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A Study on Role of Anaerobic Bacteria in Chronic Non-Healing Ulcers

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Abstract

Ulcers are defined as a break in the continuity of epithelium. Healing of an ulcer is a natural process through steps of haemostasis, inflammation, proliferation and maturation .Clinically, ulcers that take more than three months to heal are usually considered to be chronic [1].Chronicity of ulcers is usually associated with its infection [2].The normal microbial flora of the skin and mucosa account for a vast majority of organisms that may infect ulcers. Chronic ulcers are defined as wounds that have failed to proceed through the orderly process that produces satisfactory anatomic and functional integrity or that have proceeded through the repair process without producing an adequate anatomic and functional result [1].

Some common aetiologies of chronic ulcers are:-

a. Venous diseases leading to local venous hypertension(e.g.- varicose veins)
b. Arterial diseases, either large vessel(atherosclerosis) or small vessel(diabetes)
c. Arteritis, associated with autoimmune disease (rheumatoid arthritis, lupus, etc.)
d. Chronic infections like tuberculosis or syphilis
e. Neoplastic conditions like Squamous cell carcinoma, basal cell carcinoma, sarcoma, etc.
f. Tissue necrosis with ulceration due to prolonged pressure (pressure sores), generally in severely ill, hospitalized patients. [3]

Colony counts of aerobic bacteria from moist areas such as the axilla or toe web spaces can reach 10^7 bacteria /cm^2, whereas dry areas such as the forearm or trunk may harbour 10^2 or fewer bacteria/cm^2 **[4]**. Anaerobic bacteria are also present on human skin, with colony counts up to 10^6 bacteria/cm^2.So, both aerobes and anaerobes can infect an ulcer, contributing to its chronicity.

Moreover, the longer an ulcer remains unhealed, the more likely it will acquire multiple aerobic infection and significant anaerobic population. Chronic wounds have low oxygen tension that facilitates the growth of anaerobes. Common anaerobic colonizers include *Clostridium, Prevotella, Bacteroides, Peptostreptococcus* and *Porphyromonas.* More than 95% of diabetic foot infections contain anaerobes along with aerobes such as *Staphylococcus aureus, Enterococcus* spp. and coliforms. Decubitus ulcers that are most common in sacral area are mostly contaminated with faeces and thus by anaerobic bacteria. The isolation and detection of aerobic bacteria are routinely done in the microbiology laboratories, but anaerobic bacteria require special conditions for growth. So in order to properly treat a chronic non-healing ulcer, any associated infection should be ruled out first before commencement of any therapeutic intervention.

So we had planned to do a prospective study on the bacterial causes of chronic non-healing ulcers, to perform the antibiotic sensitivity of the isolates and to study the prevalence of different bacteria causing these ulcers.

Materials & Methods

This prospective, microbiological study was carried out in the Department of Microbiology, MKCG Medical College

Berhampur, from august 2016 to July 2017. The study included all patients with chronic non-healing ulcers of at least 3months duration in the surgery ward at M.K.C.G. Medical College and Hospital, Berhampur.

Inclusion criteria

1. The patients with a non-healing ulcer for at least 3 months.
2. The patients with post-operative wounds received antibiotic medication but did not heal for at least 3 months since the operation.
3. The patients with post-traumatic ulcers received antibiotic medication but did not heal for at least 3 months.
4. Any patient undergoing anti-tubercular treatment having a non-healing ulcer for more than 3 months was also included.

Sampling and sampling size

Samples were obtained from the patient only at the time of wound dressing. The pus was collected from the base of the ulcer by using a sterile cotton swab.

Sample Collection: The ulcer was thoroughly cleaned with normal saline, and the use of any kind of antiseptic was avoided. Then two swabs were taken from the base of each ulcer and contamination with normal flora and air was avoided. One swab was used for preparing smears for Gram stain; other was used for aerobic culture. For anaerobic culture, another swab was collected in Robertson's Cooked Meat medium and brought to the laboratory. It was processed immediately without refrigeration.

Macroscopic examination: The sample was observed for foul smells, colour and consistency.

Staining: The smeared slides were subjected to Gram staining and examined for pus cells, Gram positive/Gram negative bacteria.

Culture

The first swab was inoculated on Blood Agar and Mac-Conkey Agar aerobically and another swab was inoculated in Robertson's cooked meat medium first from which it is plated on Brucella Laked Blood Agar with Vitamin K and 1% Glucose and Kanamycin Vancomycin Laked Blood Agar for anaerobic culture simultaneously.

