sterile water was added to the agar slant containing a 24 h old culture of purified test microorganism and shaken carefully to harvest the organism. Subsequently, dilutions were carried out to get microbial population of 10^5 cfu/mL by comparing with $BaSO_4$, equivalent to Mcfarland 0.5 standard.

Preparation of $Baso_4$ solution standard to Mc farland 0.5 standard

To standardize the inoculums density for a susceptibility test, a $BaSO_4$, turbidity standard, equivalent to a 0.5 McFarland standard is used. The $BaSO_4$, Mcfarland 0.5 standard is prepared as follows. A 0.5 mL of 1.175% w/v of $BaCl_2.2H_2O$ is added to 99.5 mL of 1% w/v of H_2SO_4 with constant stirring to maintain suspension. The correct density of the turbidity standard is verified by using a UV-spectrophotometer by determining the absorbance. The absorbance at 625nm is 0.08-0.10 for this standard. This suspension is used to standardize the inoculums density.

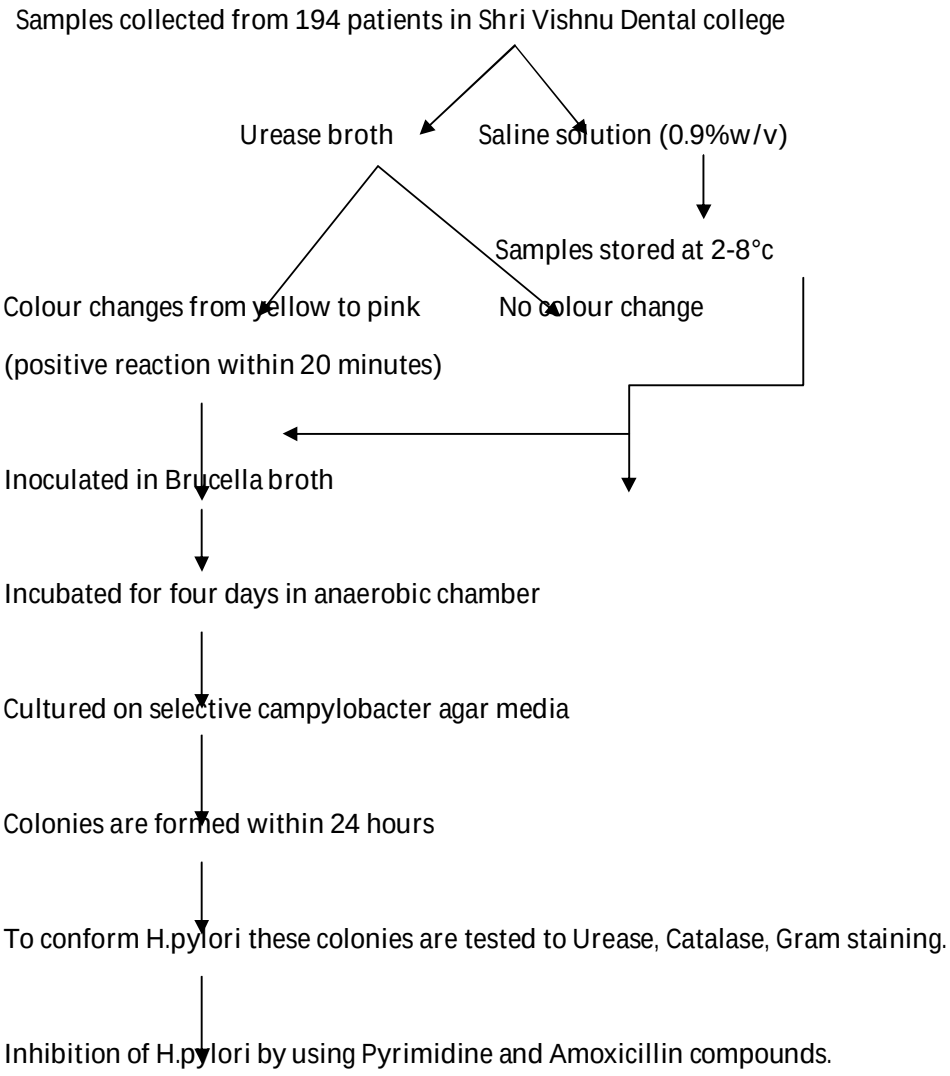
Preparation of stock solution and determination of zones of inhibition

Pyrazoline were dissolved in DMSO to obtain a stock concentration of 1 mg/ml. The required final concentrations (0.5 µg/ml, 1 µg/ml, 1.5 µg/ml, 2 µg/ml, 2.5 µg/ml) were made from the stock solution, by using the same solvent. Amoxicillin was dissolved in water and a stock concentration of 1 mg/mL was prepared. The dilutions were prepared similar to the test compounds.

Inoculums' of 100µl solution from the standardized bacterial suspension was added to the molten agar (20 ml). The mixture was poured into sterile Petri dishes, shaken slowly for uniform distribution and allowed to solidify. The plates were divided into three sections and cups were made in the agar plates, which were filled with the specific concentration of the prepared drug solution. The plates were incubated for 24 h at 35 ^{0}C in an ambient air incubator. Solvents and growth controls were kept and the zones of inhibition were measured with millimeter ruler across the cup. The zone of inhibition obtained was compared to the interpretive standard (Amoxicillin). The Petri dishes which were seeded with microorganism alone were regarded as negative controls and those dishes with bores containing reference drug solution were regarded as positive controls. The cups for each test compound were made in 3 Petridishes so as to make (n=9), and the results were reported as mean±SEM.

