CLINICAL AND MICROBIOLOGICAL DETECTION OF BIOFILM IN CHRONIC ULCERS

By

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ABSTRACT

BACKGROUND AND OBJECTIVES: Biofilms have been implicated in delayed healing. . One of the important reasons for delay in healing of an ulcer is due to formation of biofilm. It is estimated that biofilms are associated with 65-80% of non-healing ulcers leading to chronic inflammation and delayed healing. This study was conducted to detect biofilm in the chronic ulcers. To study the effect of biofilm on healing of chronic ulcers

MATERIALS AND METHODS: A prospective hospital based study was carried out on 64 cases diagnosed with chronic ulcers from October 2011 to May 2013 considering the inclusion and exclusion criteria**.**

RESULTS*:* The incidence of biofilm in chronic ulcer is 67.2%. The most common organism isolated were Staphylococcus aureus, Pseudomonas and E. coli. There was significant delay in the wound healing which contained biofilm when compared with ulcers without biofilm.

CONCLUSION*:* The study concludes that biofilm is present in 67.2% of the cases and biofilm causes significant delay in wound healing.

Key words: Chronic ulcer, biofilm, delayed wound healing, risk factors.

ABBREVIATIONS

DOA	:	Date Of Admission
DOD	:	Date of Discharge
IP No	:	In Patient No
DOW	:	Duration of Wound
DOH	:	Duration of Healing
Ppt	:	Precipitating factors
Hb	:	Hemoglobin
ESR	:	Erythrocyte Sedimentation Rate
RBS	:	Random Blood Sugar
Pus C/S	:	Pus for Culture and Sensitvity
DM	:	Diabetes mellitus
HTN	:	Hypertension
LFT	:	Liver function test
CBC	:	Complete blood count
Hb	:	Heamoglobin
i,e.	:	That is

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INTRODUCTION

A wound on the body of a person is a major concern to the patient. Some wounds heal quickly where as some don't. Wounds or ulcers which don't heal within 30-60 days are loosely termed as chronic wounds or ulcers.[1,2] The healing of an ulcer is delayed for many reasons like lack of adequate blood supply, poor sensation, inadequate nutrition and biofilm formation. One of the important reasons for delay in healing of an ulcer is due to formation of biofilm. It is estimated that biofilms are associated with 65-80% of non-healing wounds leading to chronic inflammation and delayed healing.[1]

Biofilm is an aggregation of microbes that manufacture a protective carbohydrate matrix, which allows them to adhere to each other and to a host surface. The matrix shields them from environmental factors that otherwise lead to eradication. [2]There is increasing evidence that bacteria within chronic ulcers live in biofilm communities, in which the bacteria are protected from host defences and develop resistance to antibiotic treatment. [3]The biofilms are resistant to the local treatment by hydrogen peroxide and povidone iodine cleansing as these agents cannot penetrate the biofilm.[4]

The incidence of biofilm in a chronic ulcer is 60% where as in an acute ulcer it is 6%.[1]It has been estimated that biofilms are associated with 65 % of nosocomial infections and that treatment of biofilm-associated infections costs more than $20 billion annually in the United States.[5] Detection of biofilm in chronic ulcers will help in reducing the duration of the healing as well as expenditure towards the healing process. Not many studies have taken place in India, hence the present study aims at detecting the biofilm in chronic ulcers and biofilm induced delayed wound healing.

OBJECTIVES OF THE STUDY

- To detect biofilm in the chronic ulcers.
- To study the effect of biofilm on healing of chronic ulcers

REVIEW OF LITERATURE

Biofilms were probably first recognized in 1684 by Anthony Leeuwenhoek who noticed microbial attachment to his own tooth.[6] It was forgotten for nearly 2 centuries. Later on, in 1978, a concept evolved that these surface associations were the rule (and not the exception) in all nutrient-sufficient microbial ecosystems, and that most bacteria in the biosphere grow in biofilms. [7]

It had been speculated as early as 2001 that bacteria colonizing human chronic wounds exist as biofilm communities. In a clinical study on chronic wounds, chronic wound specimens were obtained from 77 subjects and acute wound specimens were obtained from 16 subjects. Of the 50 chronic wound specimens evaluated by microscopy, 30 were characterized as containing biofilm (60%), whereas only one of the 16 acute wound specimens was characterized as containing biofilm (6%). This was a statistically significant difference ($p<0.001$).[1]

Definition of chronic ulcer

An ulcer that does not heal in an orderly set of stages and in a predictable amount of time the way most ulcers do; ulcers that do not heal within 30-60 days are termed as chronic ulcer. Chronic ulcers seem to be detained in one or more of the phases of wound healing.

The symptoms and signs of the chronic wound are pain, erythema, edema, purulent discharge, increased heat, delayed healing, increased exudates, bright red discoloration of granulation tissue, friable and exuberant tissue, new areas of slough, undermining edges and malodorous serous discharge.

Chronic wounds are rarely seen in individuals who are otherwise healthy. In fact, chronic wound patients frequently suffer from “highly branded” diseases such as diabetes and obesity. This seems to have overshadowed the significance of wounds per se as a major health problem.[8]

The process of wound-healing

Wound healing is a dynamic process consisting of four continuous, overlapping, and precisely programmed phases. The events of each phase must happen in a precise and regulated manner. Interruptions, aberrations, or prolongation in the process can lead to delayed wound healing or a non-healing chronic wound.

In adult humans, optimal wound healing involves the following the events:

(1) Rapid haemostasis;

(2) Appropriate inflammation;

(3) Mesenchymal cell differentiation, proliferation, and migration to the wound site;

(4) Suitable angiogenesis;

(5) Prompt re-epithelialization (re-growth of epithelial tissue over the wound surface); and

(6) Proper synthesis, cross-linking, and alignment of collagen to provide strength to the healing tissue. [9]

The first phase of haemostasis begins immediately after development of wound, with vascular constriction and fibrin clot formation. The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors such as

transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). Once bleeding is controlled, inflammatory cells migrate into the wound (chemo taxis) and promote the inflammatory phase, which is characterized by the sequential infiltration of neutrophils, macrophages, and lymphocytes.[9–11] The most important function of neutrophils is the clearance of invading microbes and cellular debris in the wound area, although these cells also produce substances such as proteases and reactive oxygen species (ROS), which cause some additional bystander damage.

Macrophages play multiple roles in wound healing. In the early wound, macrophages release cytokines that promote the inflammatory response by recruiting and activating additional leukocytes. Macrophages are also responsible for inducing and clearing apoptotic cells (including neutrophils), thus paving the way for the resolution of inflammation. As macrophages clear these apoptotic cells, they undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts, and angiogenesis to promote tissue regeneration[12,13]. In this way, macrophages promote the transition to the proliferative phase of healing.

T-lymphocytes migrate into wounds following the inflammatory cells and macrophages, and peak during the late-proliferative/early-remodelling phase. The role of T-lymphocytes is not completely understood. Several studies suggest that delayed T-cell infiltration along with decreased T-cell concentration in the wound site is associated with impaired wound healing, while others have reported that CD 4+ cells (T-helper cells) have a positive role in wound healing and CD8+ cells (T-suppressor-cytotoxic cells) play an inhibitory role in wound healing. [14,15] Interestingly, recent studies in mice deficient in both T- and B-cells have shown that scar formation is

diminished in the absence of lymphocytes.[16]. In addition, skin gamma-delta T-cells regulate many aspects of wound healing, including maintaining tissue integrity, defending against pathogens, and regulating inflammation. These cells are also called dendritic epidermal T-cells (DETC), due to their unique dendritic morphology. DETC are activated by stressed, damaged, or transformed keratinocytes and produce fibroblast growth factor 7 (FGF-7), keratinocyte growth factors, and insulin-like growth factor-1, to support keratinocyte proliferation and cell survival. DETC also generate chemokines and cytokines that contribute to the initiation and regulation of the inflammatory response during wound healing. While cross-talk between skin gamma-delta T-cells and keratinocytes contributes to the maintenance of normal skin and wound healing, mice, lacking or defective in skin gamma-delta T-cells show a delay in wound closure and a decrease in the proliferation of keratinocytes at the wound site [17,18]

The proliferative phase generally follows and overlaps with the inflammatory phase, and is characterized by epithelial proliferation and migration over the provisional matrix within the wound (re-epithelialization). In the reparative dermis, fibroblasts and endothelial cells are the most prominent cell types present and support capillary growth, collagen formation, and the formation of granulation tissue at the site of injury. Within the wound bed, fibroblasts produce collagen as well as glycosaminoglycans and proteoglycans, which are major components of the extracellular matrix (ECM).

Following proliferation and ECM synthesis, wound healing enters the final remodelling phase, which can last for years. In this phase, regression of many of the newly formed capillaries occurs, so that vascular density of the wound returns to

normal. One important feature of the remodelling phase is ECM remodelling to an architecture that approaches that of the normal tissue. The wound also undergoes physical contraction throughout the entire wound-healing process, which is believed to be mediated by contractile fibroblasts (myofibroblasts) that appear in the wound. [9,19]

FACTORS AFFECTING WOUND HEALING

Multiple factors can lead to impaired wound healing. The factors that influence repair can be categorized into local and systemic. Local factors are those that directly influence the characteristics of the wound itself, while systemic factors are the overall health or disease state of the individual that affect his or her ability to heal. Many of these factors are related, and the systemic factors act indirectly which in turn affect the local factors which aid wound healing.

Systemic Factors That Influence Healing

Age

The elderly population is a risk factor for impaired wound healing Delayed wound healing in the aged is associated with an altered inflammatory response, such as delayed T-cell infiltration into the wound area with alterations in chemokines production and reduced macrophage phagocytic capacity.[15] Delayed re-epithelialization, collagen synthesis, and angiogenesis have also been observed in aged mice as compared with young mice [20].Overall, there are global differences in wound healing between young and aged individuals. A review of the age-related changes in healing capacity demonstrates that every phase of healing undergoes characteristic age-related changes, including enhanced platelet aggregation, increased

secretion of inflammatory mediators, delayed infiltration of macrophages and lymphocytes, impaired macrophage function, decreased secretion of growth factors, delayed re-epithelialization, delayed angiogenesis and collagen deposition, reduced collagen turnover and remodelling, and decreased wound strength.[9]

Stress

The pathophysiology of stress results in the deregulation of the immune system, mediated primarily through the hypothalamic-pituitary-adrenal (HPA) and sympathetic-adrenal medullary axes or sympathetic nervous system (SNS). [21,22]

Diabetes

Diabetes affects millions of people worldwide. Diabetic individuals exhibit a documented impairment in the healing of acute wounds. Moreover, this population is prone to develop chronic non-healing diabetic foot ulcers (DFUs), which are estimated to occur in 15% of all persons with diabetes. A situation of prolonged hypoxia, which may be derived from both insufficient perfusion and insufficient angiogenesis, is detrimental for wound healing. Hypoxia can amplify the early inflammatory response, thereby prolonging injury by increasing the levels of oxygen radicals. Hyperglycemia can also add to the oxidative stress results from a cell or tissue failing to detoxify the free radicals that are produced during metabolic activity. Proteins that are damaged by oxidative stress have decreased biological activity leading to loss of energy metabolism, cell signalling, transport, and, ultimately, to cell death. Hyperglycemia can also add to the oxidative stress when the production of ROS exceeds the anti-oxidant capacity [23]. High levels of metalloproteases are a feature of diabetic foot ulcers, and the MMP levels in chronic wound fluid are almost

60 times higher than those in acute wounds. This increased protease activity supports tissue destruction and inhibits normal repair processes.[24,25]

Several dysregulated cellular functions are involved in diabetic wounds, such as defective T-cell immunity, defects in leukocyte chemo taxis, phagocytosis, and bactericidal capacity, and dysfunctions of fibroblasts and epidermal cells. These defects are responsible for inadequate bacterial clearance and delayed or impaired repair in individuals with diabetes.[24,26]

The neuropathy that occurs in diabetic individuals probably also contributes to impaired wound healing. Neuropeptides such as nerve growth factor, substance P, and calcitonin gene-related peptide are relevant to wound healing, because they promote cell chemo taxis, induce growth factor production, and stimulate the proliferation of cells. A decrease in neuropeptides has been associated with DFU formation. In addition, sensory nerves play a role in modulating immune defense mechanisms, with denervated skin exhibiting reduced leukocyte infiltration[27,28]

Medications

Many medications, such as those which interfere with clot formation or platelet function, or inflammatory responses and cell proliferation have the capacity to affect wound healing. The commonly used medications that have a significant impact on healing include glucocorticoid steroids, chemotherapeutic drugs and non-steroidal anti-inflammatory drugs.

Glucocorticoid Steroids

. Systemic steroids cause delay in wound healing as they impair formation of healthy granulation tissue and reduced wound contraction [(29).]

Chemotherapeutic Drugs

Most chemotherapeutic drugs are designed to inhibit cellular metabolism, rapid cell division, and angiogenesis and thus inhibit many of the pathways that are critical to appropriate wound repair. These medications inhibit DNA, RNA, or protein synthesis, resulting in decreased fibroplasia and neovascularisation of wounds.[(29)] In addition, these agents weaken the immune functions of the patients, and thereby impede the inflammatory phase of healing and increase the risk of wound infection.

Non-steroidal Anti-inflammatory Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) such as ibuprofen are widely used for pain management. There are few data to suggest that short-term NSAIDs have a negative impact on healing. Clinical recommendations suggest that individuals should discontinue NSAIDs for a time period equal to 4 to 5 times the half-life of drugs before surgery.

Obesity

Obesity increases the risk of a number of health conditions including hypertension, adverse lipid concentrations, and type 2 diabetes, dyslipidemia, stroke, sleep apnoea, respiratory problems, and impaired wound healing[(30)]. More than 35%

of U.S. men and women were obese in 2009–2010 as per Centres for Disease Control and Prevention, Atlanta Georgia.

The increase in pressure ulcers or pressure-related injuries in obese individuals is also influenced by hypovascularity, since poor perfusion makes tissue more susceptible to this type of injury. In addition, the difficulty or inability of obese individuals to reposition them further increases the risk of pressure-related injuries. Moreover, skin folds harbour micro-organisms that thrive in moist areas and contribute to infection and tissue breakdown. The friction caused by skin-on-skin contact invites ulceration. Together, these factors predispose obese individuals to the development of impaired wound healing [31–33]

The function of adipose tissue used to be considered as primarily caloric storage. However, more recent findings have documented that adipose tissue secretes a large variety of bioactive substances that are collectively named adipokines. Both adipocytes themselves as well as macrophages inside the adipose tissue are known to produce bioactive molecules including cytokines, chemokines, and hormone-like factors such as leptin, adiponectin, and resistin. Adipokines have a profound impact on the immune and inflammatory response [34–36] The negative influence of adipokines on the systemic immune response seems likely to influence the healing process, although direct proof for this is lacking. Impaired peripheral blood mononuclear cell function, decreased lymphocyte proliferation, and altered peripheral cytokine levels have been reported in obesity. Importantly, though, many of the obesity-related changes in peripheral immune function are improved by weight loss[37–39]

Alcohol

Ethanol exposure can lead to impaired wound healing by impairing the early inflammatory response, inhibiting wound closure, angiogenesis, and collagen production, and altering the protease balance at the wound site.

Smoking

The negative effects of smoking on wound-healing outcomes have been known for a long time [40–42] Nicotine stimulates sympathetic nervous activity, resulting in the release of epinephrine, which causes peripheral vasoconstriction and decreased tissue blood perfusion. Nicotine also increases blood viscosity caused by decreasing fibrinolytic activity and augmentation of platelet adhesiveness. Carbon monoxide aggressively binds to hemoglobin with an affinity 200 times greater than that of oxygen, resulting in a decreased fraction of oxygenated hemoglobin in the bloodstream. Hydrogen cyanide, a component of cigarette smoke, impairs cellular oxygen metabolism, leading to compromised oxygen consumption in the tissues. Beyond these direct tissue effects, smoking increases the individual's risk for atherosclerosis and chronic obstructive pulmonary disease, two conditions that might also lower tissue oxygen tension [41–43]

In the inflammatory phase, smoking causes impaired white blood cell migration, resulting in lower numbers of monocytes and macrophages in the wound site, and reduces neutrophil bactericidal activity. Lymphocyte function, cytotoxicity of natural killer cells, and production of IL-1 are all depressed, and macrophage-sensing of Gram-negative bacteria is inhibited [42,44].

During the proliferative phase of wound healing, exposure to smoke yields decreased fibroblast migration and proliferation, reduced wound contraction, hindered epithelial regeneration, decreased extracellular matrix production, and upset in the balance of proteases [42]

Despite the overall negative effects of smoking, some recent studies have suggested that low doses of nicotine enhance angiogenesis and actually improve healing [45,46]

Nutrition

For more than 100 years, nutrition has been recognized as a very important factor that affects wound healing. Most obvious is that malnutrition or specific nutrient deficiencies can have a profound impact on wound healing after trauma and surgery. Patients with chronic or non-healing wounds and experiencing nutrition deficiency often require special nutrients. Energy, carbohydrate, protein, fat, vitamin, and mineral metabolism all can affect the healing process [47]

Carbohydrates, Protein, and Amino Acids

Together with fats, carbohydrates are the primary source of energy in the wound-healing process. Glucose is the major source of fuel used to create the cellular ATP that provides energy for angiogenesis and deposition of the new tissues.[48]The use of glucose as a source for ATP synthesis is essential in preventing the depletion of other amino acid and protein substrates [47]

Protein is one of the most important nutrient factors affecting wound healing. A deficiency of protein can impair capillary formation, fibroblast proliferation,

proteoglycan synthesis, collagen synthesis, and wound remodelling. A deficiency of protein also affects the immune system, with resultant decreased leukocyte phagocytosis and increased susceptibility to infection .Collagen is the major protein component of connective tissue and is composed primarily of glycine, proline, and hydroxyproline. Collagen synthesis requires hydroxylation of lysine and proline, and co-factors such as ferrous iron and vitamin C. Impaired wound healing results from deficiencies in any of these co-factors[10].

Arginine is a semi-essential amino acid that is required during periods of maximal growth, severe stress, and injury. Arginine has many effects in the body, including modulation of immune function, wound healing, hormone secretion, vascular tone, and endothelial function. Arginine is also a precursor to proline, and, as such, sufficient arginine levels are needed to support collagen deposition, angiogenesis, and wound contraction.[11,48]Arginine improves immune function, and stimulates wound healing in healthy and ill individuals.[49]Under psychological stress situations, the metabolic demand of arginine increases, and its supplementation has been shown to be an effective adjuvant therapy in wound healing[10]

Glutamine is the most abundant amino acid in plasma and is a major source of metabolic energy for rapidly proliferating cells such as fibroblasts, lymphocytes, epithelial cells, and macrophages [11,47] The serum concentration of glutamine is reduced after major surgery, trauma, and sepsis, and supplementation of this amino acid improves nitrogen balance and diminishes immunosuppression. [11]Glutamine has a crucial role in stimulating the inflammatory immune response occurring early in wound healing[47] Oral glutamine supplementation has been shown to improve wound breaking strength and to increase levels of mature collagen [50]

Fatty Acids

Lipids are used as nutritional support for surgical or critically ill patients to help meet energy demands and provide essential building blocks for wound healing and tissue repair. Polyunsaturated fatty acids (PUFAs), which cannot be synthesized de novo by mammals, consist mainly of two families, n-6 (omega-6, found in soybean oil) and n-3 (omega-3, found in fish oil). Fish oil has been widely touted for the health benefits of omega-3 fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The effects of omega-3 fatty acids on wound healing are not conclusive. They have been reported to affect pro-inflammatory cytokine production, cell metabolism, gene expression, and angiogenesis in wound sites[51,52]The true benefit of omega-3 fatty acids may be in their ability to improve the systemic immune function of the host, thus reducing infectious complications and improving survival[47]

Vitamins, Micronutrients, and Trace Elements

Vitamins C (L-ascorbic acid), A (retinol), and E (tocopherol) show potent anti-oxidant and anti-inflammatory effects.

