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Visual analysis of chronic wounds and its correlation with biofilm

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Abstract
Aim: To evaluate the biofilm using visual indicators that were clinically observable to see if any significant method of clinically detecting or predicting the presence of a biofilm can be developed.
Material and methods: The prospective study was conducted from late 2019 to 2021 in the Department of Surgery at Chatrapati Shivaji Subharti Hospital, a tertiary care super speciality institute of Swami Vivekanand Subharti University among patients with chronic wounds admitted in the department through surgery outpatient department/Emergency/transferred from other departments formed the study group. Samples were subjected for aerobic bacterial culture on blood agar, chocolate agar and MacConkey agar plates. The isolated bacterial pathogen was identified using standard bacteriological procedures which included colony morphology, gram stain and battery biochemical tests both for Gram positive and negative bacteria as per Gram stain. Efficacy of visual score was calculated using diagnostic tests (Sensitivity, Specificity, Diagnostic Accuracy) considering biofilm assessment by culture method as gold standard.
Results: Visual score *viz*. 0 (None), ≤5 (Not Sure), 6-8 (Probable) and ≥9 (Predicted) was reported in 0%, 8%, 31% and 61% of the subjects respectively. Sensitivity, specificity, positive predictive value, negative predictive value and accuracy rate of visual score considering culture method as gold standard was 94.74%, 83.72%, 99.10%, 45.57% and 94.19% respectively.
Conclusion: We concluded from our findings that clinical algorithm (visual score) may serve as a nearly signal to alert clinicians that the wound has diverted off its normal healing path. Detection of early (young) biofilms enable intervention before recalcitrance and/or infection become a problem.

Keywords: Biofilm, visual score, culture, chronic wound

Introduction
The treatment of infected chronic wounds remains a global challenge for health care systems. Chronic wounds are defined by prevention or delay of the normal wound healing process. It is termed chronic when it cannot achieve anatomical and functional integrities through normal, orderly and timely repair process under influence of various internal or external factors [1].
Regardless of the type (diabetic foot, burns, venous leg ulcers, pressure ulcers) if managed inadequately, chronic wounds cause a dramatic deterioration of patient's life quality [2]. Moreover, the complications caused by microbial biofilm settled within chronic wounds pose a risk of amputation, development of a systemic infection and as a consequence, may lead to a deterioration of a patient's health or even their death [3]. Devoid of the skin's protective barrier and repeatedly flooded with nutrient-rich exudate, chronic wounds are attractive niches for microbial colonization and formation of biofilm [4]. The results of recent studies indicate that biofilm is the dominant form of microbial existence in wounds [5, 6].
Biofilm is frequently defined based on *in vitro* observations. Classic definitions often describe biofilm as bacteria attached to surfaces, encapsulated in a self-produced extracellular matrix and tolerant to antimicrobial agents (including antibiotics and topical preparations or impregnated dressings). In addition, biofilm development is often described as multi-stage, beginning with the initial attachment of single cells to a surface, maturation of the biofilm and, lastly, dispersal of bacteria from the biofilm [7-10].
Seven features have been used to indicate the presence of infection in human chronic wounds. These have included indicators such as a pale wound bed, a yellow discharge, necrotic tissue, a clear slime and a putrid smell. Being able to recognize the clinical symptoms of a wound infected with a biofilm is vital to improving the treatment of non-healing chronic wounds in both

human and animals [11]. However, clinical diagnosis is highly subjective. Hence, due to concerns in biofilm identification, continued work is required to develop techniques that can rapidly identify biofilms *in vivo*. Hence the clinical assessment of a wound biofilm is vital for diagnosis. However to date, no clear definition is used by clinicians to indicate biofilm infection.

Culture is the most common accepted technique to analyse the presence of a wound biofilm. The culture techniques needed to cultivate biofilm is not readily available in our state. This result in non-identification of biofilms and the treatment of all wounds continue in the same manner.

In this study we evaluated the biofilm using visual indicators that were clinically observable to see if any significant method of clinically detecting or predicting the presence of a biofilm can be developed. Such a clinical assessment system will greatly improve the vastly untouched area of biofilm, thereby improving wound care for chronic ulcers including more aggressive approach in case a biofilm is predicted through the clinical assessment method.

Material and Method

The prospective study was conducted from late 2019 to 2021 in the Department of Surgery at Chatrapati Shivaji Subharti Hospital, a tertiary care superspeciality institute of Swami Vivekanand Subharti University among patients with chronic wounds admitted in the department through surgery outpatient department/Emergency/transferred from other departments formed the study group.