The same procedure was repeated for the organism with respect to all the test compounds. (Robbin S et al., 2007)

PLAN OF WORK



RESULTS AND DISCUSSION

Table 6: Determination of minimum inhibitory concentration of amoxicillin by using cup plate method

Amoxicillin concentration (nm)	Zone of inhibition (CM)
1.77	1.15
3.55	1.31
5.33	1.64
7.11	1.90
8.89	2.13
10.66	2.23
12.44	2.33

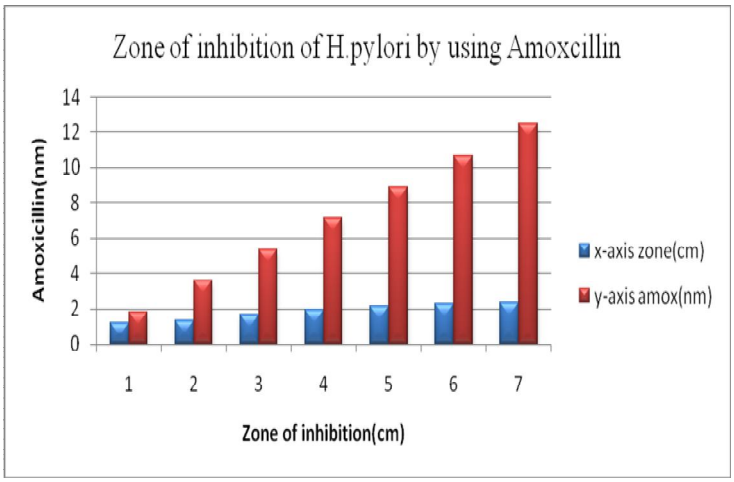


Table 6 & Fig 6: Indicates zone of inhibition (cm) of H.Pylori by amoxicillin of various concentrations

The figure represents MIC (Minimum Inhibitory Concentration) of Amoxicillin. Diffusion of Amoxicillin through the solidified Campylobacter agar layer of a Petri-dish, growth of inoculated H.pylori organism is inhibited entirely in a circular zone. Amoxicillin of 1.77 nm concentrations shows 1.15 cm zone of inhibition, 3.55 nm shows 1.31cm, 5.33nm shows 1.64cm, 7.11nm shows 1.9cm, 8.89nm shows 2.13cm, 10.66 nm shows 2.23cm, 12.44 nm shows 2.33nm, and so concentration of Amoxicillin increases zone of inhibition increases. MIC value of Amoxicillin is 1.77nm.

Table 7: Determination of minimum inhibitory concentration of pyrazoline by using cup plate method

Pyrazoline concentration (Nm)	Zone of inhibition (CM)
1.77	1.28
3.55	1.52
5.33	1.58
7.11	1.61
8.89	1.99
10.66	2.13
12.44	2.23

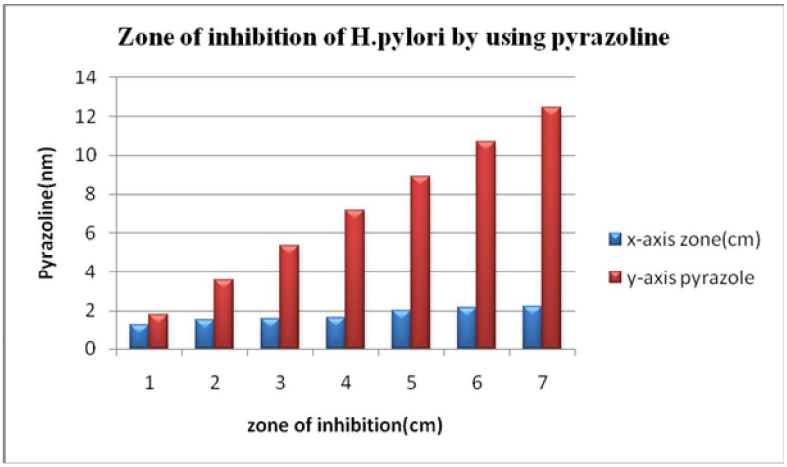


Table 7 &Fig 7: Indicates zone of inhibition (cm) by Pyrazoline compound

The figure represents MIC of Pyrazoline. Diffusion of pyrazoline through the solidified Campylobacter agar layer of a Petri-dish, growth of inoculated H.pylori organism is inhibited entirely in a circular zone. Pyrazoline of 1.77 nm concentration shows 1.28cm, 3.55nm shows 1.52cm, 5.33nm shows 1.58cm, 7.11nm shows 1.61cm, 8.89nm shows 1.99cm, 10.66nm shows 2.13cm, 12.44nm shows 2.23cm, so concentration Pyrazoline increases zone of inhibition increases. MIC value of Amoxicillin is 1.77nm.

Table 8: Determination of minimum inhibitory concentration of pyrazoline with combination of amoxicillin by using cup plate method

Pyrazoline concentration (Nm)	ZONE of inhibition (CM)
0.531	0
0.708	0
0.885	1.19
1.062	1.25
1.239	1.29
1.416	1.30
1.593	1.38
1.77	1.40
1.947	1.48
2.124	1.49
2.301	1.50
2.478	1.52
2.655	1.56
2.832	1.60
3.009	1.62
3.186	1.68
3.363	1.7
3.540	1.77
3.717	1.89
3.894	1.98
4.071	2.24
4.248	2.33
4.425	2.43
4.602	2.45
4.779	2.50
4.956	2.52
5.133	2.56
5.310	2.58

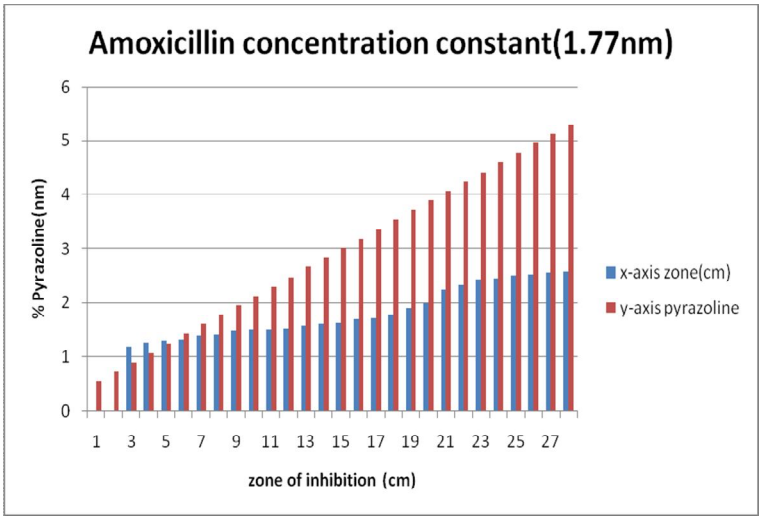


Table8 &Fig. 8: Indicates zone of inhibition of H.pylori by pyrazoline (nm) by keeping amoxicillin constant (1.77nm).The figure represents MIC of pyrazoline with the combination of amoxicillin. MIC of Amoxicillin concentration is 1.77nm. By

keeping Amoxicillin constant, concentration of Pyrazolines is varied. In these graph MIC of pyrazoline is 0.885nm, having zone of inhibition 1.19cm. So concentration of pyrazoline increases zone of inhibition increases. In these graph we can observe synergistic effect of amoxicillin. Without Amoxicillin MIC of pyrazoline is 1.77nm, but with Amoxicillin MIC of pyrazoline is 0.885nm.