Vitamin C has many roles in wound healing, and a deficiency in this vitamin has multiple effects on tissue repair. Vitamin C deficiencies result in impaired healing, and have been linked to decreased collagen synthesis and fibroblast proliferation, decreased angiogenesis, and increased capillary fragility. Also, vitamin C deficiency leads to an impaired immune response and increased susceptibility to wound infection [11,47]

Similarly, vitamin A deficiency leads to impaired wound healing. The biological properties of vitamin A include anti-oxidant activity, increased fibroblast proliferation, modulation of cellular differentiation and proliferation, increased collagen and hyaluronate synthesis, and decreased MMP-mediated extracellular matrix degradation[53]

Vitamin E, an anti-oxidant, maintains and stabilizes cellular membrane integrity by providing protection against destruction by oxidation. Vitamin E also has anti-inflammatory properties and has been suggested to have a role in decreasing excess scar formation in chronic wounds. Animal experiments have indicated that vitamin E supplementation is beneficial to wound healing[47,53] and topical vitamin E has been widely promoted as an anti-scarring agent. However, clinical studies have not yet proved a role for topical vitamin E treatment in improving healing outcomes[54]

Several micronutrients have been shown to be important for optimal repair. Magnesium functions as a co-factor for many enzymes involved in protein and collagen synthesis, while copper is a required co-factor for cytochrome oxidase, for cytosolic anti-oxidant superoxide dismutase, and for the optimal cross-linking of collagen.

Zinc is a co-factor for both RNA and DNA polymerase, and a zinc deficiency causes a significant impairment in wound healing. Iron is required for the hydroxylation of proline and lysine, and, as a result, severe iron deficiency can result in impaired collagen production[47,48,55]

As indicated above, the nutritional needs of the wound are complex, suggesting that composite nutrition support would benefit both acute and chronic

wound healing. A recent clinical research study examined the effects of a high-energy, protein-enriched supplement containing arginine, vitamin C, vitamin E, and zinc on chronic pressure ulcers and indicated that this high-energy and nutrition-enriched supplement improved overall healing of the pressure ulcer [56] In summary, proteins, carbohydrates, arginine, glutamine, polyunsaturated fatty acids, vitamin A, vitamin C, vitamin E, magnesium, copper, zinc, and iron play a significant role in wound healing, and their deficiencies affect wound healing. Additional studies are necessary to fully understand how nutrition affects the healing response.

Local Factors That Influence Healing

Oxygenation

Oxygen is important for cell metabolism, especially energy production by means of ATP, and is critical for nearly all wound-healing processes.

Due to vascular disruption and high oxygen consumption by metabolically active cells, the microenvironment of the early wound is depleted of oxygen and is quite hypoxic. Several systemic conditions, including advancing age and diabetes, can create impaired vascular flow, thus setting the stage for poor tissue oxygenation. Chronic wounds are notably hypoxic; tissue oxygen tensions have been measured transcutaneously in chronic wounds from 5 to 20 mm Hg, in contrast to control tissue values of 30 to 50 mm Hg [27]

In wounds where oxygenation is not restored, healing is impaired. Temporary hypoxia after injury triggers wound healing, but prolonged or chronic hypoxia delays wound healing[57,58]. In acute wounds, hypoxia serves as a signal that stimulates many aspects of the wound-healing process. Hypoxia can induce cytokine and growth factor

production from macrophages, keratinocytes, and fibroblasts. Cytokines that are produced in response to hypoxia include PDGF, TGF-β, VEGF, tumour necrosis factor-α (TNF-α), and endothelin-1, and are crucial promoters of cell proliferation, migration and chemo taxis, and angiogenesis in wound healing [58]

In normally healing wounds, ROS such as hydrogen peroxide (H_2O_2) and superoxide (O^2) are thought to act as cellular messengers to stimulate key processes associated with wound healing, including cell motility, cytokine action (including PDGF signal transduction), and angiogenesis. Both hypoxia and hyperoxia increase ROS production, but an increased level of ROS transcends the beneficial effect and causes additional tissue damage[58]

One therapeutic option that can sometimes overcome the influence of tissue hypoxia is hyperbaric oxygen therapy (HBOT). [58]While HBOT can be an effective treatment for hypoxic wounds, its availability is limited.

Infections

Once skin is injured, micro-organisms that are normally sequestered at the skin surface obtain access to the underlying tissues. The state of infection and replication status of the micro-organisms determines whether the wound is classified as having contamination, colonization, local infection/critical colonization, and/or spreading invasive infection.

Contamination is the presence of non-replicating organisms on a wound, while colonization is defined as the presence of replicating micro-organisms on the wound without tissue damage. Local infection/critical colonization are an intermediate stage, with micro-organism replication and the beginning of local tissue responses. Invasive

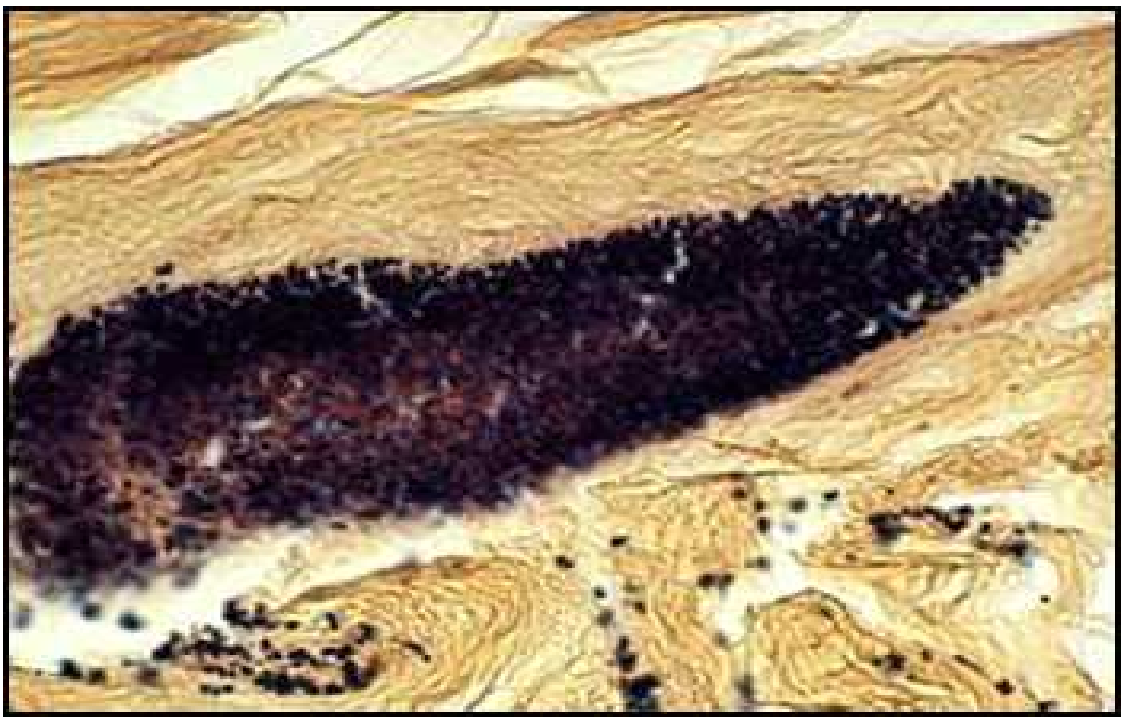
infection is defined as the presence of replicating organisms within a wound with subsequent host injury. [3]

Inflammation is a normal part of the wound-healing process, and is important to the removal of contaminating micro-organisms. In the absence of effective decontamination, however, inflammation may be prolonged, since microbial clearance is incomplete. Both bacteria and endotoxins can lead to the prolonged elevation of pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α and elongate the inflammatory phase. If this continues, the wound may enter a chronic state and fail to heal. This prolonged inflammation also leads to an increased level of matrix metalloproteases (MMPs), a family of proteases that can degrade the ECM. In tandem with the increased protease content, a decreased level of the naturally occurring protease inhibitors occurs. This shift in protease balance can cause growth factors that appears in chronic wounds to be rapidly degraded [3,59].

Similar to other infective processes, the bacteria in infected wounds occur in the form of biofilms, which are complex communities of aggregated bacteria embedded in a self-secreted extracellular polysaccharide matrix (EPS).[3] Mature biofilms develop protected microenvironments and are more resistant to conventional antibiotic treatment. Staphylococcus aureus (S. aureus), Pseudomonas aeruginosa (P. aeruginosa), and β-haemolytic streptococci are common bacteria in infected and clinically non-infected wounds.[3, 60]

P. aeruginosa and Staphylococcus appear to play an important role in bacterial infection in wounds. Many chronic ulcers probably do not heal because of the presence of biofilms containing P. aeruginosa, thus shielding the bacteria from the

phagocytic activity of invading polymorphonuclear neutrophils (PMNs). This mechanism may explain the failure of antibiotics as a remedy for chronic wounds.[61]



Light micrographs of Gram-stained tissue sections from chronic wounds showing biofilms of Gram-positive cocci.

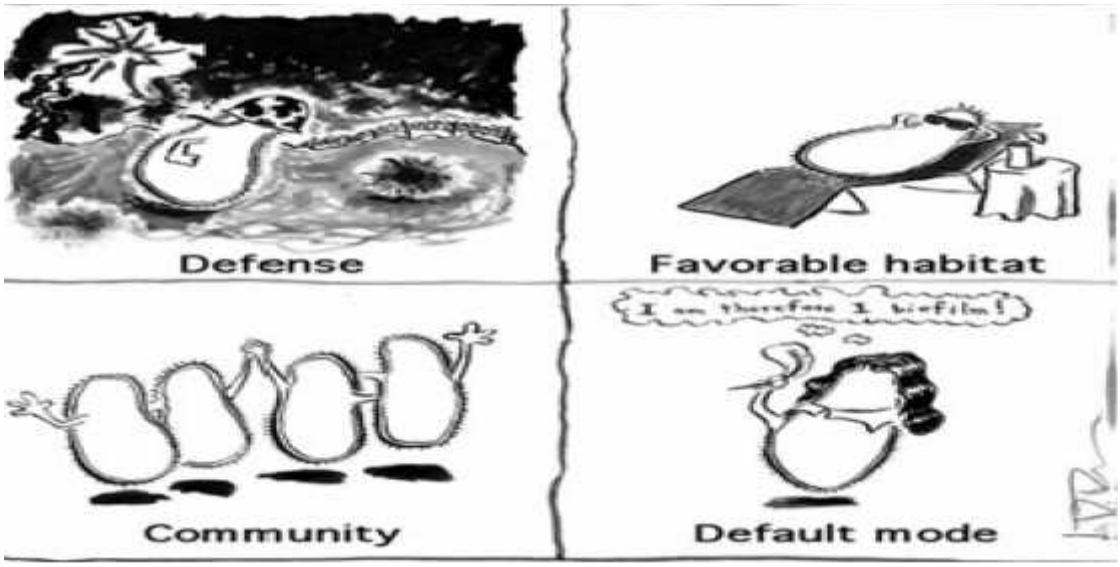
Definition of biofilm

Biofilm is a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription.[62]

Biofilm associated cell is differentiated from other suspended counterparts by reduced growth rate, up and down regulation of gene and regulation of extracellular polymeric matrix.[63]

Formation of biofilm

Four driving forces as depicted by Jefferson[64] are shown below which are necessary for the development of biofilm.



The formation of biofilm confers the bacteria a defense against various antimicrobials and the bacteria reside in favourable habitat undisturbed by the effect of local antimicrobial agents. Together they form a community of favourable organism and form a biofilm.

Development of biofilm

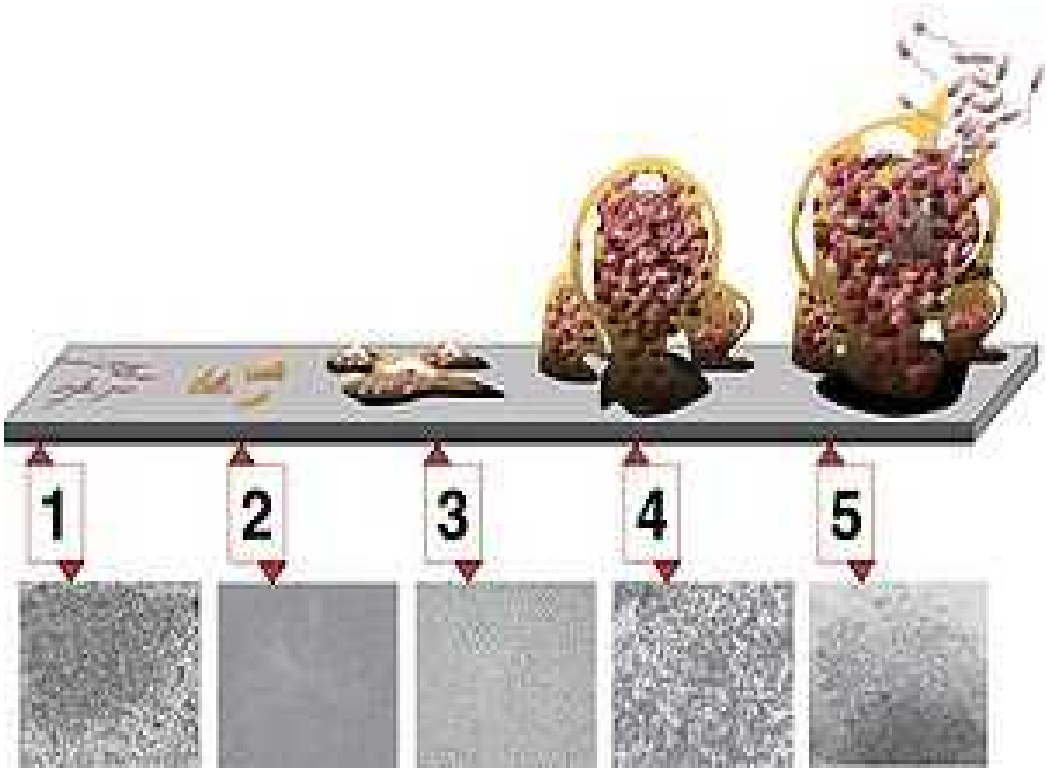
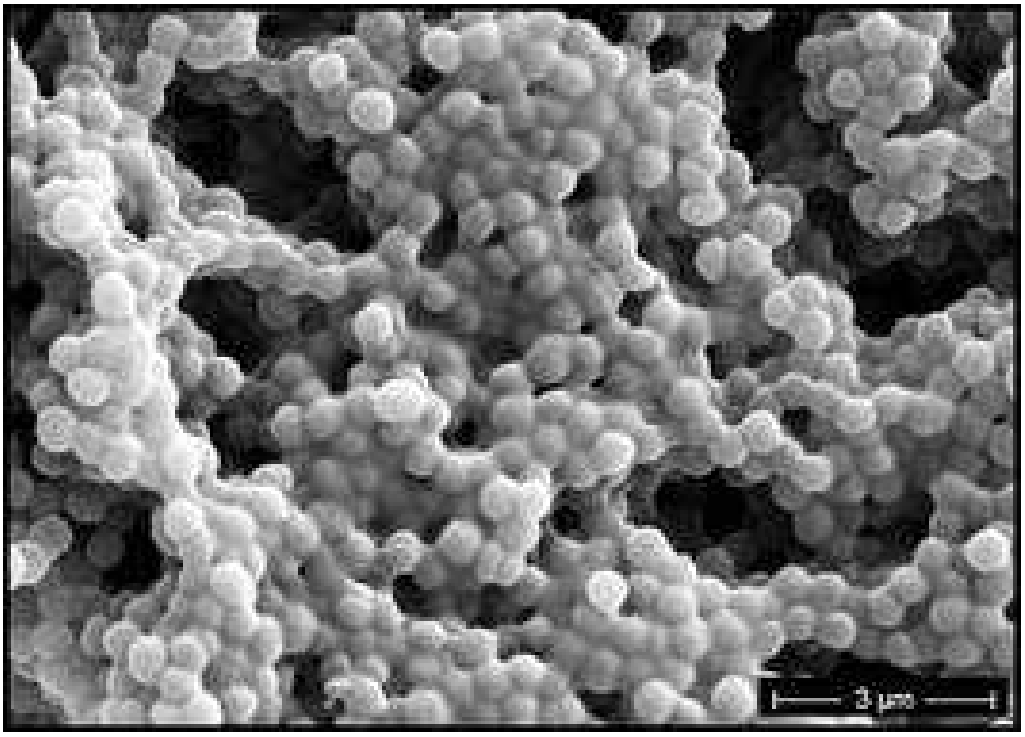


Fig 2:-- Five stages of biofilm development: (1) Initial attachment, (2) Irreversible attachment, (3) Early Maturation, (4) Late Maturation , and (5) Dispersion. Each stage of development in the diagram is paired with a photomicrograph of a developing P. aeruginosa biofilm.

Initial aggregation is probably a default mechanism whereby bacteria stick to each other. Further biofilm formation progresses by adaptation to the available nutritional and environmental conditions.

Any type of aggregation demands a physical attachment or attractive forces between individual particles within an aggregate, or the aggregate will disintegrate, and bacterial aggregates are no exception. It is generally believed that bacteria are immobilized in aggregates by the matrix or EPS components. Extracellular polymeric substances consist of polysaccharides[65–67], extracellular DNA[68–71] and other

macromolecular components such as proteins[72–74] , lipids[75],bio surfactants [76,77], flagella and pili[78,79]. Thus, the matrix has been referred to as the 'house of biofilm cells.[80]. The initial interaction among bacteria, or between bacteria and a surface, is most often mediated via flagella and/or pili. Bacteria in biofilms are then encapsulated in the EPS, which is either produced by the bacteria or sometimes additionally adapted from the host. Extracellular polymeric substances seems to constitute the scaffolding component for bacteria aggregating in the biofilm[68,81] and it acts as a scavenger of free oxygen radicals[82], as well as binding many classes of antibiotics, such as amino glycosides[83]. Apart from this, very little is known about the biofilm matrix, and no complete biochemical profiles exist because different bacteria seem to produce different matrix components.



Electron microscopic picture of biofilm.

Factors favoring biofilm formation

Formation of biofilm initially begins with the attachment of free-floating microorganisms to a surface. If the biofilm is not initially disrupted, it uses cell adhesion structures such as flagella and pili to permanently anchor on to the surface.