The aerobic culture plates were incubated aerobically for 24 hrs. The anaerobic culture plates were incubated in an anaerobic gas pack jar for 3 days. A metronidazole disc (5µg) was placed at the junction of primary and secondary streaking lines.

After incubation for the required time, another Gram's stain from all the discrete colony types was performed. The culture from Brucella Laked Blood Agar and Kanamycin Vancomycin Laked Blood Agar was inoculated again into Robertson Cooked Meat Medium for further subcultures and biochemical testing

Results and discussion

Out of total 128 cases, 71.1% were male patients. Among both the sexes majority belonged to 61-70 years age group (Table-1).

Age(In years)	Male No. (%)	Female No. (%)	Total No. (%)
<40	5 (3.9)	3 (2.3)	8 (6.3)
41-50	16 (12.5)	5 (3.9)	21 (16.4)
51-60	21 (16.4)	10 (7.8)	31 (24.2)
61-70	29 (22.6)	16 (12.6)	45 (35.1)
>70	20 (15.7)	3 (2.3)	23 (18.0)
Total	91 (71.1)	37 (28.9)	128 (100)

The most common associated factor of chronic ulcers was diabetes mellitus (29.7%). (Table-2)

Table-2:-Associated factors with growth of organisms

Associated factors	No. of patients (%)	Growth (%)	No growth (%)
Diabetes mellitus	38 (29.7)	35 (92.1)	3 (7.9)
Squamous cell carcinoma	7 (5.4)	6 (85.7)	1 (14.3)
Tuberculosis	4 (3.1)	3 (75.0)	1 (25.0)
HIV/AIDS	5 (3.9)	4 (80.0)	1 (20.0)
Venous ulcers	18 (14.1)	15 (83.3)	3 (16.7)
Trauma	26 (20.3)	22 (84.6)	4 (15.4)
Post-operative	18 (14.1)	16 (88.8)	2 (11.2)
Pressure ulcers	12 (9.4)	11 (91.6)	1 (8.4)
Total	128(100)	112(87.5)	16(12.5)

All the samples were cultured, among which bacterial growth was detected in 112 (87.5%) cases. No growths could be found in rest 12.5% cases. Total of 157 organisms were isolated.

Table-3:-Type of organisms isolated

Type of organism	No. of organism	Percentage
Aerobic	95	60.5
Anaerobic	62	39.5
Total	157	100

Among 112 culture positive cases monomicrobial growth was noticed in 47 cases while polymicrobial growth in 65 cases. Out of 47 monomicrobials isolated, 34 were aerobic and 13 were anaerobic. *Staphylococcus aureus* was the most common (38.2%) aerobic isolate. On the other hand, *Clostridium perfringens* (30.7%) was found to be the leading anaerobic agent of chronic ulcers. Polymicrobial growth was described under the groups like mixed aerobes (24.6%), mixed anaerobes (6.1%) and mixed aerobes and anaerobes (69.2%). (Table-4).

Table-4:-Pattern of isolates in 112 culture positive cases

Monomicrobial	Aerobic isolates		Anaerobic isolates	
	Organisms	No. (%)	Organisms	No. (%)
	Staphylococcus aureus	13 (38.2)	*Clostridium perfringens*	4 (30.7)
	Klebsiella spp.	8 (23.5)	*Peptostreptococcus spp.*	3 (23.1)
	Pseudomonas aeruginosa	5 (14.7)	*Bacteroides fragilis*	2 (15.4)
	Proteus spp.	4 (11.8)	*Porphyromonas spp.*	2 (15.4)
	Streptococcus pyogenes	2 (5.9)	*Peptococcus spp.*	1 (7.7)
	Escherichia coli	2 (5.9)	*Prevotella spp.*	1 (7.7)
Total 47(41.9%)		34(72.3%)		13(27.7%)