Table 9: Determination of minimum inhibitory concentration of amoxicillin with combination of pyrazoline by using cup plate method

Amoxicillin (nm)	Zone of inhibition(cm)
0.531	0
0.708	1.3
0.885	1.35
1.062	1.36
1.239	1.40
1.416	1.49
1.593	1.52
1.770	1.59
1.947	1.60
2.124	1.63
2.301	1.68
2.478	1.69
2.655	1.72
2.832	1.78
3.009	1.79
3.186	1.82
3.363	1.85
3.540	2.10
3.717	2.34
3.894	2.36
4.071	2.39
4.248	2.50
4.425	2.52
4.602	2.56
4.779	2.58
4.956	2.60
5.133	2.63
5.310	2.65

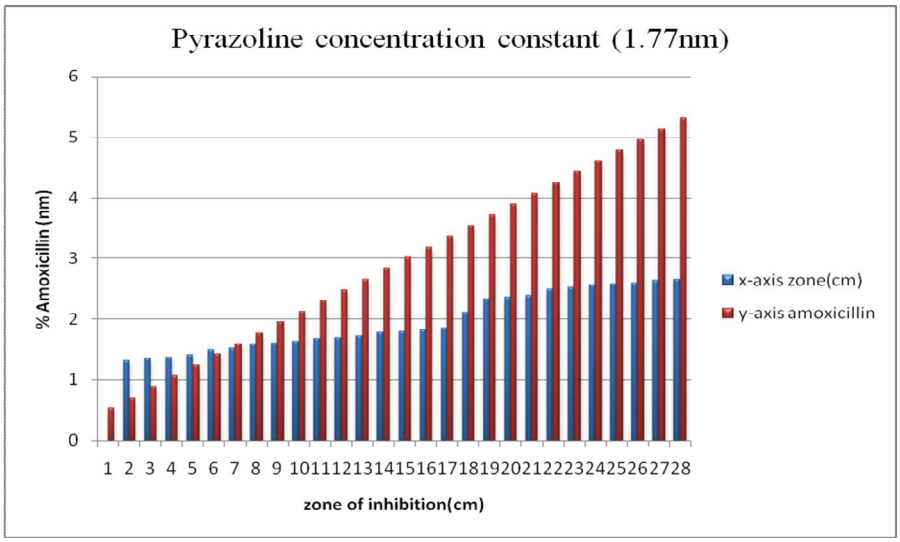


Table9 & Fig. 9: Indicates zone of inhibition of H.pylori by Amoxicillin (nm) by keeping pyrazoline constant(1.77nm)

The figure represents MIC of Amoxicillin with the combination of pyrazoline. MIC of pyrazoline concentration is 1.77nm. By keeping Pyrazoline constant, concentration of Amoxicillin is varied. In these graph MIC of Amoxicillin is 0.708nm, having zone of inhibition 1.3cm. so concentration of pyrazoline increases zone of inhibition increases. In these graph we can observe synergistic effect of pyrazoline. Without pyrazoline MIC of Amoxicillin is 0.708nm, but with pyrazoline MIC of Amoxicillin is 0.70.

DISCUSSION

In this unquenchable thirst for conquering the disease, man has focused his research at the cellular and molecular levels to understand the disease process better. Even since the discovery of H.pylori by Marshall and Warren in 1982, its role in gastric pathophysiology represents a fundamental change in the understanding of peptic ulcer diseases.

Parronet *et al*., 1998 reported that H.pylori was the most common infection caring agent in human. The rate of acquisition of H.pylori infection was higher in developing than in developed countries. Even within the developed countries, the prevalence varies between racial groups, and could be due to differences in cultural background, social and environmental factors. However, the disease only occurs in about 15% of infected persons.

Two main mechanisms were suggested by which H.pylori may produce gastric inflammation. The organism may interact with surface epithelial cells, producing either direct cell damage or liberation of epithelial pro inflammatory mediators; and H.pylori products may gain access to the underlying mucosa, there by directly stimulating host `nonspecific and specific immune responses involving the liberation of variety of cytokines (TNFα, IL-6, IL-7, IL-10 and IL-12). Direct mucosal damage may be due to adherence of the organism to the gastric epithelium, vaculating cytotoxin which includes vacuole formation in epithelial cells, variety of enzymes like urease, which by producing ammonia not only protect the organisms from gastric acid but also have toxic effects on the mucosa.

H.pylori is the first bacterial infection recognized as the human carcinogen. It is associated with gastric carcinoma, asthma, cerebrovascular and cardiovascular disease. H.pylori infections are particularly difficult to eradicate. To control the infection there is need to know about the routes of entry and various reservoirs. Various modes of transmission like oral-oral, feco-oral, and spread by water and through food have been implicated.

Dental plaque has been implicated as a possible source and route of transmission of H.pylori. Krajen first reported on the presence of H.pylori in dental plaque. Dental plaque can be defined as soft, deposits that form the Biofilm adhering to the tooth surface. The significance of the Biofilm environment has been increasingly recognized in recent years because the environment itself may alter the properties of the organisms. The Biofilm community is initially formed through bacterial interactions with the tooth and then through physical and physiologic interactions among different species, within the microbial mass. Newer microscopic heterogeneous in structure, with clear evidence of open fluid-filled channels running through the plaque mass.

These channels may provide for circulation within plaque to facilitate movement of soluble molecules such as nutrients or waste products. This Biofilm acts as barrier. Substances produced by bacteria within the Biofilm are retained within the Biofilm. The resistance of bacteria to antibacterial agents is significantly increased in a Biofilm environment.