The ability of the organisms to adhere to surfaces, as well as the rate of adherence, will influence biofilm formation. Biofilm formation also depends on availability of nutrients. Increased amounts of nutrients enhance the production of quorum-sensing molecules, enzymes, and other essential amino acids necessary for the formation and growth of biofilm and a lack of nutrients causes biofilm to detach and then disperse more efficiently.

Bacteria monitor and respond to the types and amounts of nutrients in their environment. The largest role in biofilm formation belongs to quorum-sensing. It allows bacteria to coordinate gene expression and help the biofilm transition along during the formation process.

Effect of biofilm on human body

Biofilms stimulate a chronic inflammatory response which results in abundant neutrophils and macrophages surrounding biofilms. These inflammatory cells secrete high levels of reactive oxygen species (ROS) and proteases (matrix metalloproteinases (MMPs) and elastase). The proteases can help to break down the attachments between biofilms and the tissue, dislodging the biofilms from the wound. However, the ROS and proteases also damage normal and healing tissues, proteins and immune cells and have ‘off target’ effects that impair healing. The chronic inflammatory response is not always successful in removing the biofilm. The bacterial

biofilm communities interfere with the human immune system in numerous ways. This interference facilitates establishment of further bacterial communities and inflammation of the chronic wound, and prevents healing.[61]

Role of biofilm in delayed wound healing

In 2007, an in vitro multispecies Lubbock chronic wound biofilm model was proposed. They noticed that multispecies biofilms were becoming increasingly recognized as the naturally occurring state in which bacteria reside. One of the primary health issues that was recognized to be exacerbated by biofilms are chronic, nonhealing wounds such as venous leg ulcers, diabetic foot ulcers, and pressure ulcers. Three of the most important species associated with multispecies biofilms were Pseudomonas aeruginosa, Enterococcus faecalis, and Staphylococcus aureus.[84]

The biofilm mode of growth confers on the associated organisms a measurable decrease in antimicrobial susceptibility. For example, Ceri et al. found that biofilm-associated Escherichia coli required 1500 times the MIC of ampicillin to provide a 3-log reduction.[85] Williams et al. found that Staphylococcus aureus biofilms required 110 times the MBC of vancomycin to provide a 3-log reduction.[86]The effect on susceptibility may beintrinsic (i.e., inherent in the biofilm mode of growth) or acquired (i.e., caused by the acquisition of resistance plasmids).

There are at least 3 reasons for the intrinsic antimicrobial resistance of biofilms.

1. Antimicrobial agents must diffuse through the EPS matrix to contact and inactivate the organisms within the biofilm. EPSs retard diffusion either by chemically reacting with the antimicrobial molecules or by limiting their rate of transport. Hoyle et al. showed that the EPSs of Pseudomonas aeruginosa were capable

of binding tobramycin; dispersed cells were 15 times more susceptible to this agent than were cells in intact biofilms.

2. Biofilm-associated organisms have reduced growth rates, minimizing the rate that antimicrobial agents are taken into the cell and therefore affecting inactivation kinetics.[87] DuGuid et al. found that an increase in growth rate resulted in an increase in susceptibility of Staphylococcus epidermidis biofilms[88]. DuGuid et al. also showed that ciprofloxacin activity was influenced by the cell cycle; newly formed daughter cells were more susceptible than other populations in the biofilm.

3. The environment immediately surrounding the cells within a biofilm may provide conditions that further protect the organism.[89]Tresse et al found that agar-entrapped E. coli demonstrated a decreased susceptibility to aminoglycoside antibiotics as a result of decreased uptake of the antibiotic by the oxygen-deprived cells.[90]

Biofilms reside within the chronic wound and represent an important mechanism underlying the observed, delayed healing and infection. The reasons for this include both protease activity and immunological suppression. Furthermore, a lack of responsiveness to an array of antimicrobial agents has been due to the biofilms' ability to inherently resist antimicrobial agents[91]

A growing body of evidence suggests that in addition to hypoxia, ischemia-reperfusion injury, and intrinsic host factors, bacterial biofilms represent a fourth major pillar in chronic wound pathogenesis.

In a study done by Gurjala AN etal , dermal punch wounds were created in New Zealand rabbit ears, and used as uninfected controls, or inoculated with green fluorescent protein-labelled Staphylococcus aureus to form wounds with bacteria

predominantly in the planktonic or biofilm phase. Epifluorescence and scanning electron microscopy revealed that S. aureus rapidly forms mature biofilm in wounds within 24 hours of inoculation, with persistence of biofilm viability over time seen through serial bacterial count measurement and laser scanning confocal imaging at different time points post wounding and inoculation. Inflammatory markers confirmed that the biofilm phenotype creates a characteristic, sustained, low-grade inflammatory response, and that over time biofilm impairs epithelial migration and granulation tissue in-growth, as shown histologically[92]

Diabetic patients exhibit dysregulated inflammatory and immune responses that predispose them to chronic wound infections and the threat of limb loss. Diabetic wounds had significantly less neutrophil oxidative burst activity. This translated into a log-fold greater bacterial burden and significant delay of wound epithelisation for biofilm-impaired diabetic wounds at 10 days post wounding[93]

The in vivo antimicrobial assay was used to demonstrate that both mupirocin cream and the triple antibiotic ointment were effective in reducing planktonic S. aureus but had reduced efficacy against biofilm-embedded S. aureus. These biofilm-like communities also demonstrated increased antimicrobial resistance when compared with their planktonic phenotype in vivo[60]

Biofilms have been associated with chronic infections in wounds because these organisms often resist host mechanisms and antimicrobial interventions.[1,61] The biofilm organisms are encased in extracellular polymeric matrix and are able to resist phagocytic action and impede the action of the host immune system and antimicrobials.[61,94] Quorum-sensing molecules are required for biofilm formation and increase production of virulence factors such as cytotoxic enzymes. Increased

production of toxins further drains the immune mechanism of the patient and reduces the healing process. In 2007, Loryman and Mansbridge observed the effect of quorum-sensing molecules on inhibition of keratinocyte migration.[95] All of these factors explain the inability of wounds with biofilms to heal.

Furthermore, biofilms frequently show resistance towards antimicrobials. Organisms within biofilm are able to resist antimicrobials through various mechanisms due to the architecture and composition of biofilm. Biofilm forms and proliferates rapidly; the turnover rate is quite slow. A period of 24 to 48 hours is required for biofilm formation. This is a factor that enhances its ability to resist host immune mechanisms and antimicrobial interventions.

Resistance starts at the 'attachment' phase and increases as biofilm develops. The components of extracellular polysaccharides (EPS) of biofilm act as a barrier and physically restrict diffusion of antimicrobial agents into the biofilm, thus protecting the organisms from the effect of antimicrobials[94]

Another reason for antimicrobial resistance can be due to close cell-to cell contact that permits bacteria to transfer plasmids to one another more effectively than in the planktonic state. These plasmids can then encode for resistance to several different antimicrobial agents. [95] Also, the heterogenous environments within biofilm such as pH, oxygen tension, and other chemical substances have been shown to reduce the activities of antimicrobials.[96]

The biofilm also provides a physical protection to bacteria because antimicrobial agents are also ineffective at penetrating the biofilm, decreasing the concentration acting on the bacterial cells within the biofilm and therefore their efficacy.[97] In addition to resistance to antimicrobials, biofilms also appear to have an antiphagocytic property which makes the leukocytes within the matrix ineffective.[94]

Detection of biofilm

Early biofilm formation detection might result in a greater success in the treatment, because in long standing cases, they may be very damaging and may produce immune complex sequelae.[98]

There are two methods for the detection of biofilms –

1. The Phenotypic method

a. The tissue culture plate (TCP) method – The wells of the tissue culture plates are inoculated with a bacterial suspension along with positive and negative controls and these are incubated for 24 to 48 hours. Planktonic cells are removed by washing with phosphate buffered saline. Biofilms are fixed with 2% sodium acetate and are stained with 0.1% crystal violet. The excess dye is washed away with deionised water. The plates are dried properly and the optical densities of the stained biofilms are obtained spectrophotometrically.

b. The tube method(TM) – 10 ml of Tripticase soy broth with 1% glucose is inoculated with a loopful of test organisms, along with positive and negative controls. The broths are incubated at for 24 – 48 hours. The culture supernatants are decanted and the tubes are washed with phosphate buffered saline. The tubes are dried and are stained with 0.1% crystal violet. The excess stain is washed away with deionised water. The tubes are dried in an inverted position.

c. The Congo red agar (CRA) method – The Congo red stain is prepared as a concentrated aqueous solution and is autoclaved at 121 ^{0} C for 15 minutes. This is added to autoclaved Brain heart infusion agar with sucrose at 55^{0} C. The plates are

inoculated with the test organisms along with positive and negative controls and are incubated at 37^0 C for 24 to 48 hours aerobically. Black colonies with a dry crystalline consistency indicate biofilm production.

Various studies have established that TCP is a better screening test for biofilm production than the TM and the CRA methods. The test is easy to perform and to assess biofilms, both qualitatively and quantitatively.[99]

2. The Genotypic method

Sonications and PCR amplification methods have been shown to improve the detection of biofilms. Biofilm non producers are negative for ica A and ica D and lack the entire ica ADBC operon. But this requires specialized equipments and techniques[100]

In University of Estadual Paulista, Department of Microbiology and Immunology, Biosciences Institute Bacteriology Laboratory, Botucatu, SP, Brazil "a comparative study of different methods to detect biofilms was conducted which showed that the sensitivity and specificity of the tube adherence test were 100% sensitivity and 100% specificity of the tube test when compared to PCR (concomitant presence of the icaA and icaD or icaACD genes).[63]

Safranin, ConA, and immunofluorescent staining with confocal laser scanning microscopy (CLSM) was used to study and demonstrate the presence of S. aureus biofilms in specimens collected from patients with the skin diseases bullous impetigo, atopic dermatitis, and pemphigus foliaceus[100]

In 2009, in a study, fluorescent in situ hybridization (FISH) was used in combination with CSLM to detect and characterize the spatial distribution of biofilm-forming bacteria which predominate within human chronic skin wounds (Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus species and Micrococcus species.). The application of this standardized procedure makes available an assay for identification of single- or multi-species bacterial populations in tissue biopsies.[101]

Management of biofilm

It is tempting for the clinician to start antibiotic treatment, but in case of established, mature biofilm this treatment often has only temporary effect on both inflammation and healing. In addition the clinician has to rely on the results from a swab or biopsy, which rarely reflects all specimens present in the wound. The bacteria in biofilm are up to 1000 times less susceptible to antibiotics and MIC is not reached in the chronic wound fluid. Even silver treatment, as incorporated in several wound dressings, has limited effect in biofilm in vitro. With this in mind the clinician should exercise restraint in admission of antibiotics. Administering antibiotics favours biofilm capable bacteria and promotes resistance to the administered antibiotic. Mechanical removal of wound debris and even granulation tissue is an effective way of diminishing the bacterial load and is an important part of treatment protocols.

The application of antimicrobials in the management of wounds is a complex procedure requiring appropriate clinical decision making, judgment and a thorough understanding of antimicrobial therapies, together with their potential disadvantages. There is considerable direct and indirect evidence for the presence of bacterial biofilms in the chronic wound bed, and it has been demonstrated that bacteria within these biofilms may exhibit both specific and nonspecific antimicrobial tolerance. The

antimicrobial tolerance of biofilms is a major concern in the treatment of both infected and nonhealing chronic wounds and an understanding of the mechanisms involved is of fundamental importance in managing wound infections and developing future wound management strategies.[137]

It must to be noted that biofilm antibiotic tolerance should not be confused with antibiotic resistance because, although bacteria within a biofilm tend to survive antibiotic treatment, they become susceptible to the treatment when the biofilm is disrupted[123]

Topical negative pressure dressing was effective in killing the tested bacteria evident in both the tested mono and polymicrobial biofilms, which provides valuable evidence that this dressing may have an effect on biofilms found in recalcitrant chronic wounds.[80]

Even in the case of extensive surgical debridement in combination with split skin transplant the presence of P. aeruginosa prior to surgery seams to influence the healing (Hoegsberg et al., unpublished results). This indicates that the bacteria reside deep in what is thought to be normal tissue, probably protected in biofilm.

TIME acronym for Tissue, Infection/Inflammation, Moisture, Edge was developed in 2002 by a group of wound care experts, as a practical guide for use when managing patients with wounds.[105] The TIME comprises of four main components of wound bed preparation:

1. Tissue management
2. Control of infection and inflammation
3. Moisture imbalance
4. Advancement of the epithelial edge of the wound.

- **Tissue management**

  Wound healing is delayed whenever a tissue is non-viable or deficient. It also provides a focus for infection, prolongs the inflammatory response, mechanically obstructs contraction and impedes re-epithelialisation.[106] Repeated debridement should be done to make the margins of the ulcer healthy.

  If debridement is effective, the T of TIME is removed and wounds can progress through the remaining phases of wound healing.

- **Control of infection and inflammation**

  All wounds contain bacteria at levels ranging from contamination, through critical colonisation (also known as increased bacterial burden or occult infection), to infection. The infection should not be treated aggressively with systemic antibiotics as there is increase tolerance or resistance to antibiotics. Only if there are signs of acute infection, then it should be treated with antibiotics. The best treatment would be mechanical debridement.

- **Moisture balance**

  Creating a moisture balance at the wound interface is essential if wound healing is to be achieved. Exudate is produced as part of the body's response to tissue damage and the amount of exudate produced is dependent upon the pressure gradient within the tissues A wound which progresses through the normal wound healing cycle produces enough moisture to promote cell proliferation and supports the removal of devitalized tissue through autolysis. If, however, the wound becomes inflamed and gets stuck in the inflammatory phase of healing, exudate production increases as the blood vessels dilate. If a wound bed becomes too dry, however, a crust will form which then impede healing and wound contraction. The underlying collagen matrix and the surrounding tissue at the wound edge become desiccated [107]

If a wound produces excessive amounts of exudate the wound bed becomes saturated and moisture leaks out onto the peri-wound skin causing maceration and excoriation. This in turn could lead to an increased risk of infection.

- **Edge -Advancement of the epithelial edge of the wound.**

The final stage of wound healing is epithelialization, which is the active division, migration, and maturation of epidermal cells from the wound margin across the open wound. There are many factors which need to be present in order for epithelialization to take place. The wound bed must be full of well vascularised granulation tissue in order for the proliferating epidermal cells to migrate. The vascularised granulation tissue also ensures that there is adequate oxygen and nutrients to support epidermal regeneration. There should be a rich source of viable epidermal cells which can undergo repeated cell division particularly at the edge of the wound,where cells have become senescent the process slows down or stops completely. Wounds that have a significant number of fibroblasts that are arrested due to senescence, damaged DNA or enduring quiescence do not heal.[108]

Other factors, such as bacteria or the presence of devitalised tissue, which interfere with epidermal cell growth should be absent.

In 2007, a study of biofilm-based wound management in subjects with critical limb ischemia; it was found that when they compared the healing frequency in this study with a previously published study, Biofilm Based Wound Control strategies significantly improved healing frequency. These findings demonstrate that effectively managing the biofilm in chronic wounds is an important component of consistently transforming 'non-healable' wounds into healable wounds.[112]

Economic burden of biofilm

Chronic wounds represent a silent epidemic that affects a large fraction of the world population and poses major and gathering threat to the public health and economy of the United States. In developed countries, it has been estimated that 1 to 2% of the population will experience a chronic wound during their lifetime [(109)]. In the United States alone, chronic wounds affect 6.5 million patients.[(110)] In the Scandinavian countries, the associated costs account for 2–4% of the total health care expenses.[(111)]

The burden of treating chronic wounds is growing rapidly due to increasing health care costs, an aging population and, in the United States and beyond, a sharp rise in the incidence of diabetes and obesity worldwide. It is claimed that an excess of US$25 billion is spent annually on treatment of chronic wounds[(112)]. To that add the rapidly expanding need for wound care of our veterans, and the need to prioritize wound care and research would appear to be compelling. At present, over 1000 outpatient wound centres are in operation in the United States, not including all the wound care rendered by clinicians in their offices, by inpatient acute care hospitals, long term facilities and nursing homes. According to a new report by Global Industry Analysts, the annual wound care products market would reach $15.3 billion by 2010. The United States represents the worlds largest and the fastest growing market. The amount of money spent on wound care, the loss of productivity for afflicted individuals and the families that care for them and their diminished quality of life come at great cost to our society.[(8)]

The cost to the NHS of caring for patients with a chronic wound is conservatively estimated at 2.3billion–3.1billion per year (at 2005–2006 costs),

around 3% of the total estimated out-turn expenditure on health (89.4billion) for the same period.[(113)]

MATERIALS AND METHODS

SOURCE OF DATA

All patients attending the surgery OPD &/or admitted patients in Shri B. M. Patil Medical College Hospital & Research Centre, Bijapur with symptoms / clinical features of chronic ulcer during the period of October 2011 to May 2013 were taken for the study.

METHOD OF COLLECTION OF DATA:

Patients with symptoms and/ or clinical features of chronic ulcer were taken up for study.

History of patients was noted.

Sampling:

It was a prospective study. The time period of this study was from October 2011 to May 2013.

Sample size was calculated using formula

$$n = \frac{(Z\alpha)^2 pq}{e^2}$$

n = sample size,

$Z\alpha$ = Z score

p = prevalence

q = 1- prevalence

e = sampling error

As the prevalence is 60% and for an allowable sampling error of 10%, the Z score is 1.642 with 90% confidence interval and therefore the sample size is 64.

Statistical analyses were done using

a) Diagrammatic presentation
b) Mean +/- SD
c) T test
d) Z test or Chi Square test

Inclusion criteria

1. Patients having chronic ulcer.
2. Patients having diabetic foot ulcers, venous ulcers and pressure ulcers.

Exclusion criteria

1. Patients whose ulcer was less than 30 days.
2. Patients having malignant skin lesions.

Investigations or interventions required in this study were routine standardized procedures.

Investigations

Blood:

a) Hb%
b) TC
c) DC
d) ESR

Urine:

a) Albumin
b) Sugar
c) Microscopy

Tests for HIV and HBSAg (done for universal precautions)

Random blood sugar:

X ray of the part involved.

Pus for Gram stain,

Culture and sensitivity

Biochemical test

a) Indole test,
b) Methyl red test,
c) Voges-Proskauer test,
d) Citrate test,
e) Triple sugar iron test,
f) Urease test
g) Catalase test,
h) Coagulase test,
i) Nitrate reduction test.