Inclusion criteria

- All patients presenting with venous ulcer, pressure ulcer, diabetic foot, pressure wounds, bed sores, burns.

Exclusion Criteria

1. Patient not giving consent
2. Pregnant women

Investigations

The patients were worked up thoroughly and subjected to:

- Detailed history and clinical examination.
- Routine hematological investigation: Hb, TLC,DLC, RBS
- Viral markers: HCV, HBsAg, HIV 1 & 2
- Culture
- Detection of biofilm
- Aerobic bacterial culture:

Sample collection

Pus sample; that is either frank pus or discharge was collected with all aseptic precautions and was transported to the clinical microbiology laboratory.

Sample Processing

a) **Direct Microscopy**

- The smear was made from the sample.
- Gram staining was performed on the dried fixed smear.

b) **Culture**

- Samples were subjected for aerobic bacterial culture on blood agar, chocolate agar and MacConkey agar plates.
- All culture plates and BHI broth were incubated at 37 degree celcius for 48 hours. If any growth is seen on culture media, the colony morphology of bacterial pathogen/pathogens were observed and documented.
- The isolated bacterial pathogen was identified using standard bacteriological procedures which included colony morphology, gram stain and battery biochemical tests both for Gram positive and negative bacteria as per Gram stain.
- The following are used as Quality control stains:
- Escheriichia coli ATCC 25922
- Pseudomonas aeruginosa ATCC 27853
- Staphylococcus aureus ATCC 25923

Identification and antibiotic susceptibility testing

- The isolated bacterial pathogens were identified using standard bacteriological procedures.
- Antibiotic susceptibility testing was done as per recommended CLSI guidelines.

Detection of biofilm by tissue culture method

- This quantitative test described by (Christensen *et al*. is considered the gold-standard method for biofilm detection.
- Organisms isolated from fresh agar plates were inoculated in 10 mL of trypticase soy broth with 1% glucose.
- Broths were incubated at 37oC for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well- flat bottom polystyrene tissue culture treated plates (Sigma- Aldrich, Costar, USA) were filled with 200 µL of the diluted cultures.
- The control organisms were also be incubated, diluted and added to tissue culture plate. Negative control wells contained inoculated sterile broth.
- The plates were incubated at 37oC for 24 h. After incubation, contents of each well was be removed by gentle tapping.
- The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times. This removes free floating bacteria.
- Biofilm formed by bacteria adherent to the wells were fixed by 2% sodium acetate and stained by crystal violet (0.1%).
- Excess stain was removed by using deionized water and plates were then kept for drying.
- Optical density (OD) of stained adherent biofilm was obtained by using micro ELISA autoreader (model 680, Biorad, UK) at wavelength 570 nm. The experiment was performed in triplicate and repeated three times.

Visual score to detect biofilm

Does the surface have any friable granulation tissue?	No	Yes	Grade 1
Does the surface have any necrotic tissue?	No	Yes	Grade 2
Does the surface have high moisture?	No	Yes	Grade 2
Does the surface have a slimy layer?	No	Yes	Grade 2
Does the surface substance detach easily and a traumatically from the underlying would bed using physical removed techniques such as swabs, pads or sharp debridement?	No	Yes	Grade 1
Does the surface substance persist despite use of autolytic or enzymatic debridement?	No	Yes	Grade 3

Does the surface substance re-form quickly (in 1-2 days) in the absence of frequent intervention (e.g. cleansing, debridement)?	No	Yes	Grade 3
Does the wound respond poorly to topical or systemic antibiotics?	No	Yes	Grade 1
Does the wound respond poorly or slowly to dressings than contain antiseptic agents (e.g. silver, iodine, PHMB), including products that may control biofilm *in vitro* (e.g., cadexomer iodine, nanocrystalline silver or ionic silver containing carboxymethyl cellulose dressings)?	No	Yes	Grade 1
Does the wound respond favourably to multi-modal strategies such as physical debridement, cleansing, and topical antimicrobial agents and dressings?	No	Yes	Grade 1

- Score 0- no
- Score 1- yes
- Na- not applicable to this patient
- Score less than 5: not sure
- Score between 6-8: probable
- Score more than 9: confirmed

The data was collected and subjected to statistical analysis. Then we compared findings of biofilm by clinical algorithm and culture in our microbiology lab.

Statistical analysis

Data was analysed using SPSS software version 24. Efficacy of visual score was calculated using diagnostic tests (Sensitivity, Specificity, and Diagnostic Accuracy) considering biofilm assessment by culture method as gold standard.