In our study, after isolating samples, immediately placed in transport media i.e. saline and samples brought to the laboratory within 2 hours and stored under cold conditions, same samples which are placed in urea broth, within 30 minutes colour of urea broth changes from yellow to pink. It indicates presence of H.pylori in isolated samples. For further conformation other biochemical tests such as catalase, motility, gram staining tests should be done. If the samples passed urease test, catalase test, motility test, gram-staining test, samples which are gram negative organism,

having curved, rod shaped bacteria are considered as H.pylori organism.

These organisms innoculated in brucella broth which is considered as liquid media, brucella broth is standardized by using Macfen solution. H .pylori in brucella broth streaked on Campylobacter agar which is selective media for H.pylori. Although success in H.pylori isolation and growth depends on many factors such as method of collection time, composition of culture media, transport conditions. Samples are incubated at 37°c under microaerophillic conditions, examined for 4 to 7 days, characteristic colonies of H.pylori were confirmed by Gram staining, Catalase and Motility tests.

Over a study period of 6 months, dental plaque samples were collected from 194 patients in and around Bhimavaram who visited Shri Vishnu dental college. Among 194 patients, 138 patients were male, 56 patients were female. Comparatively male patients are more compared to female patients because factors such as Smoking, Drinking and Tobacco chewers are mainly influenced the formation of plaque. 21 patients visited to periodontology lab in the month of November, 16 patients in December, 24 patients in January, 34 patients in February, 41 patients in March, 58 patients in April. Stress levels are more between 30-50 years of age compared with 20-30 and 50-60. Stress levels are directly proportional to prevalence of H.pylori. Acutely developing gastric mucosal defects that may appear after severe physiologic stress are called stress ulcers.

Prevalence of H.pylori depend upon various factors such as smoking which affects 47% (91.18) patients having H.pylori in dental plaque, Drinking affects 38% (73.72) patients having H.pylori in dental plaque, Tobacco chewers affects 27% (52.28) patients having H.pylori in dental plaque, Tap water affects only 12% (23.38) patients having H.pylori in dental plaque, Ground water affects 80% (155.2) patients having H.pylori in dental plaque, Ineffective brushing affects 88% (170.72) patients having H.pylori in dental plaque, Stress affects 40% (77.6) patients having H.pylori in dental plaque, Gastritis, duodenal ulcer affects 7% (13.58) patients having H.pylori in dental plaque.

To inhibit H.pylori organism pyrazoline compound are used. For determination of bacterial susceptibility test gram negative H.pylori is used. To standardize the organism 10 ml volume of sterile water was added, subsequently dilutions were carried out to get a microbial population of 10^5 cfu/ml by comparing with $Baso_4$ equivalent to Mcfarland 0.5 standard. To standardize the inoculums density for a susceptibility test, a $Baso_4$, turbidity standard equivalent to 0.5 Mcfarland is prepared.

Pyrazoline compound is dissolved in DMSO to obtain a stock concentration of 0.5µg/ml, 1µg/ml, 1.5µg/ml, 2µg/ml, 2.5µg/ml, 3µg/ml, were made from same stock solution, by using the same solvent. Amoxicillin is dissolved in water and a stock concentration of 1mg/ml is prepared. Inoculums of 100µl solution from the standardized bacterial suspension was streaked on the surface of Campylobacter agar base. After 45 minutes bacteria was diffused completely into the agar. Cups were made in the agar plates, which are filled with specific concentrations of drug and amoxicillin. The plates were incubated for 24 h at 35°c in an ambient air incubator. Zone of inhibition were measured with millimeter ruler across the cup. By using cup plate method we can determine MIC of Amoxicillin is 1.77nm, MIC of Pyrazoline is 1.77nm, in the combination of Amoxicillin and pyrazoline, MIC of Amoxicillin is 0.708nm and MIC of pyrazoline is 0.885nm.So we can observe synergistic effect of Amoxicillin and Pyrazoline.

CONCLUSION

In conclusion, H.pylori is a micro-organism that causes or is associated with a curable chronic infection. In some patients the infection can be difficult to eradicate and there is a significant rate of recurrence, whether the rate of recurrence can be reduced by concomitant emphasis on improving oral hygiene and periodontal disease remains to be clarified. Knowledge about the reservoirs and modes of transmission could help to explain the high prevalence and risk factors for H.pylori infection.

H.pylori has been found in feaces, and transmission via feaces-contaminated water can occur. Although culture of H.pylori is a tedious procedure, it is reliable and almost all clinical microbiology laboratory can perform it. Whatever specimen is cultured, careful consideration of the transport conditions, culture media, and incubation conditions are necessary for effective recovery of the organism. Samples should be transported to the microbiology laboratory in sterile saline at room temperature. Cultures should be performed on freshly prepared Skirrow campylobacter agar. Plates should be incubated at 37°c for five to seven days under micro aerobic conditions. Biochemical identification requires Gram's staining, rapid urease test, catalase test, positive should be tentatively identified as H.pylori.

Demonstration of oral carriage of H.pylori, transient, permanent may have immediate applications with recommendations to prevent person-to-person transmission via the oral-oral route. Further studies are needed to confirm whether removal of plaque can cause any change in the recurrence rate.

Table 1: A view on how data was collected and month-wise record of various patients under the study

MONTHS	NO. OF PATIENTS
November	21
December	16
January	24
February	34
March	41
April	58
TOTAL	194

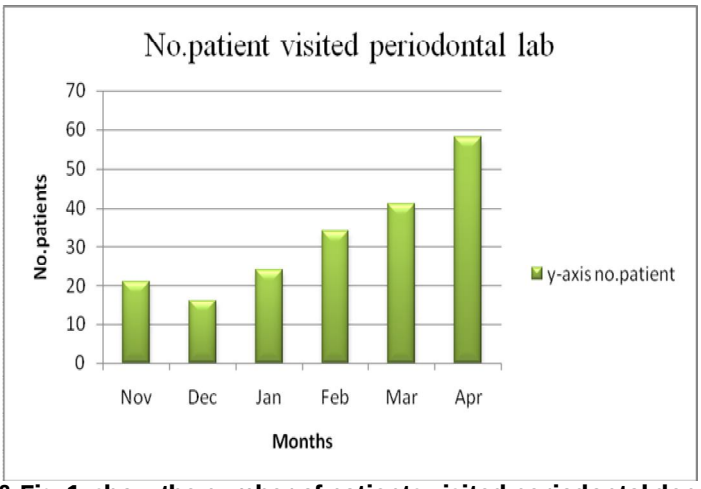


Table1 & Fig. 1: show the number of patients visited periodontal department month –wise during November 2009 and April 2010.