Polymicrobial	Mixed aerobes only		Mixed anaerobes only		Mixed (Aerobe+Anaerobe)	
	Organisms	No.	Organisms	No.	Organisms	No.
	Staphylococcus aureus Klebsiella spp. Pseudomonas aeruginosa	3	*Clostridium perfringens Peptostreptococcus spp.*	2	*Staphylococcus aureus Escherichia coli Clostridium perfringens*	8
	Staphylococcus aureus Klebsiella spp	3			*Staphylococcus aureus Clostridium perfringens*	6
	Staphylococcus aureus Pseudomonas aeruginosa	3	*Bacteroides fragilis Peptostreptococcus spp.*	2	*Staphylococcus aureus Bacteroides fragilis*	5
	Klebsilla spp. Proteus spp. Escherichia coli	2			*Klebsiella spp. Peptostreptococcus spp.*	5
	Klebsiella spp. Escherichia coli	2	*Porphyromonas spp Peptococcus spp.*	1	*Klebsiella spp. Pseudomonas aeruginosa Bacteroides fragilis*	4
	Pseudomonas aeruginosa Proteus spp.	2			*Pseudomonas aeruginosa Clostridium perfringens*	4
	Escherichia coli Streptococcus pyogenes	1			*Staphylococcus aureus Pseudomonas aeruginosa Peptostreptococcus spp.*	3
					Klebsiella spp. Proteus spp. Peptococcus spp	3
					Klebsiella spp. Bacteroides fragilis	3
					Pseudomonas aeruginosa Prevotella spp.	2
					Escherichia coli Peptococcus spp.	2
Total 65(58.1%)	Total	16(24.6%)	Total	4(6.1%)	Total	45(69.2%)

Most of Staphylococcus aureus showed sensitivity towards Ceftriaxone (87.2%), while gram negative bacilli showed maximum sensitivity towards Cefoperazone and Ceftazidime (Table-5).

TYPE	Organisms	TOTAL NO.	A/S	AMC	OX	G	LE	CTR	CAZ	CPZ	TOTAL NO.	VA	LZ	TOTAL NO.	I/C	PIT
GPC	*Staphylococcus aureus*	47	27(57.4)	19(40.4)	27(57.4)	22(46.8)	25(53.2)	41(87.2)	–	40(85.1)	20	20(100)	20(100)	–	–	–
	Streptococcus pyogenes	3	3(100)	2(66.7)	–	2(66.7)	–	3(100)	–	–	–	–	–	–	–	–
GNR	*Klebsiella spp.*	33	–	0(0)	–	10(30.3)	18(54.5)	15(45.4)	5(15.1)	–	–	–	–	13	13(100)	13(100)
	Pseudomonas aeruginosa	26	–	0(0)	–	11(42.3)	22(84.6)	12(46.1)	17(65.3)	–	–	–	–	15	15(100)	15(100)
	Escherichia coli	17	–	3(17.6)	–	–	13(76.5)	0(0)	4(23.5)	15(88.2)	–	–	–	12	12(100)	12(100)
	Proteus spp.	11	–	0(0)	–	4(36.3)	9(81.8)	7(63.6)	9(81.8)	5(45.4)	–	–	–	5	5(100)	5(100)

All the anaerobic isolates were found to be sensitive to metronidazole. *Bacteroides fragilis* was found to be resistant to Kanamycin and Vancomycin whereas Prevotella spp. was found to be resistant to Vancomycin alone. Peptostreptococcus spp. was found to be resistant to kanamycin alone. This study demonstrates the infection of chronic ulcers and their antibiotic susceptibility pattern. In this study males (71.1%) suffered more from chronic ulcers than females (28.9%). The median age of the patients was 60.5years. In a study, 71.3% of the patients were female and 28.7% were males and the median age was 60.5years. [5] The factors associated with the chronic non-healing ulcer were found to be diabetes mellitus (29.7%), trauma (20.3%), post-operative wounds (14.1%), venous ulcers (14.1%), squamous cell ca. (5.4%), Tuberculosis (3.1%), HIV/AIDS (3.9%) and pressure ulcers (9.4%). Maximum cases are in people suffering

from diabetes mellitus. This is inconsistent with a study in which 54.2% patients had venous ulcer, 15% had arterial ulcers and 3.3% had ulcers due to a malignancy. [6]

From 157 isolates obtained from 112 infected cases, 60.5% were aerobic and 39.5% were anaerobic. This correlates with the study in which 51% aerobic and 49% anaerobic out of 220 isolates from 44 infected ulcers. [7], but in contrast to the study of chronic leg ulcers in which out of 325 isolates only 22% were anaerobic [8].The anaerobic isolates were more common in diabetic ulcer scrapings similar to the study showing 45% anaerobic isolates. [9] The predominant aerobic isolates were *S.aureus* (34.3%), *Klebsiella* (24.1%) and *Pseudomonas* (18.9%) and the predominant anaerobic isolates were *Clostridium perfringens* (35.5%), *Bacteroides fragilis (*22.6%*)* *Peptostreptococcus* spp. (17.7%) and *Porphyromonas* spp. (3.2%). This is consistent with a study of 538 isolates from diabetic foot ulcers in 130 patients, found *Staphylococcus*, *Enterococcus* and *Peptostreptococcus* as the predominant isolates [7]; and of 285 isolates from 43 diabetic foot ulcers, found *Peptostreptococcus*, *Bacteroides*, and *Prevotella* as the predominant isolates [10] and also with a study of 711 isolates from 340 trauma patients found *Bacteroides fragilis*, *Clostridium* spp., *Peptostreptococcus* and *Staphylococcus aureus*. [11].similar distribution was also seen in various studies. [12-17]