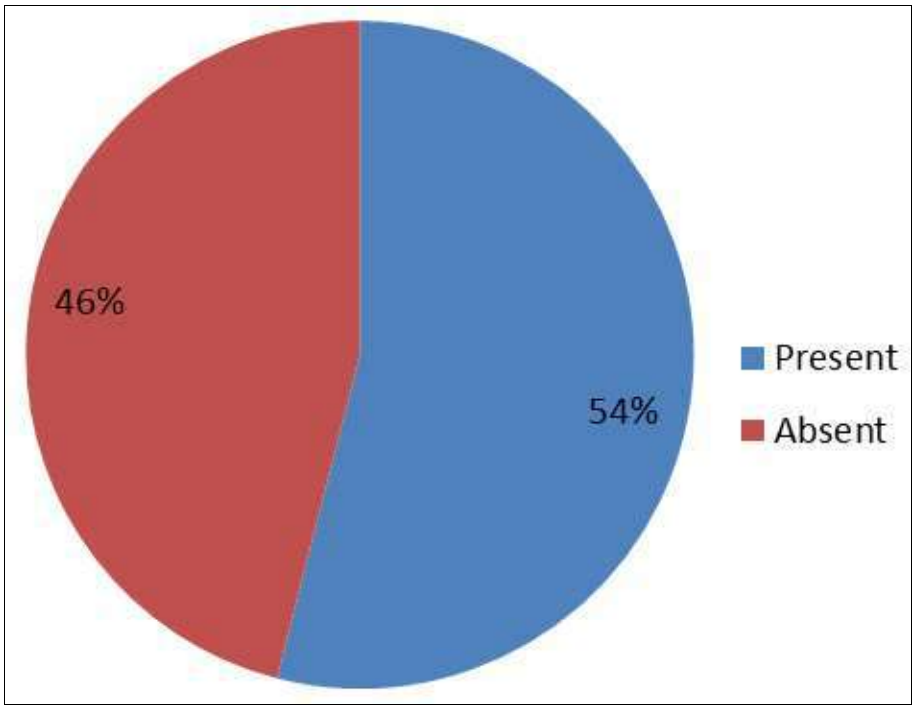
Results

Out of 100 subjects, maximum were from the age group of >60 years (23%) followed by 51-60 years (20%) and 31-40 years (19%). Minimum were from the age group of 10-20 years (7%) followed by 41-50 years (14%). Hence chronic wounds probability was more in old age group. Out of 100 subjects, there were 57 males and 43 females. Most of the subjects live in rural area (68%) as compared to urban area. Hence injury was related more with rural area, poor socioeconomic background. Co-morbidities *viz.*diabetes, hypertension and tuberculosiswas reported among 29%, 18% and 7% of the subjects respectively (table 1). Hence diabetes was associated with chronic wounds.

Table 1: Gender, location and co-morbidities among the study subjects

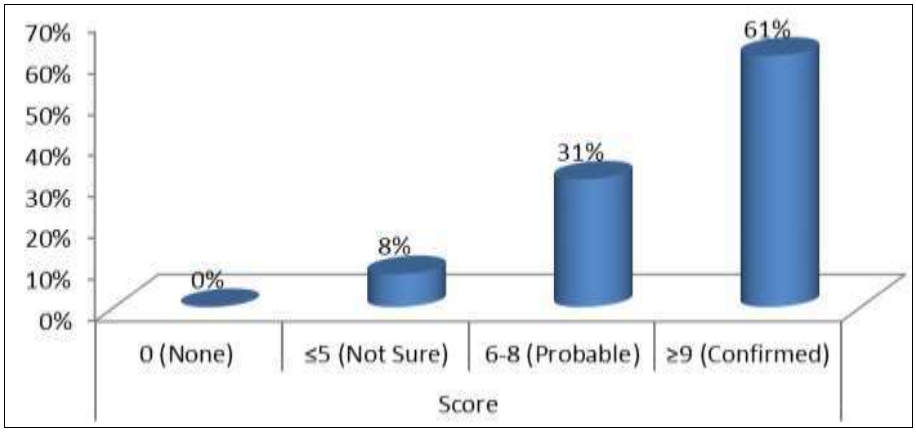
	N=100	%
Gender		
Male	53	53
Female	47	47
Location		
Rural	68	68
Urban	32	32
Co-morbidities		
Diabetes	29	29
Hypertension	18	18
Tuberculosis	7	7

According to culture method, biofilm was present in 54 samples and absent in 46 samples (graph 1).