The figure1 & Table1 represents the month-wise analysis of number of patients visited periodontal lab, No. of patients visited in November: 21, December: 16, January: 24, February: 34, March: 41 and April: 58. Comparatively more no. of patients visited in April, than November, December, January and March 2009-2010

\Table 2: Comparative studies of stress and H.pylori found in different age groups of patients

Age group	stress	% H.pylori found
20-30	II stage	24
30-40	III stage	34
40-50	III stage	40
50-60	I stage	18

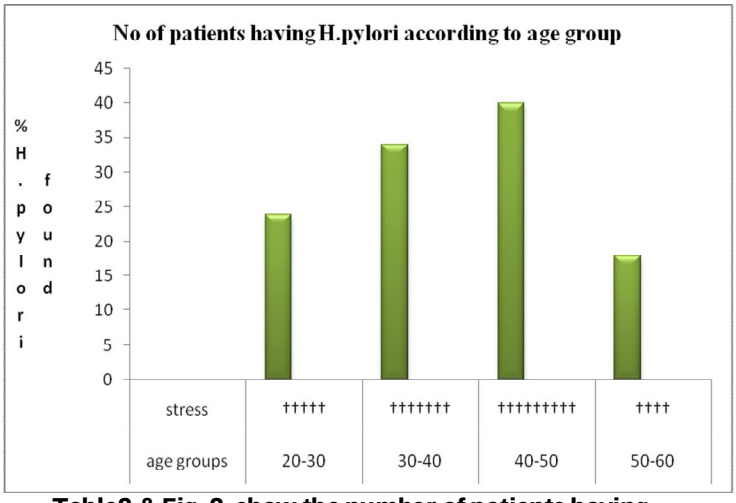


Table2 & Fig. 2: show the number of patients having H.pylori according to age group due to stress

The figure2 & Table2 represent the correlation between the age group and stress. It can be observed that the stress increases the patients are more prone to H.pylori.

Table 3: Analysis of male patients visited during the period of study

MONTH	NO. of males
November	16
December	9
January	20
February	21
March	32
April	40
TOTAL	138

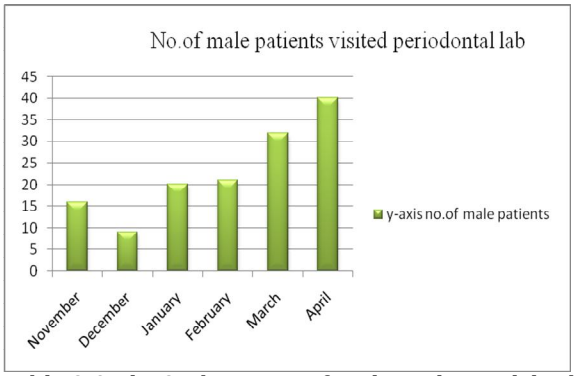


Table 3 & Fig. 3: shows no. of male patients visited periodontology lab month –wise

The figure 3 and Table3 represents the month-wise analysis of number of male patients visited periodontal lab, No of male patients visited in November are 16, in December 9, January 20, February 21, March 32, April 40. In our study male patients are more compared to female patients.

Table 4: Analysis of female subjects visited during the period of study

Month	No. female patients
November	5
December	7
January	4
February	13
March	9
April	18
Total	56

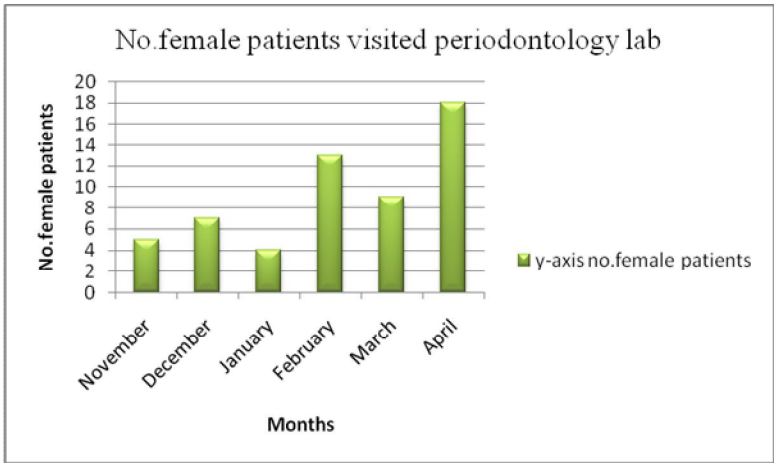


Table4 & Fig. 4: Shows no. of female patients visited periodontology lab month- wise

The figure represents the month-wise analysis of number of female patients visited periodontal lab. No of female patients visited in November: 5, December: 7, January: 4, February: 13, March: 9, April: 18. Comparatively more number of male patients visited in April than November, December, January, February, and March.

Table 5: Comparative analysis of the various factors affecting the presence of H.pylori Total no. of patients visited:194

Factors	% H.Pyroli found	Number of patients having H.Pyroli
Smoking	47%	91.18
Drinking	38%	73.72
Tobacco	27%	52.38
Tap water	12%	23.28
Ground water	80%	155.2
Ineffective brushing	88%	170.72
Stress	40%	77.6
Gastritis / duodenal ulcers	7%	13.58

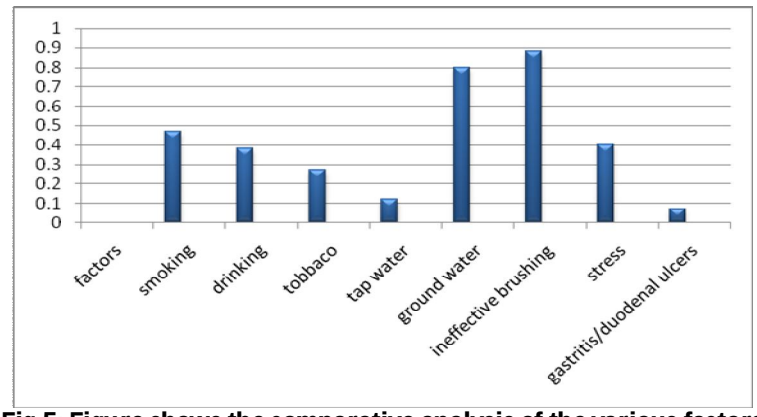


Fig 5: Figure shows the comparative analysis of the various factors affecting the presence of H.pylori