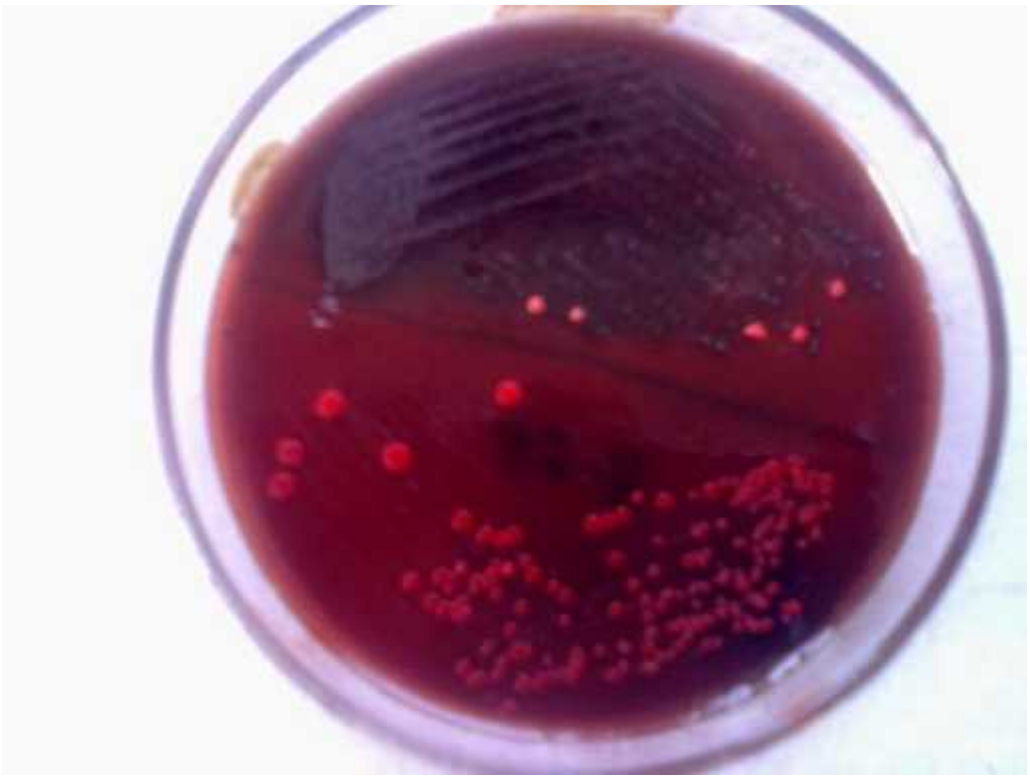
Tube adherence method to detect biofilm

PROCEDURE

Informed consent from the patient was taken prior to the study.

The ulcer was inspected for a transparent membrane and pus. It was excised and/ or pus was collected in a sterile container and transported to the microbiology department and processed further immediately. In those cases, where the pus specimen was not available, the specimens were be collected with sterile swab and processed immediately. The specimens were inoculated on blood agar and Mc Conkey agar, incubated at 37^0 C for 18-24 hrs. The plates were observed for colony morphology and the bacterial isolates further identified by conventional method using biochemical tests such as Indole test, Methyl Red test, Voges-Proskauer test, Citrate test, Triple Sugar Iron test, Urease, Catalase, Coagulase, Nitrate Reduction test. The isolates were further subjected to antimicrobial susceptibility testing by disc diffusion technique according to CLSI guidelines.

The isolates were further tested for biofilm by tube method[16], which is a qualitative method for biofilm detection. A loopful of test organisms was inoculated in 10 mL of trypticase soy broth with 1% glucose in test tubes. The tubes were incubated at 37ºC for 24 hours. After incubation, tubes were decanted and washed with phosphate buffer saline (pH 7.3) and dried. Tubes were then stained with crystal violet (0.1%). Excess stain was washed with deionized water. Tubes were dried in inverted position. The scoring for tube method will be done according to the results of the control strains. Biofilm formation was considered positive when a visible film lined the wall and the bottom of the tube. The amount of biofilm formed was scored as 1-weak/none, 2-moderate and 3-high/strong. The experiment was performed in triplicate and repeated three times.



The above photograph shows the growth of the colonies obtained from a chronic ulcer on a blood agar plate.

The above photograph shows the biofilm which has coated the walls of the test tube.

(violet colour).

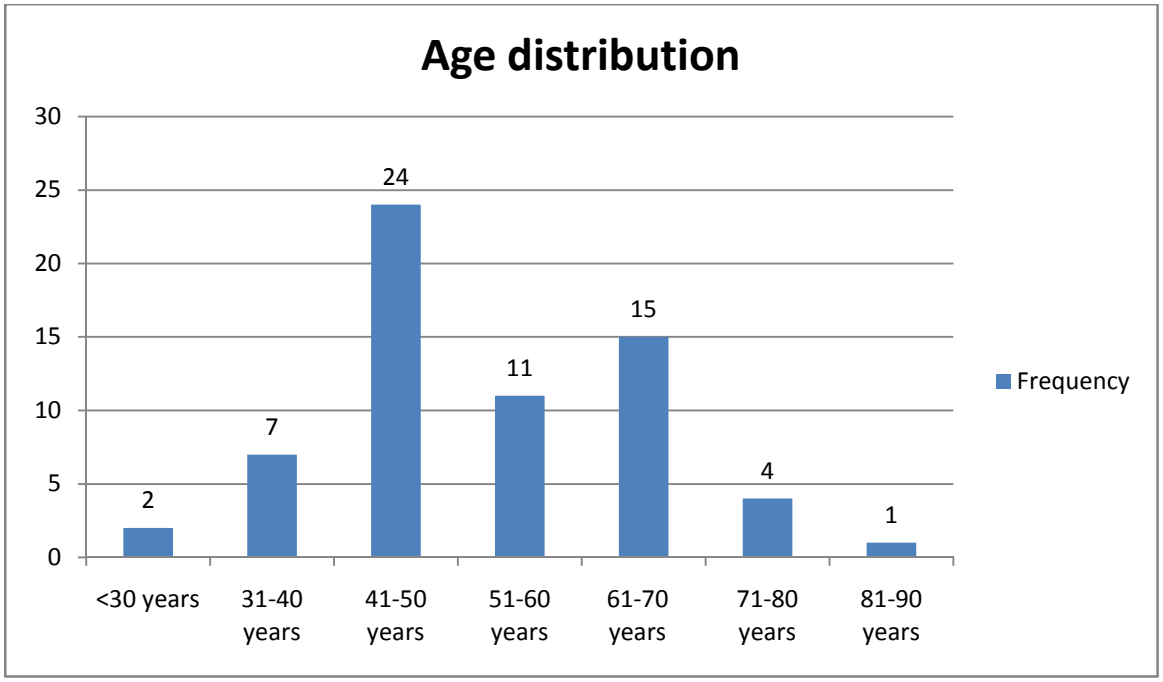
OBSERVATIONS AND RESULTS

The observations made in the current study and the inferences drawn are highlighted in the following pages.

Table 1: Age distribution of cases in years

Age	Frequency	Percent	Cumulative Percent
<30	2	3.1	3.1
31-40	7	10.9	14.1
41- 50	24	37.5	51.6
51-60	11	17.2	68.8
61-70	15	23.4	92.2
71-80	4	6.3	98.4
81-90	1	1.6	100.0
Total	64	100.0	

Most of the patients were between 41-70 years of age (n= 50). The mean age of the patients was 60yrs.

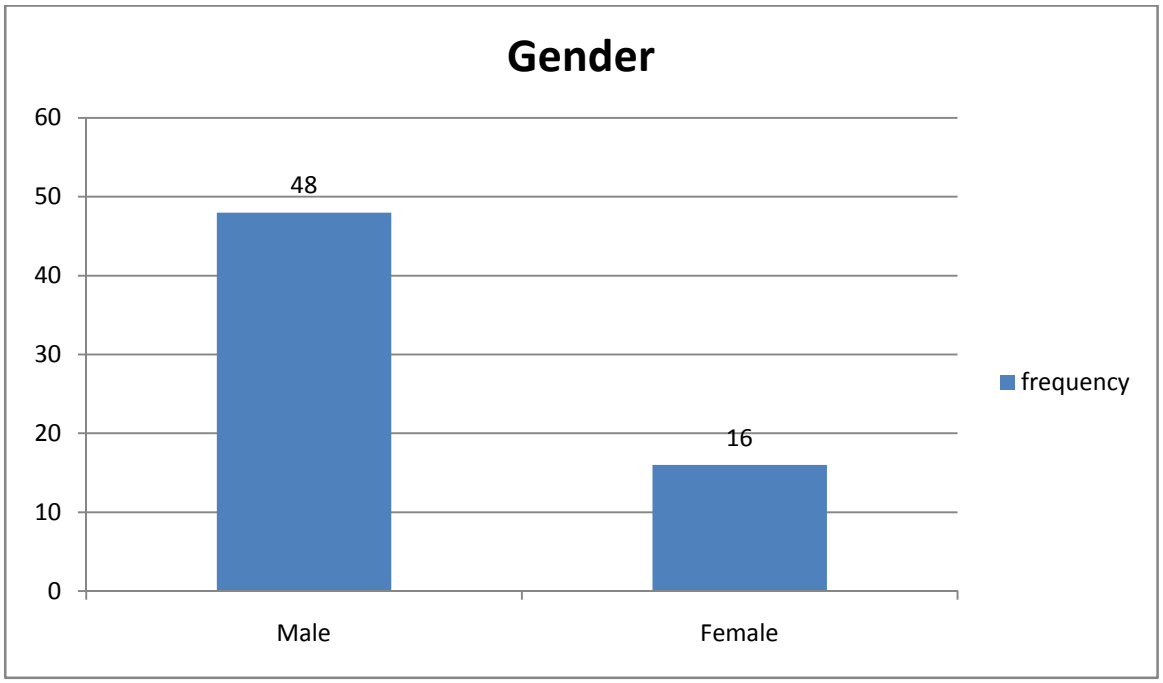


Graph 1:- Shows distribution of cases by age

Table 2: Showing distribution of cases according to sex.

Gender	Frequency	Percent
Male	48	75
Female	16	25

The above table shows that the predominant patients were male i.e 48 patients out of 64 patients had chronic wound.



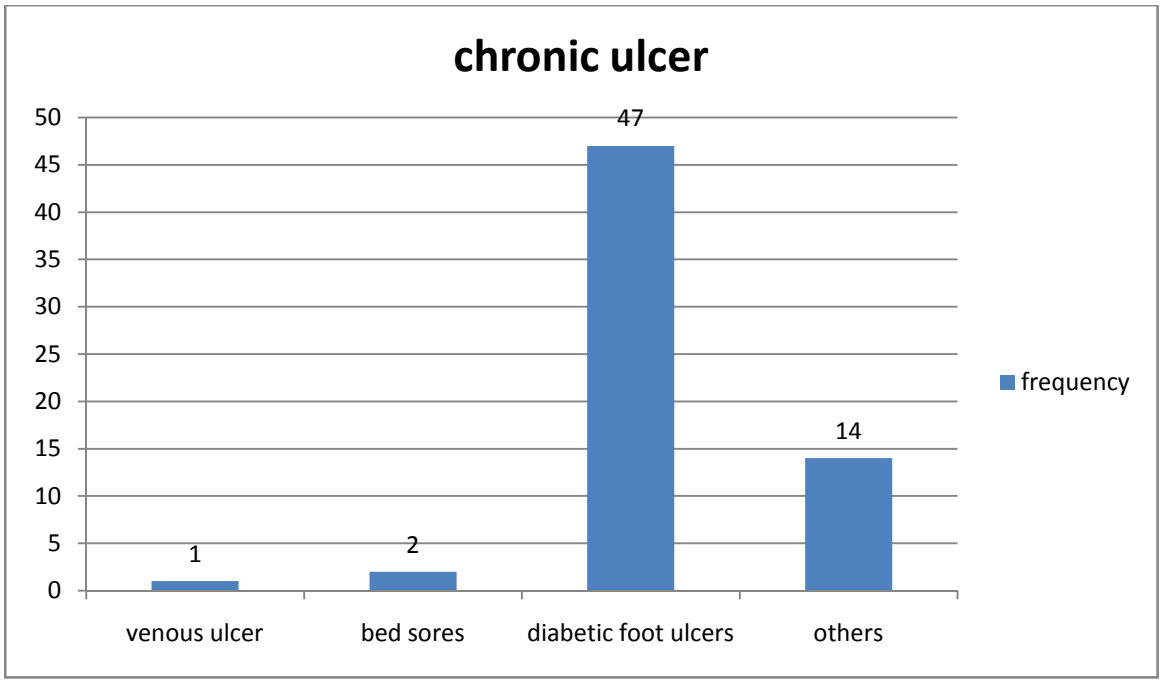
Graph 2:- Shows distribution of cases by gender

The above graph shows that males are predominantly having chronic ulcer.

Table 3 Showing the pattern of distribution of causes of chronic wound

Chronic wound	Frequency	Percentage
Venous ulcer	1	1.56
Bed sores	2	3.12
Diabetic foot ulcers	47	73.43
Others	14	21.89

Out of the 64 patients having chronic ulcer , 47 were diabetic, 2 had bed sores, 1 had venous ulcer.

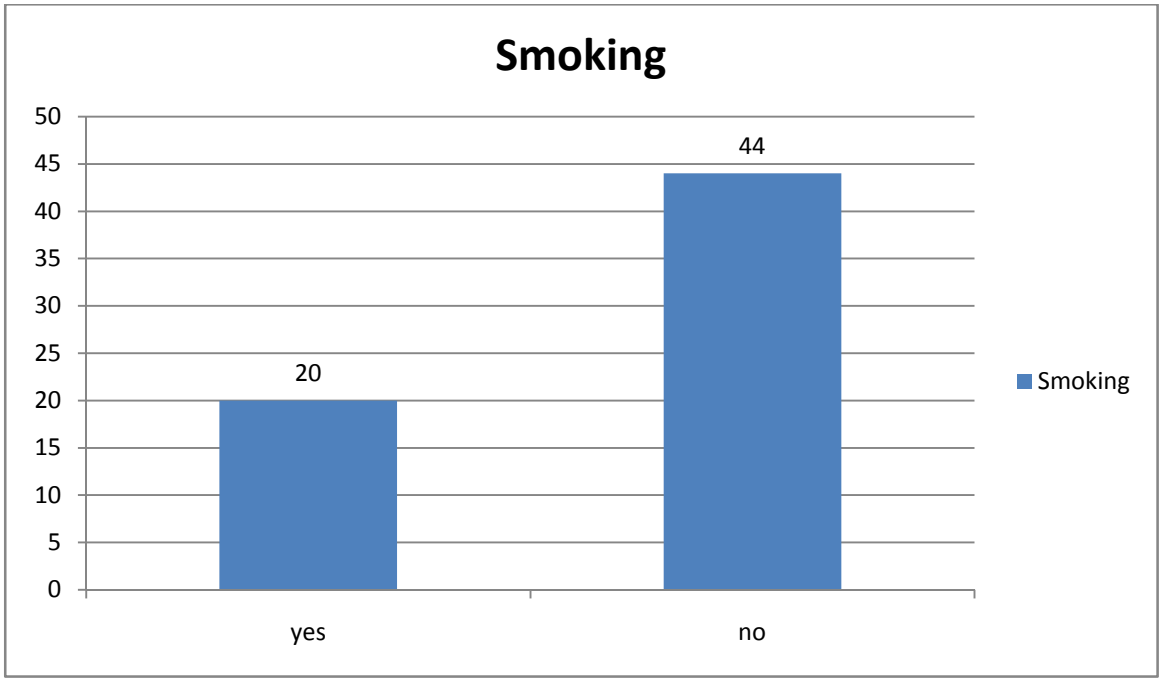


Graph 3 showing the pattern of distribution of chronic ulcers according to cause

Table 4: Showing the distribution of cases among the smokers

Smokers	Frequency	Percent
Yes	20	31.3
No	44	68.6

The above table shows that the most of the patients were non smokers.

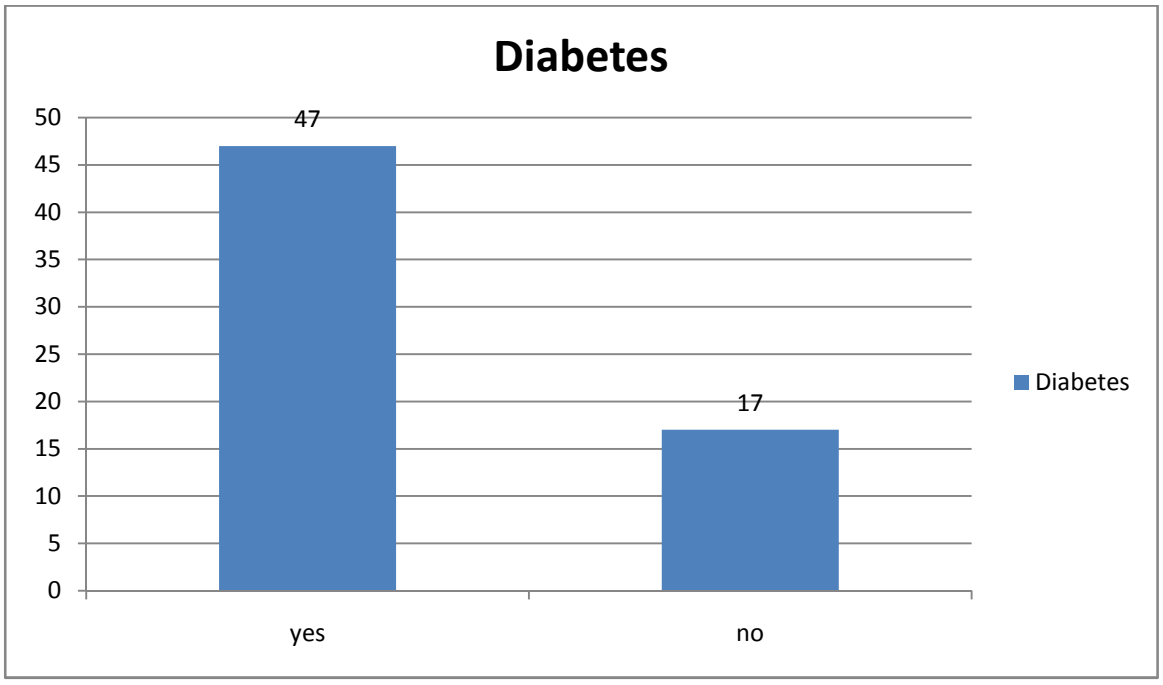


Graph 4: The above graph shows smoking pattern of the patients with chronic ulcer.

Table 5: Showing the occurrence of diabetes mellitus in chronic ulcer.

Diabetes	Frequency	Percent
Yes	47	73.4
No	17	26.6

The above table shows that most of the patients who had a chronic wound had diabetes mellitus i.e 73.4% of the cases.



Graph 5 The above graphs the pattern of distribution among patients based on the presence or absence of diabetes mellitus.