Graph 1: Detection of biofilm by culture method

Visual score *viz*. 0 (None), ≤5 (Not Sure), 6-8 (Probable) and ≥9 (Predicted) was reported in 0%, 8%, 31% and 61% of the subjects respectively (graph 2).



Graph 2: Visual Score among the study subjects

Sensitivity, specificity, positive predictive value, negative predictive value and accuracy rate of visual score considering culture method as gold standard was 94.74%, 83.72%, 99.10%, 45.57% and 94.19% respectively (table 2).

Sensitivity was 90%. 90% samples were identified as truly positive by the screening test.

Specificity was 4% i.e. 4% samples were identified as truly negative by the screening test (table 2).

Table 2: Necrotic tissue according to biofilm outcome by culture

Necrotic Tissue	Biofilm				Total	
	Absent		Present			
	N	%	N	%	N	%
Absent	2	4.3	5	9.3	7	7
Present	44	95.7	49	90.7	93	93
Total	46	46	54	54	100	100

Sensitivity was 94%. 94% samples were identified as truly positive by the screening test. Specificity was 6% i.e. 6% samples were identified as truly negative by the screening test (table 3).

Table 3: Re-form quickly according to biofilm outcome by culture

Necrotic Tissue	Biofilm				Total	
	Absent		Present			
	N	%	N	%	N	%
Absent	3	6.5	3	5.6	8	8
Present	43	93.5	51	94.4	94	94
Total	46	46	54	54	100	100

Sensitivity, specificity, positive predictive value, negative predictive value and accuracy rate of visual score considering culture method as gold standard was 94.74%, 83.72%, 99.10%, 45.57% and 94.19% respectively (table 4).

Table 4: Diagnostic efficacy of visual score considering culture method as gold standard

Parameters	Value	95% CI (Lower to Upper Bound)
Sensitivity	94.74%	85.38% to 98.90%
Specificity	83.72%	69.30% to 93.19%
Positive Predictive Value	99.10%	98.25% to 99.54%
Negative Predictive Value	45.57%	21.64% to 71.74%
Accuracy Rate	94.19%	87.64% to 97.88%

Discussion

Elgharably *et al.* [12] provide the first direct evidence of biofilm involvement in human deep sternal wound infection. It has been demonstrated that biofilm impairs key healing processes such as the inflammatory immune response, granulation tissue formation and epithelialization. Culture is the most common accepted technique to analyse the presence of a wound biofilm. But due to time consuming and cost involvement, search was still going to find the cost effective and quick method. In this study we evaluated the biofilm using visual analysis score method and correlated it with the culture outcome.

In this study, 100 subjects were enrolled and with help of visual scoring of chronic wounds, sensitivity and specificity of each clinical parameters was calculated and correlated with culture and was observed that visual score can detect biofilm and sensitive and specific markers were differentiated.

Sensitive Markers for Detection of Biofilm	Sensitivity/ Specificity	Specific Markers for Detection of Biofilm	Sensitivity/ Specificity
Does the Surface have Any hyperganulation tissue?	79%/23% 90%/4%	Does the surface discharge persist despite use 10%povidone iodine Dressings?	29%69%
	96%/0%		12%95%
Does the Surface have any necrotic tissue?	61%/41%		
Does the surface have significant wound discharge?		Does the wound respond to topical antibiotics?	
Does the Surface have a slimy layer?	94%/6%	Does the surface substance detach easily and Atraumatically from the underlying would bed using physical removal via back f Scalpel or Forceps?	14%61%
	77%/21%		
Does the surface Discharge re- form quickly (1- 2 days) in the absence of frequent intervention?	92%13%		
Does the Wound have foul odor?			
Does the surrounding skin is Inflamed?			

The formation of granulation tissue is central to the proliferative phase of wound healing, however in some cases the formation of granulation tissue continues without the migration of epithelial cells across the wound bed. As this occurs, the granular tissue

increases to a level higher than surrounding healthy tissues. This forms areas of friable, irregular heaped tissues referred to as hypergranulation. In our study, hypergranulation tissue on the surface of wound was noted on day1 by the dark red, heaped up granulation tissue rising above the floor of the ulcer, 78 wound surfaces had hypergranulation tissue, the sensitivity was observed to be 79%. Hence it's a sensitive visual marker to detect biofilm in chronic wounds. Problems like differentiating between hypergranulation tissue and basal cell carcinoma and fungal infections were also seen during the study.