The figure represents the various factors such as Smoking, Drinking, Tobacco, Tap water, Ground water Ineffective brushing, Stress, and Gastritis affecting the prevalence of H.pylori. Smoking impairs mucosal blood flow, healing and inflammatory processes and is an important risk factor for development of H.pylori. Smoking affects the vasculature, immune system Alcohol cause peptic ulceration and alcoholic cirrhosis is associated with incidence of peptic ulcer. Stress ulcers are most commonly encountered in certain conditions such as NSAID induced ulcers, Extensive burns, Corticosteroids, Gastric irritant drugs. In our study we also found that chronic stress lead to change in daily habits, such as poorer oral hygiene, clenching, grinding, decreased saliva flow and suppressed immunity. Ineffective Brushing causes more accumulation of plaque on supragingival and sub gingival regions of tooth, so availability of H.pylori also more. Gastric ulcer patients having hypercalcemia stimulates gastric production and acid secretion.

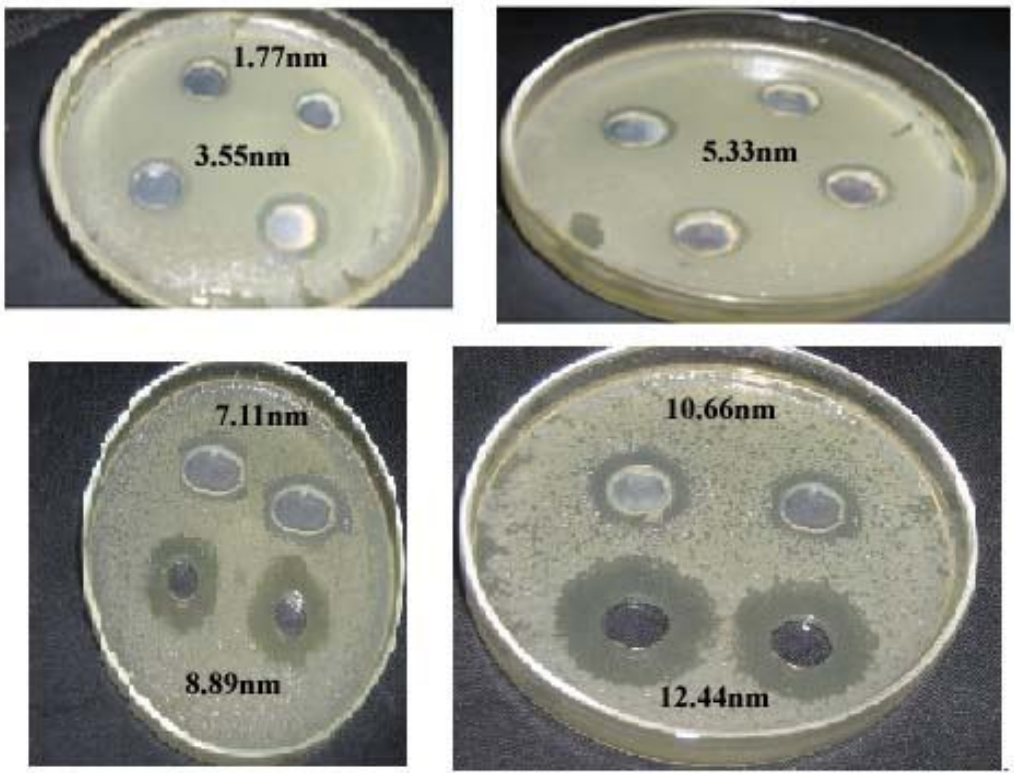


Fig. 8: Figure shows Minimum inhibitory concentration of Amoxicillin

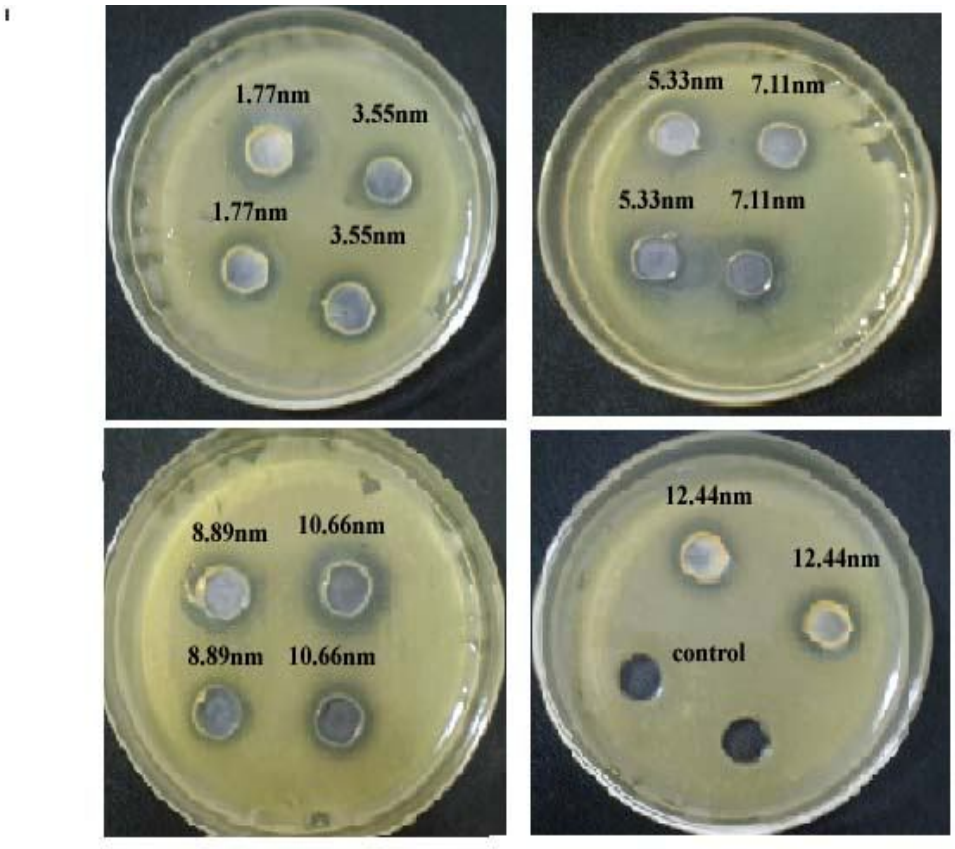


Fig. 7: Figure shows Minimum inhibitory concentration of Pyrazolines

Fig. 8: Figure shows samples which pass urease test

Table 9: Plaque samples which are positive to urease test to conform H.pylori according to various factors.