Table 6 Showing the duration of healing with diabetes mellitus

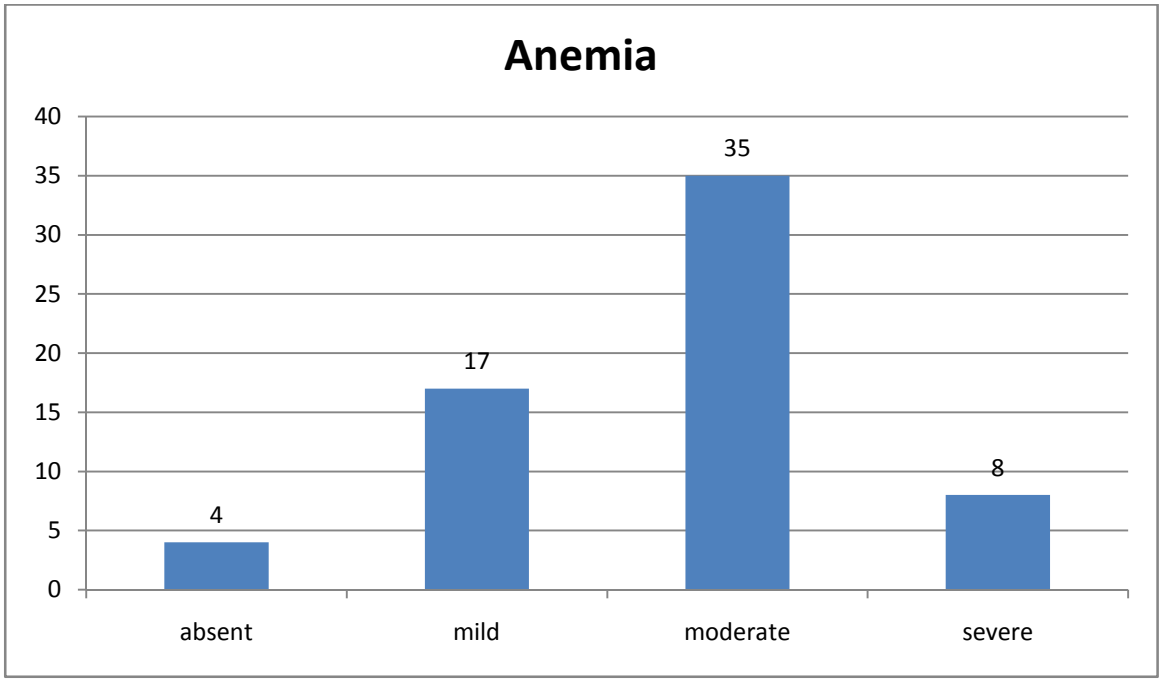
Diabetes	Mean duration of healing in days	P value
Yes	53.21	0.0175
No	43.1	

The mean duration of the healing of the ulcers in diabetic patients was 53.21 days when compared to non diabetics ie 43.1 days, which was clinically significant (p value = 0.0175)

Table 7 shows the presence of anemia in chronic ulcer patients.

Variable		Frequency	Percent
Anaemia	Absent	4	6.3
	Mild	17	26.6
	Moderate	35	54.7
	Severe	8	12.5

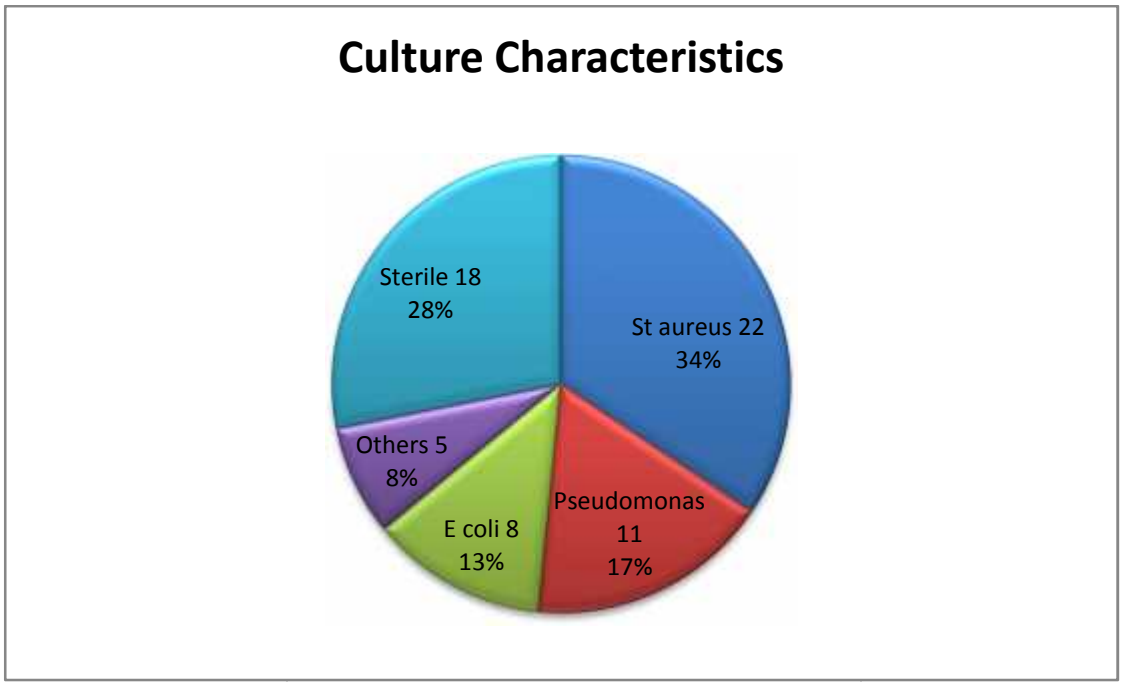
The above table shows that most of the patients had anemia. And 54.7% of the patients had moderate anemia, 26.6% had mild anemia and 12.5% had severe anemia.



Graph 6 : The above graph shows the degree of anemia in chronic ulcers

Table 8 : Showing the isolated bacteria on culture studies of chronic ulcers.

Organisms	**Number**	**Percentage**
Staphylococcus aureus	22	34
Pseudomonas aeruginosa	11	17
Eschreschia coli	8	12.5
Klebsiella oxytoca	3	4.5
Citrobacter	1	1.5
Proteus vulgaris	1	1.5
Sterile	18	28

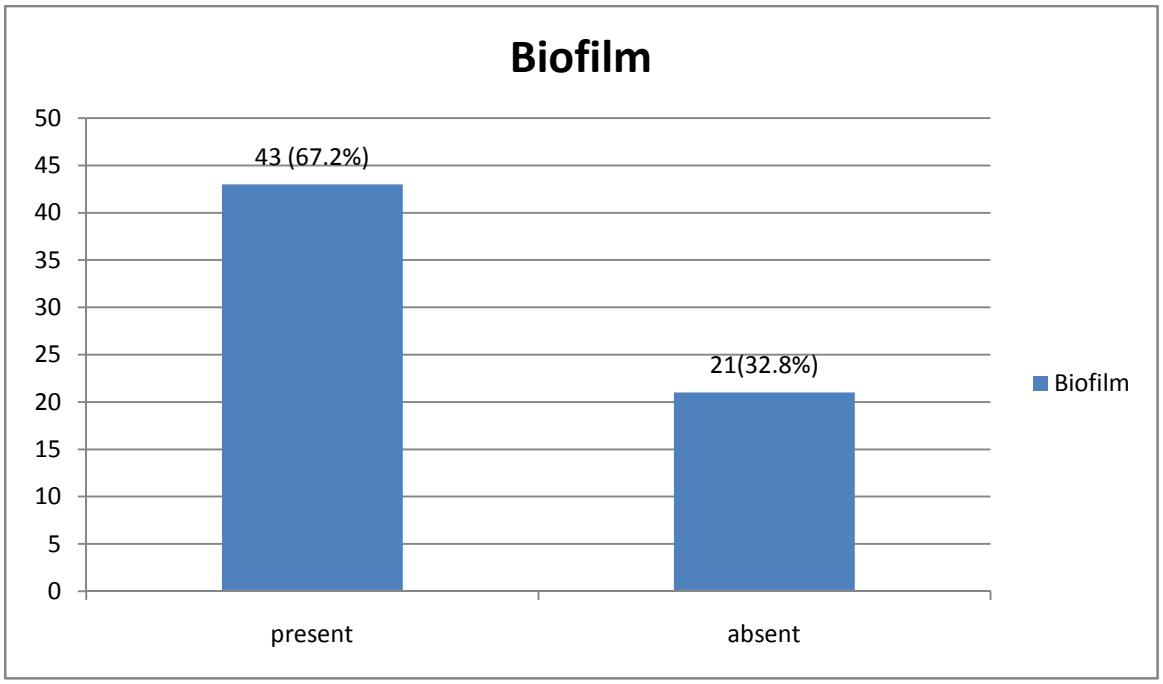


Graph : 7 The above table and graph show that the most common organism isolated was S aureus, followed by Pseudomonas and E coli.

Table 9: Tube test to detect biofilm

	Result	Frequency	Percent
Tube test	Positive	43	67.2
	Negative	21	32.8

The above table shows that 43 patients out of 64 patients ie 67.2 % of the patients having chronic ulcers had biofilm.



Graph 8 : Showing the results of the tube test.

Table 10: Tube test and duration of healing

Tube test	Mean duration in days	P value
Positive	52.31	0.002
Negative	42.56	

The above table shows that if the tube test is positive then healing will be delayed. Since p value is less than 0.05, there is a significant difference between the mean duration of healing and presence of biofilm.

The mean duration of healing in the wound which had biofilm was more when compared to the wound which didn't contain biofilm.

Table 11 showing correlation of diabetic status and biofilms in wound healing.

Total no of diabetics: 47

Biofilm	Number	Mean duration of healing in days	P value
Presence	31	54.387	0.006
Absence	16	43.687	

Out of total number of patients [64] forty seven were diabetics .Among the diabetics [47] thirty five of them had biofilm. It was observed that in individuals who were diabetic and were positive for biofilms had significant delayed wound healing with p value of 0.006 ,in comparison with diabetic patients who did not have biofilm.

DISCUSSION

Chronic wounds are those that have failed to proceed through an orderly and timely reparative process to produce anatomic and functional integrity of the injured site.[114] In developed countries, it has been estimated that 1 to 2% of the population will experience a chronic wound during their lifetime.[115] In the United States alone, chronic wounds affect 6.5 million patients [116]. It has been found that chronic ulcers do not heal as a result of biofilms present in it. Biofilm is an aggregation of microbes that manufacture a protective carbohydrate matrix, which allows them to adhere to each other and to a host surface. The presence of biofilm delays the healing of the wound.

Biofilm was present in 67.18% of the chronic wounds in our study and consistent with the studies done by James et al , [1] where the incidence of biofilm in chronic ulcers was 60% and when compared to study done by SR Swarna et al [117], where the incidence of biofilm was 70.73%.

The age group in our study is 30yrs to 90 yrs and is comparable with the studies done by SR Swarna et al[117] where the age group was 30yrs to 80 yrs.

Biofilm formation is more common in chronic wounds such as those with diabetic foot ulcers, pressure ulcers and venous leg ulcers, and similarly seen in studies done by James et al 2007[118]

In our study there were 2 cases of pressure sores, 1 case of venous leg ulcer and 47 cases of diabetic foot ulcers.

In our study most common organism isolated was Staphylococcus aureus followed by Pseudomonas aeruginosa and consistent with the study in 2010, were the most common organism isolated was staph aureus.[119,120], S.aureus and P.aeruginosa are the bacteria most frequently isolated from these wounds[121,122]

A similar study from Puducherry reported Klebsiella species,Pseudomonas and Staphylococcus species as predominant bacteria[123] Predominance of Gram negative bacteria was documented in other studies also.[124,125]

In our study, the mean duration of healing of chronic ulcers with biofilm was 52.31 days and the mean duration of healing of chronic ulcers without biofilm was 42.56 days which was statistically significant (P value of 0.002). The healing of the ulcer in patients with biofilm was delayed by 10 days. Similar study done by Zhao G et al showed a delay of 2 weeks in healing of wounds with biofilm compared to wounds without biofilm. [62]

It was interesting to note that there was significant delay in the wound healing of diabetic patients who had biofilms compared to diabetic patients who didn't have biofilm. Studies co-relating delay in wound healing due to biofilms in diabetics are scarce. However a study from Montana University observed that there was 2 weeks delay in the wound healing in biofilm challenged mouse model.

More studies regarding the effect of biofilm in diabetic individuals which appear to increase significantly are required in human beings for confirmation.

Bacteria forming biofilms plays a major role in developing multi-drug resistance in chronic infections[126]

Biofilm mediated infections are difficult to eliminate resulting in treatment failure. It is suggested that the development of biofilm in chronic wounds are associated with increased synthesis of exopolysaccharides that leads to poor

penetration of antibiotics. Slime production has been reported in strains of Staphylococcus species and P. aeruginosa associated with the infection of biomedical devices[99]

SUMMARY

It was a prospective study done from October 2011 to June 2013, a total of 64 cases were studied. The study group consisted of patients varying from 30 yrs to 90yrs with a mean age of 60yrs.Out of these 75% were male and 25% were females. Most of the patients who had a chronic wound had diabetes mellitus (73%). Biofilm was detected by a tube test. Out of 64 samples tested, 43 samples were positive with the incidence of biofilm was 67.2%. Our study showed that if biofilm was present, then duration of healing was more, suggesting that biofilm is one of the causative factors for delay in wound healing. It was also observed that diabetic patients having biofilm had significant delay in wound healing when compared with diabetic patients who didn't have biofilm in their wounds.

So whenever the healing of an ulcer is delayed significantly despite of adequate treatment, testing for biofilm should be considered.

CONCLUSION

Biofilm is a relatively new concept which has grasped the attention of the surgeons treating chronic wounds. Not many studies have been done in India about the presence and effects of biofilm and its influence on healing of ulcers. The current prospective study shows that presence of biofilm in a chronic ulcer plays significant role in delaying the wound healing in patients. Patients taking treatment for an ulcer for prolonged duration without any improvement in the outcome might have undetected biofilm which causes economic burden in both ways i.e. monetary expenditure as well as absence from work. The detection of biofilm in a chronic ulcer helps in management of the ulcer in a better way by reducing the antibiotic resistance, improving wound healing and reducing economic burden for the patient. Suspecting and investigating for the presence of biofilm in a patient with chronic non healing ulcer, helps us manage such patients better.

REFERENCES

1. James GA, Swogger E, Wolcott R, Pulcini E deLancey, Secor P, Sestrich J, et al. Biofilms in chronic wounds. Wound Repair Regen [Internet]. 2008 [cited 2013 Oct 15];16(1):37–44. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1524-475X.2007.00321.x/abstract

2. Black CE, Costerton JW. Current Concepts Regarding the Effect of Wound Microbial Ecology and Biofilms on Wound Healing. Surg Clin North Am [Internet]. 2010 Dec [cited 2013 Oct 16];90(6):1147–60. Available from: http://www.sciencedirect.com/science/article/pii/S003961091000109X

3. Edwards R, Harding KG. Bacteria and wound healing. Curr Opin Infect Dis. 2004 Apr;17(2):91–6.

4. Cochran WL, McFeters GA, Stewart PS. Reduced susceptibility of thin Pseudomonas aeruginosa biofilms to hydrogen peroxide and monochloramine. J Appl Microbiol. 2000 Jan;88(1):22–30.

5. Cowan LJ, Stechmiller JK, Phillips P, Yang Q, Schultz G. Chronic Wounds, Biofilms and Use of Medicinal Larvae. Ulcers [Internet]. 2013 Feb 14 [cited 2013 Oct 15];2013. Available from: http://www.hindawi.com/journals/ulcers/2013/487024/abs/

6. Růzicka F, Holá V, Votava M, Tejkalová R, Horvát R, Heroldová M, et al. Biofilm detection and the clinical significance of Staphylococcus epidermidis isolates. Folia Microbiol (Praha). 2004;49(5):596–600.

7. Costerton JW, Geesey GG, Cheng KJ. How bacteria stick. Sci Am. 1978 Jan;238(1):86–95.

8. Sen CK, Gordillo GM, Roy S, Kirsner R, Lambert L, Hunt TK, et al. Human Skin Wounds: A Major and Snowballing Threat to Public Health and the Economy. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc [Internet]. 2009 [cited 2013 Oct 16];17(6):763–71. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2810192/

9. Gosain A, DiPietro LA. Aging and wound healing. World J Surg. 2004 Mar;28(3):321–6.

10. Broughton G 2nd, Janis JE, Attinger CE. The basic science of wound healing. Plast Reconstr Surg. 2006 Jun;117(7 Suppl):12S–34S.

11. Campos ACL, Groth AK, Branco AB. Assessment and nutritional aspects of wound healing. Curr Opin Clin Nutr Metab Care. 2008 May;11(3):281–8.

12. Meszaros AJ, Reichner JS, Albina JE. Macrophage-induced neutrophil apoptosis. J Immunol Baltim Md 1950. 2000 Jul 1;165(1):435–41.

13. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. Nat Rev Immunol. 2008 Dec;8(12):958–69.

14. Park JE, Barbul A. Understanding the role of immune regulation in wound healing. Am J Surg. 2004 May;187(5A):11S–16S.

15. Swift ME, Burns AL, Gray KL, DiPietro LA. Age-related alterations in the inflammatory response to dermal injury. J Invest Dermatol. 2001 Nov;117(5):1027–35.

16. Gawronska-Kozak B, Bogacki M, Rim J-S, Monroe WT, Manuel JA. Scarless skin repair in immunodeficient mice. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2006 Jun;14(3):265–76.

17. Jameson J, Havran WL. Skin gammadelta T-cell functions in homeostasis and wound healing. Immunol Rev. 2007 Feb;215:114–22.

18. Mills RE, Taylor KR, Podshivalova K, McKay DB, Jameson JM. Defects in skin gamma delta T cell function contribute to delayed wound repair in rapamycin-treated mice. J Immunol Baltim Md 1950. 2008 Sep 15;181(6):3974–83.

19. Campos ACL, Groth AK, Branco AB. Assessment and nutritional aspects of wound healing. Curr Opin Clin Nutr Metab Care. 2008 May;11(3):281–8.

20. Swift ME, Kleinman HK, DiPietro LA. Impaired wound repair and delayed angiogenesis in aged mice. Lab Investig J Tech Methods Pathol. 1999 Dec;79(12):1479–87.

21. Godbout JP, Glaser R. Stress-induced immune dysregulation: implications for wound healing, infectious disease and cancer. J Neuroimmune Pharmacol Off J Soc NeuroImmune Pharmacol. 2006 Dec;1(4):421–7.

22. Boyapati L, Wang H-L. The role of stress in periodontal disease and wound healing. Periodontol 2000. 2007;44:195–210.

23. Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. Endocr Rev. 2004 Aug;25(4):612–28.

24. Woo K, Ayello EA, Sibbald RG. The edge effect: current therapeutic options to advance the wound edge. Adv Skin Wound Care. 2007 Feb;20(2):99–117; quiz 118–119.

25. Loots MA, Lamme EN, Zeegelaar J, Mekkes JR, Bos JD, Middelkoop E. Differences in cellular infiltrate and extracellular matrix of chronic diabetic and venous ulcers versus acute wounds. J Invest Dermatol. 1998 Nov;111(5):850–7.

26. Gallagher KA, Liu Z-J, Xiao M, Chen H, Goldstein LJ, Buerk DG, et al. Diabetic impairments in NO-mediated endothelial progenitor cell mobilization and homing are reversed by hyperoxia and SDF-1 alpha. J Clin Invest. 2007 May;117(5):1249–59.

27. Galkowska H, Olszewski WL, Wojewodzka U, Rosinski G, Karnafel W. Neurogenic factors in the impaired healing of diabetic foot ulcers. J Surg Res. 2006 Aug;134(2):252–8.

28. Gary Sibbald R, Woo KY. The biology of chronic foot ulcers in persons with diabetes. Diabetes Metab Res Rev. 2008 Jun;24 Suppl 1:S25–30.