Monomicrobial isolates were encountered from 47 samples (41.9%) and Polymicrobial isolates from 65 samples (58.1%).Most common association in polymicrobial infections is of aerobe and anaerobe organisms (69.2%) with *S.aureus*, *E.coli*, *Clostridium perfringens* being most frequent (17.8%).This is contrary to a study in which out of 96 samples, 54(56.2%) were monomicrobial and the most common association is of *Staph.aureus and Pseudomonas spp*. [18] and also to the study 86 cases with 41% showing polymicrobial growth.[19]

S.aureus was tested for first line antibiotics. The sensitivity pattern was ampicillin-sulbactam (57.4%), co-amoxiclav (40.4%), oxacillin (57.4%), gentamicin (46.8%), levofloxacin (53.2%), ceftriaxone (87.2%). 20 isolates resistant to oxacillin were considered as methicillin resistant *S.aureus*(MRSA), which accounted for 42.6% of all S.aureus were further tested for 2nd line antibiotics, the sensitivity pattern of which was vancomycin(100%) , linezolid(100%). In a study ,out of 150 isolates of *S.aureus* isolated from different clinical specimens, the sensitivity pattern of *S. aureus* to the following antibiotics; Gentamicin, Amoxycillin/clavulanate, Streptomycin, Cloxacillin, Erythromycin, Chloramphenicol, Cotrimoxazole, Tetracycline, Penicillin, Ciprofloxacin, Ofloxacin, Levofloxacin, Ceftriaxone, Amoxycillin and vancomycin were 92.4%, 63.0%, 44.2%, 35.8%, 52.4%, 61.9%, 15.5%, 31.2%, 7.1%, 78.9%, 76.6%, 100%, 71.4%, 30.7% and 100% respectively. [19]*Streptococcus pyogenes* showed 100% sensitivity to ampicillin, amoxicillin and 3rd generation cephalosporins.

Strains of *Pseudomonas* showed 100% sensitivity to Imipenem-Cilastatin, and Piperacillin-Tazobactam, followed by 84.6% to Levofloxacin. It showed total resistance towards amoxicillin. *Proteus* spp. showed maximum sensitivity of 100% towards Imipenem-Cilastatin and Piperacillin-Tazobactam, followed by Levofloxacin and Ceftazidime at 81.8%. It showed total resistance towards Amoxicillin. Klebsiella spp. showed maximum sensitivity of 100% towards Imipenem-Cilastatin, Piperacillin-Tazobactam, followed by Levofloxacin at 54.5% and Ceftriaxone at 45.4%.

Among the anaerobic organisms, all of them were found to be sensitive to metronidazole. *Bacteroides fragilis* and

Prevotella spp. were found to be resistant to kanamycin and vancomycin. *Porphyromonas* spp. were found to be resistant to kanamycin but sensitive to vancomycin. *Peptostreptococcus* spp. was found to be resistant to kanamycin alone.

Non healing ulcers that are left unattended for prolonged time are prone to malignant transformation and may include both squamous cell carcinoma and basal cell carcinoma [1]. Chronic persistence of ulcer may lead to gangrene, ultimately needing amputation.

With the increasing incidence of obesity, chronic medical conditions, and an increasing life expectancy, the healthcare cost of non-healing ulcers has recently been estimated to be high [18]. Infection is a major reason for the chronic persistence of any ulcer. The increasing resistance of the bacteria to various antibiotics is also a great concern. In this study, 42.6% of the S.aureus isolates were found to be MRSA, but none were VRSA. Among gram negative bacteria, many were resistant towards ampicillin-sulbactam and amoxicillin-clavulanate and other drugs like fluoroquinolones and cephalosporins. Considering this sensitivity pattern, proper antibiotic prophylaxis of any patient with an ulcer should be taken to either prevent the chronicity altogether, or, to limit the duration of the chronic ulcer.

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