% urease test	Factors affecting H.pylori
49	Smoking
41	Drinking
30	Tobacco chewers
12	Tap water
89	Ineffective brushing
85	Ground water
40	Stress
9	Gastric/Duodenal ulcers

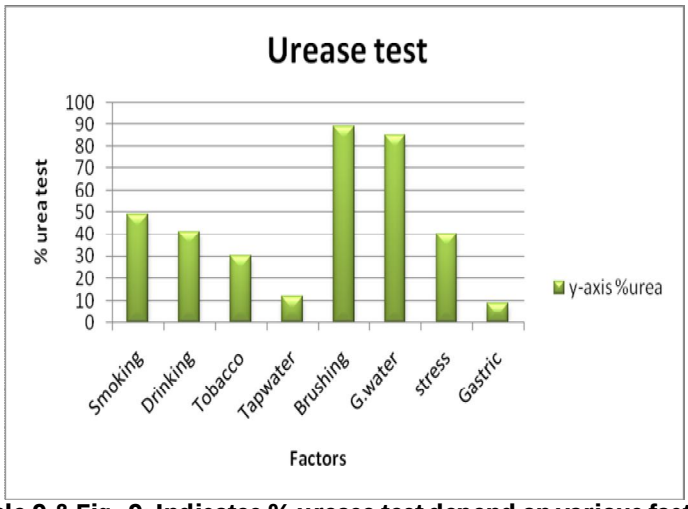


Table 9 & Fig. 9: Indicates % urease test depend on various factors

The figure represents samples which are positive to urease test to conform H.pylori in plaque samples. A sample which is placed into small quantity (1ml) of urea broth with a phenyl red indicator, that detects alkalinity resulting from the formation of ammonia by urease. So from graph we can represent that patients who are smokers give a positive result (49%) within 30 minutes, who are drinkers give positive result (41%) within 30 minutes, Tobacco chewers give positive result (30%) within 30 minutes, tap water give positive result (12%) within 30 minutes, Ineffective Brushing give result (89%) within 30 minutes, Groundwater give result (85%) within 30 minutes, Stress give result (40%) within 30 minutes, Gastric give result (9%) within 30 minutes. Among all factors Ineffective brushing, Ground water affects more prevalence of H.pylori, when compared to other factor.

Table 10: Plaque samples which are positive to catalase test to conform H.pylori according to various factors

% catalase test	Factors
47	Smoking
38	Drinking
27	Tobacco
12	Tap water
88	Ineffective brushing
80	Ground water
40	Stress
7	Gastric/duodenal ulcer

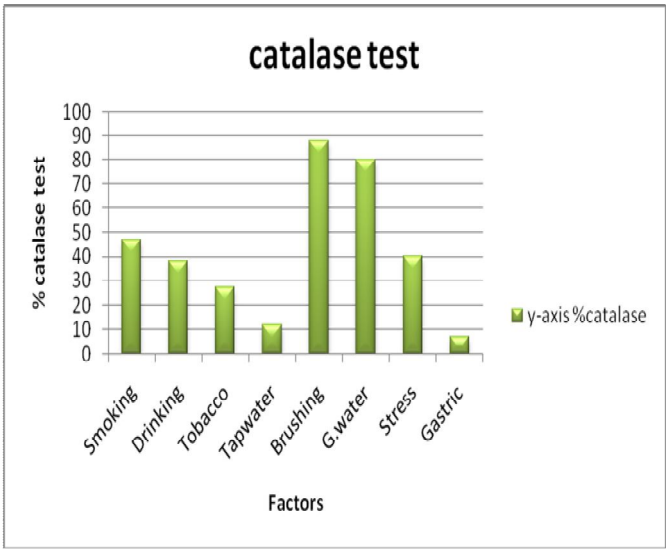


Table10 & Fig10: Indicates % catalase test depend on various factors

The figure represents samples which are positive to catalase test to conform H.pylori in plaque samples. This test is simple, cheap. It is an alternative method to determine the prevalence of H.pylori. A plaque sample is placed into a small quantity (1ml) of H_2O_2 in test tube. Bubbles of oxygen are observed. Among all factors ineffective brushing, Ground water affects more prevalence of H.pylori, when compared to other factors.

Table 11: Plaque samples which are positive to motility test to conform H.pylori according to various factors

% motility test	Factors
50	Smoking
40	Drinking
28	Tobacco chewers
13	Tap water
93	Ineffective brushing
84	Ground water
75	Stress
76	Gastric/duodenal ulcer

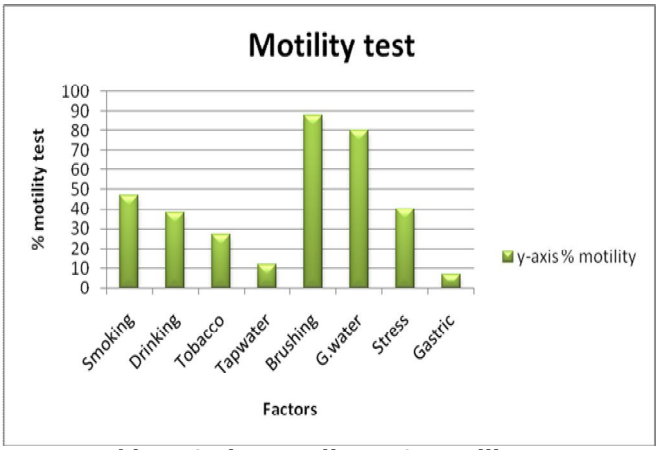


Table11 & Fig11: Indicates % motility test depend upon various factors

The figure represents samples which are positive to motility test to conform H.pylori in plaque samples. These cells have a single flagellum at one or both poles. Transmission from person to person affects with Ground water is more when compared with tap water. Ineffective Brushing is the factor of poor oral-hygiene.