29. Franz MG, Steed DL, Robson MC. Optimizing healing of the acute wound by minimizing complications. Curr Probl Surg. 2007 Nov;44(11):691–763.

30. CDC - Obesity - Facts - Adolescent and School Health [Internet]. [cited 2013 Oct 19]. Available from: http://www.cdc.gov/healthyyouth/obesity/facts.htm

31. Wilson JA, Clark JJ. Obesity: impediment to postsurgical wound healing. Adv Skin Wound Care. 2004 Oct;17(8):426–35.

32. Anaya DA, Dellinger EP. The obese surgical patient: a susceptible host for infection. Surg Infect. 2006 Oct;7(5):473–80.

33. Greco JA 3rd, Castaldo ET, Nanney LB, Wendel JJ, Summitt JB, Kelly KJ, et al. The effect of weight loss surgery and body mass index on wound complications after abdominal contouring operations. Ann Plast Surg. 2008 Sep;61(3):235–42.

34. Juge-Aubry CE, Henrichot E, Meier CA. Adipose tissue: a regulator of inflammation. Best Pract Res Clin Endocrinol Metab. 2005 Dec;19(4):547–66.

35. Calabro P, Yeh ET. Obesity, inflammation, and vascular disease: the role of the adipose tissue as an endocrine organ. Subcell Biochem. 2007;42:63–91.

36. Wozniak SE, Gee LL, Wachtel MS, Frezza EE. Adipose tissue: the new endocrine organ? A review article. Dig Dis Sci. 2009 Sep;54(9):1847–56.

37. Nieman DC, Henson DA, Nehlsen-Cannarella SL, Ekkens M, Utter AC, Butterworth DE, et al. Influence of obesity on immune function. J Am Diet Assoc. 1999 Mar;99(3):294–9.

38. Fontana L, Eagon JC, Colonna M, Klein S. Impaired mononuclear cell immune function in extreme obesity is corrected by weight loss. Rejuvenation Res. 2007 Mar;10(1):41–6.

39. De Mello VDF, Kolehmainen M, Schwab U, Mager U, Laaksonen DE, Pulkkinen L, et al. Effect of weight loss on cytokine messenger RNA expression in peripheral blood mononuclear cells of obese subjects with the metabolic syndrome. Metabolism. 2008 Feb;57(2):192–9.

40. Siana JE, Rex S, Gottrup F. The effect of cigarette smoking on wound healing. Scand J Plast Reconstr Surg Hand Surg Nord Plast Foren Nord Klubb Handkirurgi. 1989;23(3):207–9.

41. Jensen JA, Goodson WH, Hopf HW, Hunt TK. Cigarette smoking decreases tissue oxygen. Arch Surg Chic Ill 1960. 1991 Sep;126(9):1131–4.

42. Ahn C, Mulligan P, Salcido RS. Smoking-the bane of wound healing: biomedical interventions and social influences. Adv Skin Wound Care. 2008 May;21(5):227–236; quiz 237–238.

43. Siana JE, Rex S, Gottrup F. The effect of cigarette smoking on wound healing. Scand J Plast Reconstr Surg Hand Surg Nord Plast Foren Nord Klubb Handkirurgi. 1989;23(3):207–9.

44. McMaster SK, Paul-Clark MJ, Walters M, Fleet M, Anandarajah J, Sriskandan S, et al. Cigarette smoke inhibits macrophage sensing of Gram-negative bacteria and lipopolysaccharide: relative roles of nicotine and oxidant stress. Br J Pharmacol. 2008 Feb;153(3):536–43.

45. Jacobi J, Jang JJ, Sundram U, Dayoub H, Fajardo LF, Cooke JP. Nicotine accelerates angiogenesis and wound healing in genetically diabetic mice. Am J Pathol. 2002 Jul;161(1):97–104.

46. Morimoto N, Takemoto S, Kawazoe T, Suzuki S. Nicotine at a low concentration promotes wound healing. J Surg Res. 2008 Apr;145(2):199–204.

47. Arnold M, Barbul A. Nutrition and wound healing. Plast Reconstr Surg. 2006 Jun;117(7 Suppl):42S–58S.

48. Shepherd AA. Nutrition for optimum wound healing. Nurs Stand R Coll Nurs Gt Br 1987. 2003 Oct 22;18(6):55–8.

49. Tong BC, Barbul A. Cellular and physiological effects of arginine. Mini Rev Med Chem. 2004 Oct;4(8):823–32.

50. Da Costa MAR, Campos ACL, Coelho JCU, de Barros AM, Matsumoto HM. Oral glutamine and the healing of colonic anastomoses in rats. JPEN J Parenter Enteral Nutr. 2003 Jun;27(3):182–185; discussion 185–186.

51. McDaniel JC, Belury M, Ahijevych K, Blakely W. Omega-3 fatty acids effect on wound healing. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2008 Jun;16(3):337–45.

52. Shingel KI, Faure M-P, Azoulay L, Roberge C, Deckelbaum RJ. Solid emulsion gel as a vehicle for delivery of polyunsaturated fatty acids: implications for tissue repair, dermal angiogenesis and wound healing. J Tissue Eng Regen Med. 2008 Oct;2(7):383–93.

53. Burgess C. Topical vitamins. J Drugs Dermatol JDD. 2008 Jul;7(7 Suppl):s2–6.

54. Khoosal D, Goldman RD. Vitamin E for treating children's scars. Does it help reduce scarring? Can Fam Physician Médecin Fam Can. 2006 Jul;52:855–6.

55. Campos ACL, Groth AK, Branco AB. Assessment and nutritional aspects of wound healing. Curr Opin Clin Nutr Metab Care. 2008 May;11(3):281–8.

56. Heyman H, Van De Looverbosch DEJ, Meijer EP, Schols JMGA. Benefits of an oral nutritional supplement on pressure ulcer healing in long-term care residents. J Wound Care. 2008 Nov;17(11):476–8, 480.

57. Bishop A. Role of oxygen in wound healing. J Wound Care. 2008 Sep;17(9):399–402.

58. Rodriguez PG, Felix FN, Woodley DT, Shim EK. The role of oxygen in wound healing: a review of the literature. Dermatol Surg Off Publ Am Soc Dermatol Surg Al. 2008 Sep;34(9):1159–69.

59. Menke NB, Ward KR, Witten TM, Bonchev DG, Diegelmann RF. Impaired wound healing. Clin Dermatol. 2007 Feb;25(1):19–25.

60. Davis SC, Ricotti C, Cazzaniga A, Welsh E, Eaglstein WH, Mertz PM. Microscopic and physiologic evidence for biofilm-associated wound colonization in vivo. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2008 Feb;16(1):23–9.

61. Bjarnsholt T, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, et al. Why chronic wounds will not heal: a novel hypothesis. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2008 Feb;16(1):2–10.

62. Zhao G, Hochwalt PC, Usui ML, Underwood RA, Singh PK, James GA, et al. Delayed wound healing in diabetic (db/db) mice with Pseudomonas aeruginosa biofilm challenge: a model for the study of chronic wounds. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2010 Oct;18(5):467–77.

63. Malic S, Hill KE, Hayes A, Percival SL, Thomas DW, Williams DW. Detection and identification of specific bacteria in wound biofilms using peptide nucleic acid fluorescent in situ hybridization (PNA FISH). Microbiol Read Engl. 2009 Aug;155(Pt 8):2603–11.

64. Jefferson KK. What drives bacteria to produce a biofilm? FEMS Microbiol Lett. 2004 Jul 15;236(2):163–73.

65. Zogaj X, Nimtz M, Rohde M, Bokranz W, Römling U. The multicellular morphotypes of Salmonella typhimurium and Escherichia coli produce cellulose as the second component of the extracellular matrix. Mol Microbiol. 2001 Mar;39(6):1452–63.

66. SCHRAMM M, HESTRIN S. Factors affecting production of cellulose at the air/liquid interface of a culture of Acetobacter xylinum. J Gen Microbiol. 1954 Aug;11(1):123–9.

67. Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. Assembly and development of the Pseudomonas aeruginosa biofilm matrix. PLoS Pathog. 2009 Mar;5(3):e1000354.

68. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. Mol Microbiol. 2006 Feb;59(4):1114–28.

69. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science. 2002 Feb 22;295(5559):1487.

70. Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, Chandramohan L, et al. Modulation of eDNA release and degradation affects Staphylococcus aureus biofilm maturation. PloS One. 2009;4(6):e5822.

71. Rice KC, Mann EE, Endres JL, Weiss EC, Cassat JE, Smeltzer MS, et al. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. Proc Natl Acad Sci U S A. 2007 May 8;104(19):8113–8.

72. Borlee BR, Goldman AD, Murakami K, Samudrala R, Wozniak DJ, Parsek MR. Pseudomonas aeruginosa uses a cyclic-di-GMP-regulated adhesin to reinforce the biofilm extracellular matrix. Mol Microbiol. 2010 Feb;75(4):827–42.

73. Fexby S, Bjarnsholt T, Jensen PØ, Roos V, Høiby N, Givskov M, et al. Biological Trojan horse: Antigen 43 provides specific bacterial uptake and survival in human neutrophils. Infect Immun. 2007 Jan;75(1):30–4.

74. Klemm P, Hjerrild L, Gjermansen M, Schembri MA. Structure-function analysis of the self-recognizing Antigen 43 autotransporter protein from Escherichia coli. Mol Microbiol. 2004 Jan;51(1):283–96.

75. Matsuyama T, Kaneda K, Ishizuka I, Toida T, Yano I. Surface-active novel glycolipid and linked 3-hydroxy fatty acids produced by Serratia rubidaea. J Bacteriol. 1990 Jun;172(6):3015–22.

76. Davey ME, Caiazza NC, O'Toole GA. Rhamnolipid surfactant production affects biofilm architecture in Pseudomonas aeruginosa PAO1. J Bacteriol. 2003 Feb;185(3):1027–36.

77. Pamp SJ, Tolker-Nielsen T. Multiple roles of biosurfactants in structural biofilm development by Pseudomonas aeruginosa. J Bacteriol. 2007 Mar;189(6):2531–9.

78. Harmsen M, Yang L, Pamp SJ, Tolker-Nielsen T. An update on Pseudomonas aeruginosa biofilm formation, tolerance, and dispersal. FEMS Immunol Med Microbiol. 2010 Aug;59(3):253–68.

79. Parsek MR, Tolker-Nielsen T. Pattern formation in Pseudomonas aeruginosa biofilms. Curr Opin Microbiol. 2008 Dec;11(6):560–6.

80. Flemming H-C, Neu TR, Wozniak DJ. The EPS matrix: the “house of biofilm cells.”J Bacteriol. 2007 Nov;189(22):7945–7.

81. Nivens DE, Ohman DE, Williams J, Franklin MJ. Role of alginate and its O acetylation in formation of Pseudomonas aeruginosa microcolonies and biofilms. J Bacteriol. 2001 Feb;183(3):1047–57.

82. Simpson JA, Smith SE, Dean RT. Scavenging by alginate of free radicals released by macrophages. Free Radic Biol Med. 1989;6(4):347–53.

83. Allison DG, Matthews MJ. Effect of polysaccharide interactions on antibiotic susceptibility of Pseudomonas aeruginosa. J Appl Bacteriol. 1992 Dec;73(6):484–8.

84. Sun Y, Dowd SE, Smith E, Rhoads DD, Wolcott RD. In vitro multispecies Lubbock chronic wound biofilm model. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2008 Dec;16(6):805–13.

85. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. J Clin Microbiol. 1999 Jun;37(6):1771–6.

86. Williams I, Venables WA, Lloyd D, Paul F, Critchley I. The effects of adherence to silicone surfaces on antibiotic susceptibility in Staphylococcus aureus. Microbiol Read Engl. 1997 Jul;143 (Pt 7):2407–13.

87. Hoyle BD, Wong CK, Costerton JW. Disparate efficacy of tobramycin on Ca(2+)-, Mg(2+)-, and HEPES-treated Pseudomonas aeruginosa biofilms. Can J Microbiol. 1992 Nov;38(11):1214–8.

88. Duguid IG, Evans E, Brown MR, Gilbert P. Effect of biofilm culture upon the susceptibility of Staphylococcus epidermidis to tobramycin. J Antimicrob Chemother. 1992 Dec;30(6):803–10.

89. Duguid IG, Evans E, Brown MR, Gilbert P. Growth-rate-independent killing by ciprofloxacin of biofilm-derived Staphylococcus epidermidis; evidence for cell-cycle dependency. J Antimicrob Chemother. 1992 Dec;30(6):791–802.

90. Tresse O, Jouenne T, Junter GA. The role of oxygen limitation in the resistance of agar-entrapped, sessile-like Escherichia coli to aminoglycoside and beta-lactam antibiotics. J Antimicrob Chemother. 1995 Sep;36(3):521–6.

91. Percival SL, Hill KE, Williams DW, Hooper SJ, Thomas DW, Costerton JW. A review of the scientific evidence for biofilms in wounds. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2012 Oct;20(5):647–57.

92. Gurjala AN, Geringer MR, Seth AK, Hong SJ, Smeltzer MS, Galiano RD, et al. Development of a novel, highly quantitative in vivo model for the study of biofilm-impaired cutaneous wound healing. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2011 Jun;19(3):400–10.

93. Nguyen KT, Seth AK, Hong SJ, Geringer MR, Xie P, Leung KP, et al. Deficient cytokine expression and neutrophil oxidative burst contribute to impaired cutaneous wound healing in diabetic, biofilm-containing chronic wounds. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2013 Oct 9;

94. Leid JG, Willson CJ, Shirtliff ME, Hassett DJ, Parsek MR, Jeffers AK. The exopolysaccharide alginate protects Pseudomonas aeruginosa biofilm bacteria from

IFN-gamma-mediated macrophage killing. J Immunol Baltim Md 1950. 2005 Dec 1;175(11):7512–8.

95. Loryman C, Mansbridge J. Inhibition of keratinocyte migration by lipopolysaccharide. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2008 Feb;16(1):45–51.

96. Borriello G, Werner E, Roe F, Kim AM, Ehrlich GD, Stewart PS. Oxygen Limitation Contributes to Antibiotic Tolerance of Pseudomonas aeruginosa in Biofilms. Antimicrob Agents Chemother [Internet]. 2004 Jul [cited 2013 Oct 20];48(7):2659–64. Available from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC434183/

97. Mah TF, O'Toole GA. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 2001 Jan;9(1):34–9.

98. Danhorn T, Fuqua C. Biofilm Formation by Plant-Associated Bacteria. Annu Rev Microbiol [Internet]. 2007 [cited 2013 Oct 20];61(1):401–22. Available from: http://www.annualreviews.org/doi/abs/10.1146/annurev.micro.61.080706.093316

99. Mathur T, Singhal S, Khan S, Upadhyay D, Fatma T, Rattan A. Detection of biofilm formation among the clinical isolates of Staphylococci: An evaluation of three different screening methods. Indian J Med Microbiol [Internet]. 2006 [cited 2013 Oct 20];24(1):25. Available from: http://www.ijmm.org/article.asp?issn=0255-0857;year=2006;volume=24;issue=1;spage=25;epage=29;aulast=Mathur

100. Mallick SK] Bose S. Detection of biofilm producing staphylococci: need of the hour. Journal of clinical and diagnostic research. 2009;(3):1915–20.

101. Oliveira A, Cunha M de LR. Comparison of methods for the detection of biofilm production in coagulase-negative staphylococci. BMC Res Notes. 2010;3:260.

102. Percival SL, Hill KE, Malic S, Thomas DW, Williams DW. Antimicrobial tolerance and the significance of persister cells in recalcitrant chronic wound biofilms. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2011 Feb;19(1):1–9.

103. Bayles KW. The biological role of death and lysis in biofilm development. Nat Rev Microbiol. 2007 Sep;5(9):721–6.

104. Ngo QD, Vickery K, Deva AK. The effect of topical negative pressure on wound biofilms using an in vitro wound model. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2012 Feb;20(1):83–90.

105. Gregory S Schultz, Glenn Ladwig, Annette Wysocki. Extracellular matrix: review of its roles in acute and chronic wounds. World Wide Wounds [Internet]. Available from: http://www.worldwidewounds.com/2005/august/Schultz/Extrace-Matric-Acute-Chronic-Wounds.html

106. Baharestani M. The clinical relevance of debridement. Springer. 1999;

107. Dowsett C, Ayello E. TIME principles of chronic wound bed preparation and treatment. Br J Nurs Mark Allen Publ. 2004;13(15):16–23.

108. Vande Berg JS, Robson MC. Arresting cell cycles and the effect on wound healing. Surg Clin North Am. 2003;83(3):509–20.

109. Gottrup F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg. 2004 May;187(5A):38S–43S.

110. Crovetti G, Martinelli G, Issi M, Barone M, Guizzardi M, Campanati B, et al. Platelet gel for healing cutaneous chronic wounds. Transfus Apher Sci Off J World Apher Assoc Off J Eur Soc Haemapheresis. 2004 Apr;30(2):145–51.

111. Gottrup F, Holstein P, Jørgensen B, Lohmann M, Karlsmar T. A new concept of a multidisciplinary wound healing center and a national expert function of wound healing. Arch Surg Chic Ill 1960. 2001 Jul;136(7):765–72.

112. Brem H, Stojadinovic O, Diegelmann RF, Entero H, Lee B, Pastar I, et al. Molecular markers in patients with chronic wounds to guide surgical debridement. Mol Med Camb Mass. 2007 Feb;13(1-2):30–9.

113. January 23, 2008. The burden of chronic wounds in the UK [Internet]. [cited 2013 Oct 16]. Available from: http://www.nursingtimes.net/nursing-practice/clinical-zones/wound-care/the-burden-of-chronic-wounds-in-the-uk/527138.article

114. Lazarus GS, Cooper DM, Knighton DR, Percoraro RE, Rodeheaver G, Robson MC. Definitions and guidelines for assessment of wounds and evaluation of healing. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 1994 Jul;2(3):165–70.

115. Gottrup F. A specialized wound-healing center concept: importance of a multidisciplinary department structure and surgical treatment facilities in the treatment of chronic wounds. Am J Surg. 2004 May;187(5A):38S–43S.

116. Crovetti G, Martinelli G, Issi M, Barone M, Guizzardi M, Campanati B, et al. Platelet gel for healing cutaneous chronic wounds. Transfus Apher Sci Off J World Apher Assoc Off J Eur Soc Haemapheresis. 2004 Apr;30(2):145–51.

117. SR. Swarna, Radha Madhavan, S. Gomathi. A study of Biofilm on Diabetic Foot Ulcer. Int J Res Pharm Biomed Sci. 3(4):1809–14.

118. James GA, Beaudette L, Costerton JW. Interspecies bacterial interactions in biofilms. J Ind Microbiol [Internet]. 1995 Oct 1 [cited 2013 Oct 15];15(4):257–62. Available from: http://link.springer.com/article/10.1007/BF01569978

119. Gjødsbøl K, Christensen JJ, Karlsmark T, Jørgensen B, Klein BM, Krogfelt KA. Multiple bacterial species reside in chronic wounds: a longitudinal study. Int Wound J. 2006 Sep;3(3):225–31.