In our study, necrotic tissue was observed in 93 wounds and was noted on day 1 for any black or disscoloured tissue. The sensitivity was calculated to be 90% according to biofilm outcome by culture. The presence of necrotic tissue may prevent the clinician from making an accurate assessment of the extent and severity of the wound. Necrotic tissue serve as the source of nutrients for bacteria to form biofilm. Sometimes necrotic tissue was mistakenly refered to dry scabs and sero crust and hematomas, as they can also appear dark colour. Presence of necrotic tissue may prove formation of biofilm, hence it's a good clinical parameter to detect biofilm in chronic wounds.

Visual score *viz*. 0 (None), ≤5 (Not Sure), 6-8 (Probable) and ≥9 predicted was reported in 0%, 8%, 31% and 61% of the subjects respectively. According to visual score, biofilm was present in 61 cases while it was doubtful in 31 cases and absent in 8 cases. According to culture method, biofilm was present in 54 cases and absent in 46 cases in our study. In a study by Matthew Malone *et al*. [13], the pooled prevalence of biofilms in chronic wounds was 78.2% (CI 61.6–89, P<0.002). Biofilm prevalence varied greatly over all studies, however the percentage(s) of positive biofilm samples was no lower than 60% noted in three studies, with all remaining studies identifying 100% biofilm prevalence [13]. Given the relatively small sample size and the co-variable of 4 different chronic wound aetiologies, inferences regarding whether biofilms were more prevalent in one particular chronic wound were not possible.

Sensitivity, specificity, positive predictive value, negative predictive value and accuracy rate of visual score considering culture method as gold standard was 94.74%, 83.72%, 99.10%, 45.57% and 94.19% respectively. Hence visual score had good correlation with culture method in this study. In a review by Burmølle *et al*. (2010) [14], it was highlighted that the newest in situ detection and identification techniques indicated low bacterial diversity and overall mono-species biofilms in mixed-species infections. All methods have their limitations, including microscopy. Besides being time consuming and labor-intensive, fluorescence microscopy only reveals what you are looking for (i.e., when using species specific probes) and is sensitive toward sampling from the right area (i.e., from the area where biofilms are present). As stated by Lasse Kvich *et al*. [15], the only way to visualize the existence of bacteria in a mixed-species biofilm is to use different visualization techniques such as microscopic visualization coupled with molecular approaches, such as FISH and CLSM, or similar. Some of the disadvantages of using FISH for detection of mixed species biofilms have been reported elsewhere (Costa *et al*., 2017) [16]. Many new molecular methods, combined with microscopy, have evolved over time and are becoming more accessible, resulting in more studies reporting mixed-species biofilms.

In this study; most of the subjects live in rural area (68%) as compared to urban area (32%). Hence injury was related more with rural area, poor socioeconomic background. According to Rachel A. Fayne *et al*. [17], nutrition, financial burden, socioeconomic and education status, and acute and chronic stress are variables that have either direct (epigenetic) or indirect impact on wound healing and patient's quality of life. Wound care is costly and remains a challenge placing economic burden on patients. Individuals with low socioeconomic status are more likely to suffer from type 2 diabetes and have higher mortality risk. Furthermore, low socioeconomic status is associated with many other factors that contribute to poor clinical outcomes in diabetic patients, such as access to prevention, metabolic and infection control, and psychological stress. In addition, living in poverty, education level, and coping with depressive symptoms have been shown to have significant effects on glycemic control and hemoglobin HbA1C. Low socioeconomic status is also considered a risk factor for the development of diabetic foot problems including amputation. However, mechanisms by which socioeconomic factors affect gene expression that contributes to diabetes and development of diabetic foot ulcers remain unclear [17].

The limitation of present study is small sample size. Also as ours is the first study to use visual score for identification of biofilm, so were not able to compare our findings. Therefore we lack authentication of results.

One of the strengths of our study was that it was conducted within the context of a universal health care system with the participation of all primary care centres in the region, and it included the majority of patients receiving some type of health care.

Conclusion

We concluded from our findings that clinical algorithm (visual score) may serve as a nearly signal to alert clinicians that the wound has diverted off its normal healing path. Detection of early (young) biofilms may identify at-risk patients and enable intervention before recalcitrance and/or infection become a problem.

Further research is needed on clinical algorithm (visual score) to identify and characterize biofilms in ways that optimize their validity in diagnosing or screening patient risk of infection or delayed healing and to inform clinical decisions. This research will help validate biofilm's capacity to support wound care clinical practice decisions and establish their importance in guiding clinical practice. However, qualitative and non-quantative end points were defined and, further studies are required to validate our findings and to quantify its effects.

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