REFERENCES

1. Alikhani MY and Sadeghifard A. Evaluation of selective and nonselective media for isolation of Helicobacter pylori from gastric biopsy specimens. Pakistan J Biol Scie. 2007; 10: 4156-4159.
2. Chomvarin C and Wong PK. Comparison of media and antibiotic supplements for isolation of helicobacter pylori from gastric biopsies. Southeast Asian J Trop Med Public Health. 2006; 37: 1163-1169.
3. Czesnikiewiczguzik M, Karczewska E, Bielanski W, Guzik TJ, Kapera P, Targosz A, Konturek SJ, Loster B. Association of the presence of the Helicobacter pylori in the oral cavity and in the stomach. Indian J Pharmacol. 2004; 55: 105-115.
4. Desai HG, Gill HH, Shankaran k. Dental plaque: a permanent reservoir of Helicobacter pylori. Scan J Gastroenterol. 1991; 26: 1205-1208.
5. Dharmani P and Palit G. Exploring Indian medicinal plants for antiulcer activity. Indian J Pharmacol. 2006; 38: 95-99.
6. Dore MP, Osato MS, Malaty HM. Characterization of a culture method to recover Helicobacter pylori from the faeces of infected patients. Eur J Clin Microbiol Infect Dis., 2000; 5: 165-168.
7. Goodman KJ. A Population based study of transmission paths. American J Epidemiology. 1996; 144: 290-299.
8. Hardo PG, Tugnait AF, Lynch DA, West AP, Mapstone NP. Helicobacter pylori infection and dental care. J Periodontol. 1995; 68: 2-6.
9. Hegarty JP, Dowd MT, Baker KH. Occurrence of helicobacter pylori in surface water in the United States. J Appl Microbiol. 1990; 26: 697-701.
10. Honda K, Evans DJ, Abramovitch K, Evans DG, Graham DY. High risk of Helicobacter pylori infection in young Japanese dentists. J Gastroenterol Hepatol. 2001; 16: 862-865.
11. Hu W, Cao C, Menag H, Zhang J, Ma D, Zhang L. Detection and analysis of Helicobacter pylori in oral cavity and stomach from chronic gastritis patients. Scan J Gastroenterol. 2002; 82: 1037-1041.
12. Jones DM, Lessells AM, Eldridge J. Campylobacter like organisms on the gastric mucosa, culture, histological and serological studies. J Clin Pathol. 1984; 37: 1002-1006.
13. Kjoller M, Fischer A, Justesen T. Transport conditions and number of biopsies necessary for culture of Helicobacter pylori. Eur J Clin Infect Dis. 1991; 10: 166-167.
14. Lambert JR, Lins K, Sievert W. High prevalence of Helicobacter pylori, antibodies in an institutionalized population, evidence for person to person transmission. Am J Gastroenterol. 1995; 90: 2167-2171.
15. Lee A. The microbiology and epidemiology of Helicobacter pylori infection. Scan J Gastroenterol. 1994; 29: 2-6.
16. Leverstein MA, Vander EA, Vanmilligen M. Transmission of Helicobacter pylori via feaces. Eur J Clin Microbiol Infect Dis. 1993; 15: 378-382.
17. Madinier IM, Fosse TM, Monteil RA. Oral carriage of helicobacter pylori. J Periodontol. 1997; 68: 2-6.
18. Malaty HM, Evans DJ, Abramovitch K, Evans DG, Graham DY. Helicobacter pylori infection in dental workers. Am J Gastroenterol. 1992; 87: 1728-1731.
19. Michael GN, Henry HT, Perry RK. Carranza's clinical periodontology. 2001; 10: pp 211-214.
20. Neale KR and Logan RPH. The epidemiology and transmission of

Helicobacter pylori infection in children. J Physiol Pharmacol. 1996; 9: 77-84.
21. Newman MG, Takei HH, Klokkevold PR. Carranza's clinical periodontology. 2001; 10 : PP 211-214.
22. Osaki T, Yamaguchi H, Taguchi H. Culturing Helicobacter pylori from clinical specimens. J Pediatr Gastroenterol Nutr. 1997; 36: 615-619.
23. Parsonnet JK, Compton C, Strauss R, Wang T, Kelsey P, Ferraro MJ. Simple microbiologic detection of Campylobacter pylori. J Clin Microbiol. 1998; 26: 948-949.
24. Pytko PJ, Konturek SJ, Karczewska E, Bielanski W, Kaczmarczyk A. Oral cavity as a permanent reservoir of Helicobacter pylori and potential source of reinfection. J Physiol Pharmacol. 1996; 47: 121-129.
25. Robbin S, Abbas M, Farlo T. Basic pathology of bacteria. 2007; 8: pp 314-315.
26. Shames B, Kradjen S, Fuska M. Evidence for the occurrence of the same strain of campylobacter pylori in the stomach and dental plaque. J Clin Microbiol. 1989; 27: 2849-2850.
27. Solankee A and Lad S. Pyrazolines as antibacterial agents. Indian journal of chemistry. 2009; 48: 144-146.
28. Sudhakar U, Anusuya CN, Ramakrishnan T, Vijayalakshmi R. Isolation of Helicobacter pylori from dental plaque, a microbiological study. Journal of Indian society of periodontology. 2008; 12: 67-72.
29. Thomas E, Jiang C, Ferguson Jr DA. The role of the oral cavity in Helicobacter pylori infection. Am J Gastroenterol. 1997; 92: 2148-2154.
30. Thijs JC, Vanzwet JP, Thijis WJ. Diagnostic tests for Helicobacter pylori. Am J Gastroenterol. 1996; 91: 2125-2129.
31. Vanderende A, Rauws EAJ, Feller M, Milder CJ. Heterogenous pylori isolates from members of a family with history of peptic ulcer disease. J Gastroenterol . 1996; 111: 638-647.
32. Vandoorn LJ, Henskens Y, Nouhan TV. The efficacy of laboratory diagnosis of Helicobacter pylori infection. J Clin Microbiol. 2000; 38: 13-17.
33. Vanzwet AA, Thijs JC, Roosendaal R. Practical diagnosis of Helicobacter pylori infection. Eur J Gastroenterol Hep. 1995; 8: 501-507.