120. Thomsen TR, Aasholm MS, Rudkjøbing VB, Saunders AM, Bjarnsholt T, Givskov M, et al. The bacteriology of chronic venous leg ulcer examined by culture-independent molecular methods. Wound Repair Regen Off Publ Wound Heal Soc Eur Tissue Repair Soc. 2010 Feb;18(1):38–49.

121. James GA, Swogger E, Wolcott R, Pulcini E deLancey, Secor P, Sestrich J, et al. Biofilms in chronic wounds. Wound Repair Regen [Internet]. 2008 [cited 2013 Sep 26];16(1):37–44. Available from: http://onlinelibrary.wiley.com/doi/10.1111/j.1524-475X.2007.00321.x/abstract

122. Fazli M, Bjarnsholt T, Kirketerp-M?ller K, J?rgensen B, Andersen AS, Krogfelt KA, et al. Nonrandom Distribution of Pseudomonas aeruginosa and Staphylococcus

aureus in Chronic Wounds. J Clin Microbiol [Internet]. 2009 Dec [cited 2013 Oct 20];47(12):4084–9. Available from:

http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2786634/

123. Umadevi S, Shailesh K. microbiological study off diabetic foot infections. Indian J Med Spec. 2011;2(1):12–7.

124. Shankar E M, Mohan V. bacterial etiology of diabeetic foot infections in south india. Eur J Intern Med. 2005;(16):567–70.

125. Gadepalli R, Dhawan B, Sreenivas V, Kapil A, Ammini AC, Chaudhry R. A Clinico-microbiological Study of Diabetic Foot Ulcers in an Indian Tertiary Care Hospital. Dia Care [Internet]. 2006 Aug 1 [cited 2013 Oct 20];29(8):1727–32. Available from: http://care.diabetesjournals.org/content/29/8/1727

126. Sritharan M, Sritharan V. emerging problemns in the mangemnt of infectious disease- biofilms. Indian J Med Microbiol. 2004;22(3):140–2.

ANNEXURE –I

INFORMED CONSENT

TITLE OF THE PROJECT : CLINICAL AND MICROBIOLOGICAL STUDY OF BIOFILM IN CHRONIC ULCERS

PRINCIPAL INVESTIGATOR: Dr. HARSHAGOPAL DESHPANDE

GUIDE Dr. TEJASWINI VALLABHA

M.S (GENERAL SURGERY)

PROFESSOR AND HEAD OF DEPARTMENT

DEPARTMENT OF SURGERY.

Co GUIDE : Dr PRASHANT K PARANDEKAR

MD(MICROBIOLOGY)

PROFESSOR AND HEAD OF DEPARTMENT,

DEPARTMENT OF MICROBIOLOGY

PURPOSE OF RESEARCH:

I have been informed that this study is a clinical and microbiological study of biofilm of a chronic ulcer. I have also been given a free choice of participation in this study. This study will help in proper understanding regarding chronicity of an ulcer.

PROCEDURE:

I am aware that in addition to routine care received I will be asked series of questions by the investigator. I have been asked to undergo the necessary investigations and treatment, which will help the investigator in this study.

RISK AND DISCOMFORTS:

I understand that I may experience some pain and discomfort during the examination or during my treatment. This is mainly the result of my condition and the procedure of this study is not expected to exaggerate these feelings that are associated with the usual course of treatment.

BENEFITS:

I understand that my participation in this study will have no direct benefits to me other than the potential benefits of diagnosis & treatment which is planned to heal the ulcer. The major potential benefit is to find out what is the cause for a chronic ulcer.

CONFIDENTIALITY:

I understand that the medical information produced by this study will become a part of Hospital records and will be subject to the confidentiality and privacy regulation. Information of a sensitive personal nature will not be a part of the medical records, but will be stored in the investigator's research file and identified only by a code number. The code-key connecting name to numbers will be kept in a separate location.

If the data are used for publication in the medical literature or for teaching purpose, no name will be used and other identifiers such as photographs and audio or videotapes will be used only with my special written permission. I understand that I may see the photographs and videotapes and hear the audiotapes before giving this permission.

In this study I understand this is to be studied their relevant designated authority & the industrial sponsor are permitted to have access to my medical record

& to the date produced by this study for audit purposes however they are required to maintain confidentiality.

REQUEST FOR MORE INFORMATION:

I understand that I may ask more questions about the study at anytime. Dr Harshagopal Deshpande is available to answer my questions or concerns. I understand that I will be informed of any significant new findings discovered during the course of the study, which might influence my continued participation.

If during the study, or later, I wish to discuss my participation in or concerns regarding this study with a person not directly involved, I am aware that the social worker of the hospital is available to talk with me.

REFUSAL FOR WITHDRAWAL OF PARTICIPATION:

I understand that my participation is voluntary and that I may refuse to participate or may withdraw consent and discontinue participation in the study at any time without prejudice to my present or future care at this hospital. I also understand that Dr.Harshagopal Deshpande may terminate my participation in the study after he has explained the reasons for doing so and has helped arrange for my continued care by my own physician or physical therapist, if this is appropriate.

INJURY STATEMENT:

I understand that in the unlikely event of injury to me resulting directly from my participation in this study, if such injury were reported promptly, the appropriate treatment would be available to me, but no further compensation would be provided. I understand that by my agreement to participate in this study I am not waiving any of my legal rights.

I have explained to _____the purpose of the research, the procedures required and the possible risks and benefits to the best of my ability in patient's own language.

_____	_____	_____
Date	Dr. Harshagopal Deshpande (Investigator)	Dr.Tejaswini V (Guide)

STUDY SUBJECT CONSENT STATEMENT:

I confirm that Dr. Harshagopal Deshpande has explained to me the purpose of research, the study procedures that I will undergo, and the possible risks and discomforts as well as benefits that I may experience in my own language. I have read and I understand this consent form. Therefore, I agree to give consent to participate as a subject in this research project.

_____	_____
Participant / Guardian	Date
_____	_____
Witness to signature	Date

ANNEXURE –II

SCHEME OF CASE TAKING:

1) Case No:

2) Name

3) Age:

4) Sex

5) Religion:

6) Occupation:

7) Residence:

8) IP NO:

9) D.O.A:

10) D.O.S:

11) D.O.D:

12) Chief complaints

Wound:

13) History of Presenting Illness:

1. Duration of wound
2. Number
3. Mode of onset
4. h/o trauma
5. Precipitating factors
6. associated complaints
7. h/o discharge

14) Past History:

H/o diabetes,tuberculosis,irradiation

15) Family History:

16) Personal History:

Diet: Veg/Mixed

Alcoholic: yes/no

17) General Physical Examination

Pallor	present/absent
Icterus	present/absent
Clubbing	present/absent
Generalized Lymphadenopathy	present/absent
Build	Poor/Middle /Well
Nourishment	Poor / Middle / Well

18) Vitals

PR:

BP:

RR:

Temp:

19) Local Examination

Inspection

a) Number

b) Size

c) Shape

d) Position

e) Discharge

f) Floor

g) Edge

h) Surrounding skin

Palpation

a) Local rise of temperature

b) Tenderness

c) edge

d) base

e) bleeding

f) relation with deeper structures

g) surrounding skin

Examination of lymph nodes

Examination for vascular insufficiency

Examination for nerve lesion

Examination of bone and nearby joint

20) Other systemic examination

1. Respiratory system
2. Cardiovascular system
3. Central nervous system

21) Provisional diagnosis

22) Investigations

a) Blood:

Hb:

TC:

i. DC:

ESR:

HIV Rapid:

HBSAg Spot:

Random blood sugar:

b) Urine:

Albumin :

Sugar :

Microscopy:

c) X ray of the affected part

d) Pus for

Gram stain,

Culture and sensitivity

Biochemical tests

TEST	POSITIVE	NEGATIVE
Indole test		
Methyl red test		
Voges-Proskauer test		
Citrate test		
Triple sugar iron test		
Urease test		
Catalase test		
Coagulase test		
Nitrate reduction test		

e) Tube method to detect biofilm

Presence of biofilm yes/no

23) Final diagnosis:

Observation of the ulcer with biofilm

VARIABLES	Day 30	Day 37	Day 44	Day 51	Day 58	Day >58
Foul smell						
Discharge						
Granulation tissue						
Size of the ulcer						

Observation of the ulcer without biofilm

VARIABLES	Day 30	Day 37	Day 44	Day 51	Day 58	Day >58
Foul smell						
Discharge						
Granulation tissue						
Size of the ulcer						

Interventions done for the ulcer

Debridement

Regular dressings

Delayed primary closure

Split thickness skin grafting

Flaps

Duration required for healing:

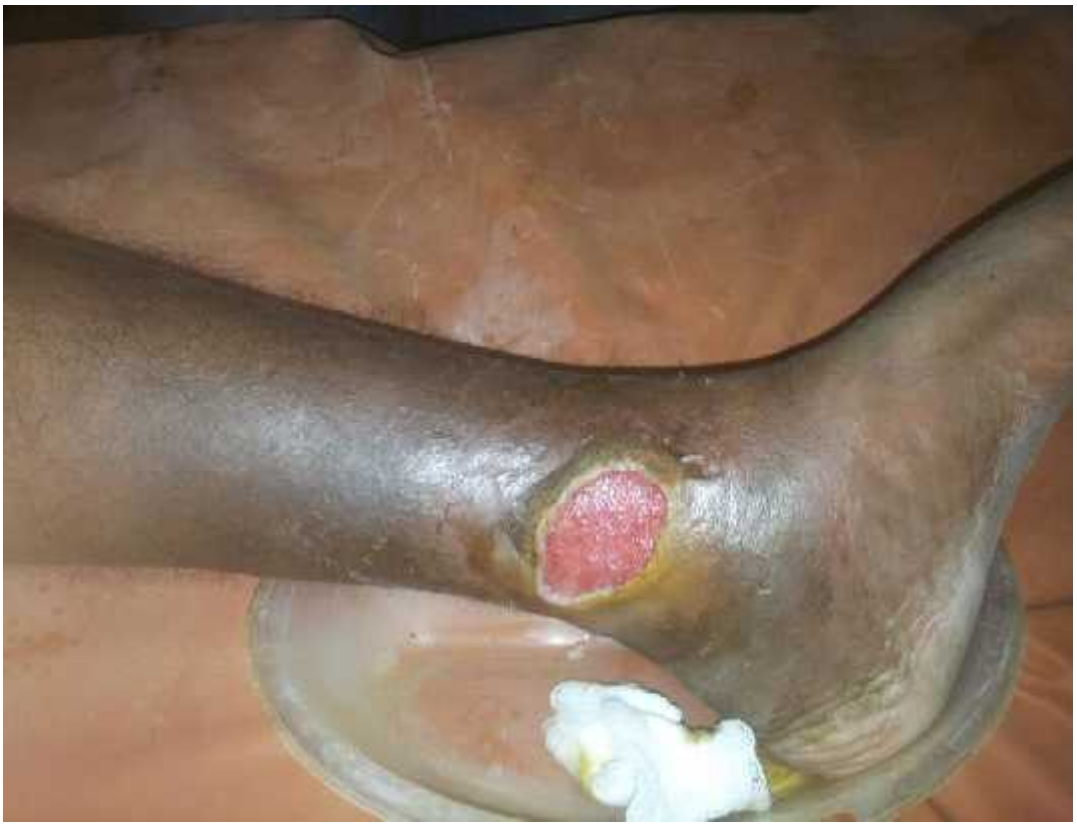
In ulcer which contains biofilm

In ulcer which didn’t contain biofilm.

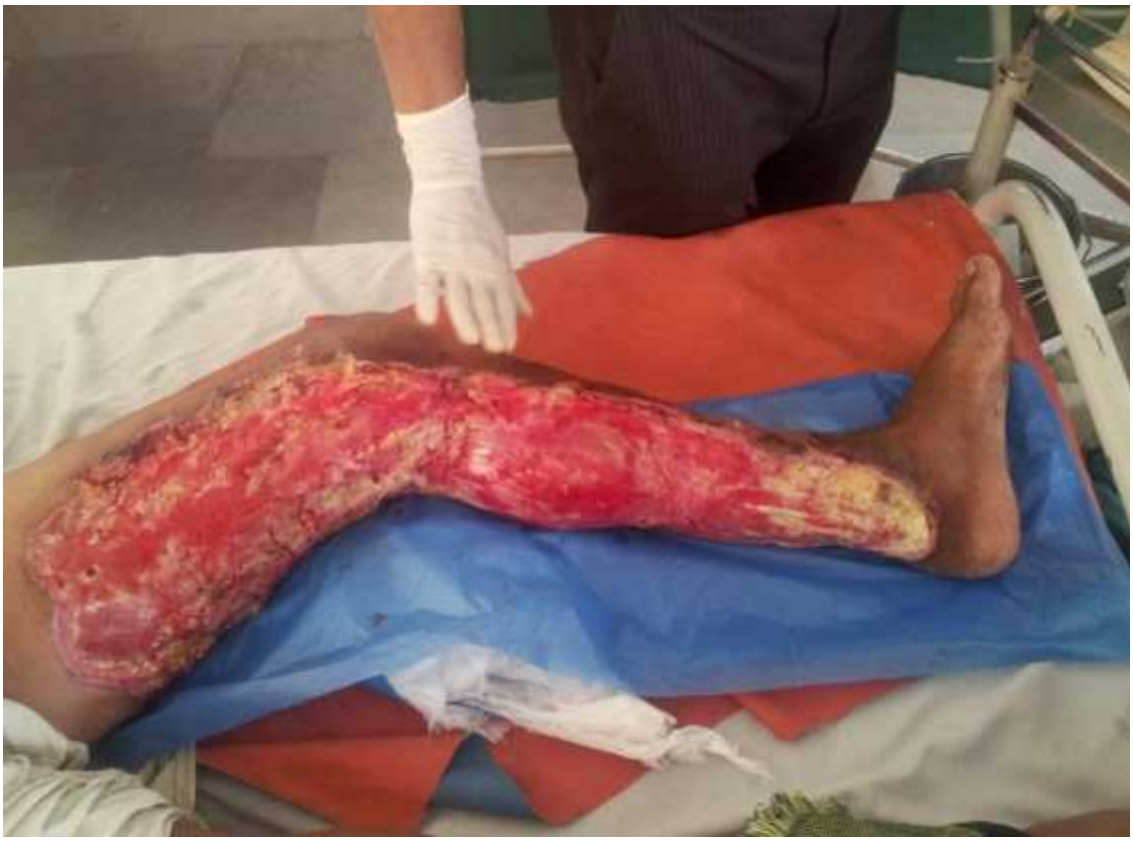
KEYS TO MASTER CHART

DOA	:	Date Of Admission
DOD	:	Date of Discharge
IP No	:	In Patient No
DOW	:	Duration of Wound
DOH	:	Duration of Healing
Ppt	:	Precipitating factors
Hb	:	Hemoglobin
ESR	:	erythrocyte Sedimentation Rate
RBS	:	Random Blood Sugar
Pus C/S	:	Pus for Culture and Sensitvity
DM	:	Diabets mellitus
HTN	:	Hypertension
Post	:	posterior
Ant	:	anterior
Med	:	medial
lat	:	lateral
r	:	right
L	:	left
M	:	male
F	:	female
PBR	:	prolonged bed rest
Blw	:	below
GT	:	granulation tisse

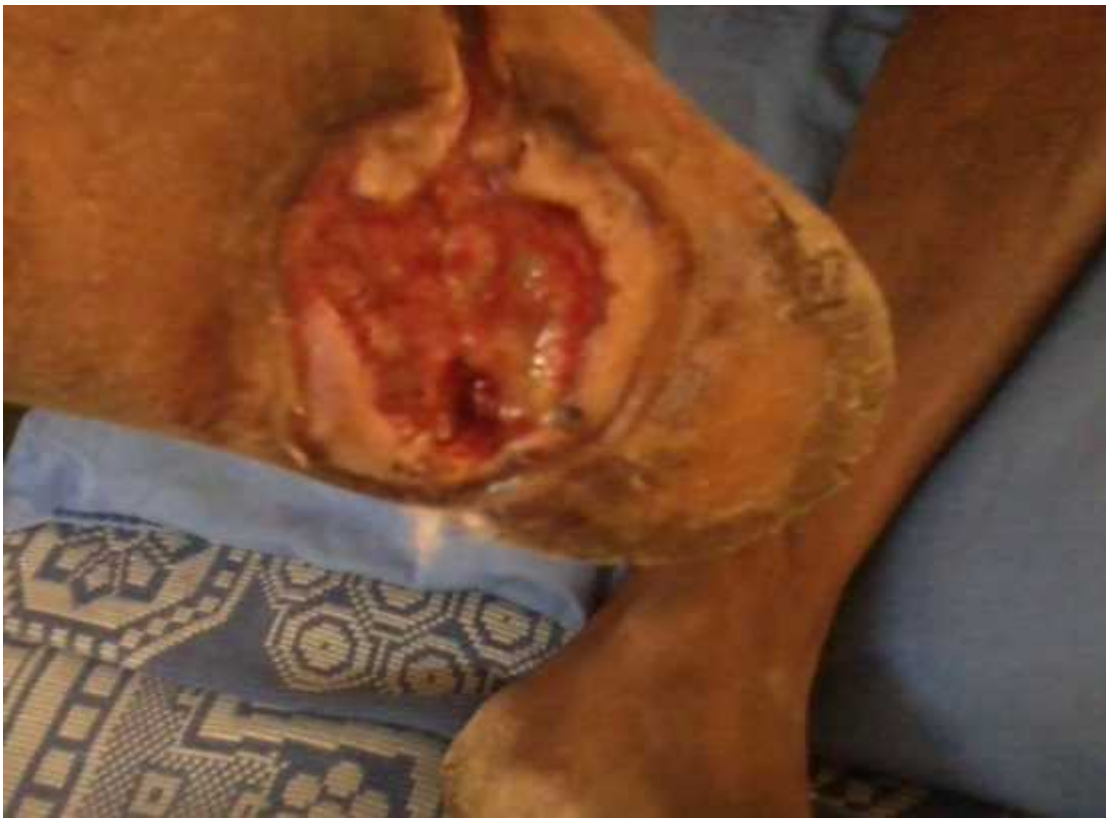
PHOTOGRAPHS



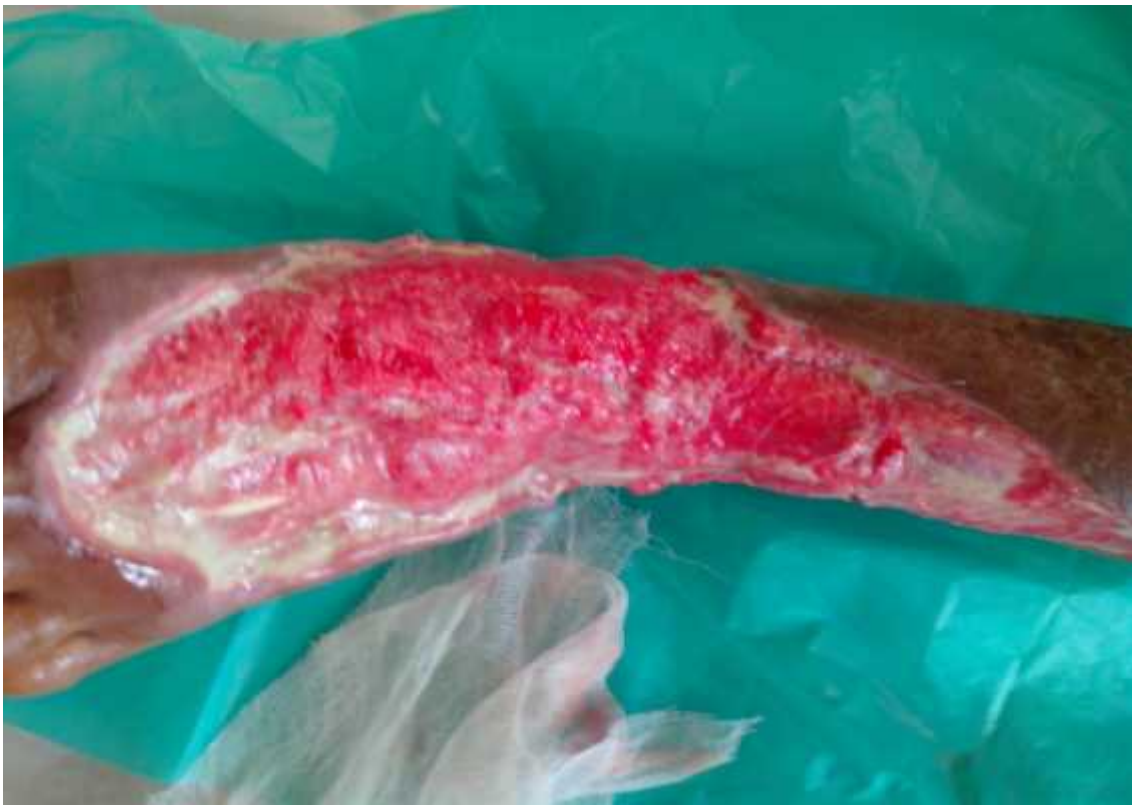
The photograph of a patient’s leg having chronic ulcer over the medial malleolus which had a biofilm.



The photograph of a patient’s leg having a chronic ulcer which had biofilm.



The photograph of a infective ulcer on sole of right foot.



The photograph of a chronic wound over the left leg

MASTER CHART

sl no	name	age	sex	Ip no	DOA	DOD	DOW IN DAYS	ppt	dm	smoking	pallor	nourishment	htn	size	position of wound	discharge	floor	edge	peripheral pulses	sensory system	bone & nearby joint	hb	esr	rbs	xray	pus c/s	tube test	DOH	wound closure
1	huvanna	70	m	24749	22/11/2011	22/01/2012	70	nil	2mths	no	present	moderate	no	7*5cm	post 1/3rd r sole	serous	ale GT with slough	ping+punch	well felt	absent	normal	12.4	70	108	normal	aeroginos	positive	70	stsg
2	laxmibai	50	f	25351	29/11/2011	28/12/2011	210	nil	20yrs	no	absent	moderate	yes	2*2cm	dorsum of l foot	serous	ale GT with slough	sloping	well felt	present	normal	9.8	90	247	normal	s aureus	positive	45	sih
3	gurubasappa	95	m	171	03/01/2012	21/01/2012	3yrs	nil	12yrs	yes	present	moderate	no	3*2cm	r forefoot	serous	pale granulation	sloping	well felt	absent	normal	11.7	65	333	normal	s aureus	positive	48	stsg
4	Irappa	75	m	734	10/01/2012	15/03/2012	60	nil	18yrs	no	present	moderate	no	4*4cm	dorsum of r foot	purulent	ale GT with slough	sloping	well felt	absent	normal	13.9	50	229	normal	bteus vulga	negative	33	stsg
5	shivappa	39	m	3243	08/02/2012	27/02/2012	60	nil	3yrs	yes	present	moderate	no	6*2 cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	11.1	100	175	normal	staph	positive	45	sih
6	nandabasappa	51	m	3670	14/02/2012	24/02/2012	30	nil	no	no	present	moderate	no	4*4cm	right heel	serous	ale GT with slough	sloping	well felt	absent	normal	11.7	65	196	normal	e coli	positive	51	sih
7	shankarappa	36	m	4600	25/02/2012	10/03/2012	1yr	nil	5yrs	yes	present	moderate	no	5*7cm	lateral asp l foot	serous	ale GT with slough	sloping	well felt	absent	normal	8.7	`135	210	normal	s aureus	positive	36	sih
8	umadevi	42	f	5244	05/03/2012	21/03/2012	30	nil	3yrs	no	present	moderate	yes	7*5cm	sole of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	9.3	75	148	normal	sterile	negative	35	stsg
9	savitri	33	f	8607	18/04/2012	26/04/2012	30	nil	8yrs	no	present	moderate	no	4*3cm	post 1/3rd r sole	serous	ale GT with slough	sloping	well felt	absent	normal	8.3	100	105	normal	sterile	negative	46	sih
10	ramachandra	52	m	10347	10/05/2012	18/06/2012	30	nil	10yrs	yes	present	moderate	no	2*2cm	medial side of l leg	serous	ale GT with slough	sloping	well felt	absent	normal	9.7	70	130	normal	k oxytocia	positive	52	stsg
11	shiavanagouda	49	m	13438	19/06/2012	07/07/2012	30	nil	no	yes	present	moderate	no	3*3cm	sole of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	10.8	120	173	normal	sterile	negative	49	sih
12	laxmibai	45	f	14118	27/06/2012	03/07/2012	45	nil	25yrs	no	present	moderate	no	2*2cm	med asp of l great toe	serous	pale GT	sloping	well felt	absent	normal	10.5	45	266	normal	sterile	negative	45	sih
13	prakash	83	m	14138	27/06/2012	01/09/2012	30	nil	5yrs	no	absent	moderate	no	9*6cm	dorsum of left foot	serous	ale GT with slough	sloping	well felt	absent	normal	10	95	180	normal	s aureus	positive	83	ss
14	sharanappa	45	m	14204	28/06/2012	18/07/2012	30	nil	4yrs	no	absent	moderate	no	6*4cm	dorsum of r foot	purulent	ale GT with slough	sloping	well felt	absent	normal	10.3	80	251	normal	s aureus	positive	46	stsg
15	shivaraya	35	m	14914	07/07/2012	25/07/2012	90	nil	no	yes	present	moderate	no	6*5cm	l forefoot	serous	ale GT with slough	sloping	well felt	absent	normal	8.3	50	188	normal	aerogenos	positive	35	sih
16	suresh	49	m	15241	11/07/2012	14/08/2012	30	nil	3yrs	yes	present	moderate	no	5*5cm	base of l great toe	serous	ale GT with slough	sloping	well felt	absent	normal	13.4	30	122	normal	s aureus	positive	49	sih
17	shankargouda	77	m	15930	27/07/2012	11/09/2012	60	nil	7yrs	no	present	moderate	no	6*5cm	base of l great toe	purulent	ale GT with slough	sloping	well felt	absent	normal	7.6	45	340	normal	baeroginosa	positive	77	sih
18	nijaguni	83	m	11083	20/04/2013	17/05/2013	30	nil	6yrs	no	present	moderate	no	6*5cm	base of r great toe	serous	ale GT with slough	sloping	well felt	absent	normal	7.6	45	340`	normal	s aureus	positive	83	sih
19	ramachandra	49	m	18132	16/08/2012	16/09/2012	120	nil	10yrs	no	absent	moderate	no	5*4cm	plantar aspect of r ft	present	ale GT with slough	sloping	well felt	absent	normal	11.2	80	142	normal	s aureus	positive	48	ss
20	mahadevappa	59	m	18388	19/08/2012	26/08/2012	45	nil	5yrs	no	present	moderate	no	3*3cm	mid 1/3rd foot	serous	ale GT with slough	sloping	well felt	absent	normal	13.5	15	106	normal	sterile	negative	45	ss
21	yamannawwa	51	f	21726	26/09/2012	15/10/2012	60	nil	12yrs	no	presnt	moderate	no	3*3cm	sole of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	9.3	30	178	normal	sterile	negative	56	stsg
22	raj kumar	63	m	2272	25/01/2013	11/03/2013	30	nil	2yrs	no	present	moderate	no	4*3cm	dorsum of foot	serous	ale GT with slough	sloping	well felt	absent	normal	11	80	182	normal	aeroginos	negative	44	ss
23	shreeshail	86	m	23107	11/10/2012	24/12/2012	30	nil	1yr	yes	presnt	moderate	no	5*5cm	sole of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	11.4	100	219	normal	e coli	positive	86	ss
24	basavaraj	59	m	23478	12/05/2012	19/07/2012	30	nil	5 yrs	no	present	moderate	no	5*4cm	med 1/3rd of r sole	serous	ale GT with slough	sloping	well felt	absent	normal	9.2	130	307	normal	aeroginos	positive	68	ss
25	mallappa	53	m	23816	19/08/2012	30/09/2012	30	nil	10yrs	no	absent	moderate	no	4*4cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	11.8	90	237	normal	e coli	positive	53	stsg
26	sonabai	35	f	20570	19/10/2012	30/10/2012	60	nil	10yrs	no	present	moderate	no	8*6cm	dorsum of foot	serous	ale GT with slough	sloping	well felt	absent	normal	10	40	192	normal	sterile	negative	35	sih
27	basvantraya	48	m	15135	10/07/2012	14/08/2012	30	nil	7yrs	no	present	moderate	no	4*3cm	dorsum of r ft	serous	ale GT with slough	sloping	well felt	absent	normal	8.7	90	123	normal	sterile	negative	55	sih
28	bhimappa	45	m	24666	30/10/2012	09/11/2012	30	nil	15yrs	no	absent	moderate	yes	4*4cm	post 1/3rd sole r ft	serous	ale GT with slough	sloping	well felt	absent	normal	11	40	216	not done	s aureus	positive	45	stsg
29	mallappa	56	m	27415	27/11/2012	31/12/2012	40	nil	17yr	yes	present	moderate	no	7*5cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	8.5	100	178	normal	k oxytocia	positive	56	stsg
30	yamannawwa	57	f	28293	05/12/2012	12/01/2013	30	nil	12yr	no	present	moderate	no	4*4cm	ant 1/3rd r foot	serous	ale GT with slough	sloping	well felt	absent	normal	7	25	240	normal	sterile	negative	59	sih
31	hanamantraya	42	m	3524	07/02/2013	12/03/2013	90	nil	8yrs	no	present	moderate	no	4*4cm	base of great toe	purulent	ale GT with slough	sloping	well felt	absent	normal	10.3	80	115	normal	e coli	positive	42	sih
32	maleppa	37	m	3641	08/02/2013	25/02/2013	60	nil	12yr	no	present	moderate	no	10*8cm	dorsum of r foot	purulent	ale GT with slough	sloping	well felt	absent	normal	10,8	66	272	normal	e coli	positive	37	sih
33	janabai	52	f	4363	15/02/2013	24/03/2013	30	nil	4yrs	no	presnt	moderate	no	4*3cm	dorsum of l foot	serous	ale GT with slough	sloping	well felt	absent	normal	9	45	162	normal	sterile	negative	56	ss
34	sarojini	91	f	3603	08/02/2013	18/03/2013	30	nil	4 yrs	no	present	moderate	no	4*4 cm	dorsum of foot	serous	ale GT with slough	sloping	well felt	absent	normal	10.5	80	145	normal	sterile	negative	32	sih
35	chayawwa	40	f	8688	28/03/2013	15/04/2013	40	nil	5mth	no	present	moderate	no	6*6cm	sole of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	7.9	80	316	normal	s aureus	positive	40	sih
36	shreemanth	45	m	11132	21/04/2013	30/04/2013	30	nil	no	yes	present	moderate	no	12*7cm	sole of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	12.3	25	217	normal	s aureus	positive	45	stsg
37	kadarsab	66	m	11329	23/04/2013	01/05/2013	240	nil	5mth	yes	present	moderate	no	4*4cm	plantar asp r foot	serous	ale GT with slough	sloping	well felt	absent	normal	8	80	148	normal	s aureus	positive	66	sih
38	shantabai	75	f	11635	27/04/2012	03/07/2012	30	nil	no	no	presetn	moderate	no	6*5cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	7.4	100	112	normal	s aureus	positive	75	sih
39	basavaraj	56	m	11815	29/04/2011	06/06/2011	30	nil	2yrs	no	present	moderate	no	4*4cm	dorsum of l foot	serous	ale GT with slough	sloping	well felt	absent	normal	8.1	120	213	normal	citrobacter	negative	35	sih
40	sidappa	64	m	12800	10/05/2013	26/06/2013	30	nil	7yrs	no	present	moderate	no	16*14cm	medial aspect of l foot	serous	ale GT with slough	sloping	well felt	absent	normal	8.9	80	168	normal	s aureus	positive	64	stsg
41	kamalabai	70	f	13064	13/05/2013	21/05/2013	30	nil	15yrs	no	present	moderate	yes	8*7cm	dorsum of l foot	serous	ale GT with slough	sloping	well felt	absent	normal	7.4	80	168	normal	s aureus	positive	70	stsg
42	mallaya	56	m	13529	17/05/2013	11/06/2013	30	nil	15yrs	no	present	moderate	no	7*6cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	9.4	120	418	normal	aerogenos	positive	56	stsg
43	channamallappa	64	m	14188	24/05/2013	16/06/2013	30	nil	7yrs	no	present	moderate	yes	15*8cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	11.2	80	201	normal	aerogenos	positive	64	stsg
44	sidappa	53	m	14595	28/05/2013	26/06/2013	30	nil	7yrs	yes	present	moderate	no	10*8cm	dorsum of r foot	serous	ale GT with slough	sloping	well felt	absent	normal	11.1	80	225	normal	aerogenos	positive	59	stsg
45	laxman	75	m	13399	21/03/2013	27/04/2013	30	nil	no	no	present	moderate	no	5*7cm	medial side of l leg	serous	pale granulation	sloping	well felt	absent	normal	8.2	135	210	normal	s aureus	positive	60	sih
46	mallangouda	30	m	21557	19/06/2012	07/07/2012	150	nil	no	yes	absent	moderate	no	8*6cm	dorsum of l foot	serous	ale GT with slough	sloping	abs blw pop	absent	normal	12.5	110	138	normal	aeroginos	positive	54	stsg
47	ashok	48	m	22924	27/06/2012	03/07/2012	60	PBR	no	no	present	moderate	no	4*3cm	r gluteal region	serous	ale GT with slough	sloping	well felt	present	normal	7.3	130	116	normal	s aureus	positive	55	sih

48	sangamesh	38	m	7178	27/06/2012	01/09/2012	30	PBR	no	no	present	moderate	no	8*6cm	lumbosacral	serous	pale granulation	sloping	well felt	absent	normal	11.6	120	113	normal	e coli	positive	30	ss
49	shivappa	65	m	20517	28/06/2012	18/07/2012	60	nil	3yrs	no	present	moderate	no	5*9cm	dorsum of r foot	serous	pale granulation	sloping	well felt	absent	normal	11	70	106	normal	s aureus	positive	45	stsg
50	sharnappa	75	m	23850	07/07/2012	25/07/2012	35	nil	no	yes	present	moderate	no	15*10cm	med asp of r foot	serous	ale GT with sloug	sloping	well felt	absent	normal	9.3	40	61	not done	sterile	negative	47	stsg
51	ramesh	32	m	25838	11/07/2012	14/08/2012	30	nil	no	no	absent	moderate	no	8*4cm	dorsum of l foot	serous	pale granulation	sloping	well felt	absent	normal	11.7	110	121	normal	aeroginos	positive	36	stsg
52	chandpasha	48	m	24657	27/07/2012	11/09/2012	30	nil	5yrs	yes	absent	moderate	no	5*4cm	dorsum of r foot	serous	pale granulation	sloping	well felt	absent	normal	9.8	70	210	normal	s aureus	positive	60	sih
53	nagesh	80	m	22986	20/04/2013	17/05/2013	30	nil	4yrs	no	absent	moderate	no	5*1cm	med asp of r leg	serous	pale granulation	sloping	well felt	absent	normal	10.8	80	98	normal	s aureus	positive	35	ss
54	ashok	45	m	13793	16/08/2012	16/09/2012	90	nil	1yr	no	absent	moderate	no	10*6cm	blw lat mallelous	serous	pale granulation	punched ou	well felt	absent	normal	9.7	115	210	normal	e coli	positive	30	ss
55	dundappa	50	m	21321	19/08/2012	26/08/2012	30	nil	no	no	present	moderate	no	7*4cm	dorsum of foot	serous	ale GT with sloug	sloping	well felt	absent	normal	12.1	100	120	normal	k oxytocia	positive	28	sih
56	manohar	50	m	21324	26/09/2012	15/10/2012	90	nil	no	no	present	moderate	no	9*4cm	lateral asp l foot	serous	ale GT with sloug	sloping	well felt	absent	normal	10.4	120	111	normal	e coli	positive	35	ss
57	veerapakshappa	68	m	23201	03/01/2012	21/01/2012	30	nil	no	yes	present	poorly	no	2*2cm	dorsum of r foot	serous	ale GT with sloug	sloping	well felt	absent	normal	10.2	100	106	normal	sterile	negative	28	stsg
58	muthappa	45	m	876	10/01/2012	15/03/2012	30	nil	3yrs	no	present	moderate	no	3*3cm	dorsum of l foot	serous	ale GT with sloug	sloping	well felt	absent	normal	10.6	100	208	osteomyelitis	sterile	negative	43	ss
59	rajkumar	38	m	912	08/02/2012	27/02/2012	35	nil	no	no	present	moderate	no	15*10cm	med asp of leg	serous	ale GT with sloug	sloping	well felt	absent	normal	9.3	40	61	not done	sterile	negative	39	sih
60	hanumanth	67	m	1331	14/02/2012	24/02/2012	30	nil	no	no	absent	moderate	no	8*4cm	dorsum of l foot	serous	pale granulation	slpoing	well felt	absent	normal	11.7	110	121	normal	aeroginos	positive	37	sih
61	gurubasappa	56	m	1214	25/02/2012	10/03/2012	30	nil	no	no	present	moderate	no	5*6cm	dorsum of r foot	serous	ale GT with sloug	sloping	well felt	absent	normal	8.7	90	123	normal	sterile	negative	25	stsg
62	ramappa	45	m	1311	05/03/2012	21/03/2012	30	nil	5yrs	yes	absent	moderate	no	5*4cm	dorsum of l foot	serous	pale granulation	sloping	well felt	absent	normal	9.8	70	210	normal	sterile	negative	45	ss
63	tukaram	50	m	5921	18/04/2012	26/04/2012	45	nil	5yrs	no	present	moderate	no	3*3cm	mid 1/3rd l foot	serous	ale GT with sloug	sloping	well felt	absent	normal	13.5	15	106	normal	sterile	negative	35	sih
64	suresh	45	m	8972	10/05/2012	18/06/2012	30	nil	4yrs	no	absent	moderate	no	5*1cm	med asp of r leg	serous	pale granulation	sloping	well felt	absent	normal	10.8	80	98	normal	s aureus	positive	38	stsg