MICROBIOTA OF PURULENT NECROTIC LESIONS IN PATIENTS WITH DIABETIC FOOT SYNDROME

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Abstract: The Diabetic Foot Syndrome (DFS) is one of the most serious complications of the diabetes mellitus (DM). Infections play a minor role in the genesis of a diabetic foot lesion but assume considerable importance in existing injuries as a risk factor for progression to amputation. Optimal treatment of these diabetic foot infections requires recognizing which foot ulcers are infected and prescribing pathogen-appropriate antibiotic therapy. The main goal of this study was the identification of microbiota's structure of purulent-necrotic lesions in 34 patients with a severe form of DFS. The certain amount of affected area was taken as material for research. The microbial composition was identified by sequencing of DNA fragments of 16S rRNA gene including V3 and V4 variable sequences. As a result, the microbiota of purulent-necrotic lesions was characterized by poly-microbial association. The high degree of the anaerobe bacteria which are generally responsible for the development of intrahospital infectious diseases was observed.

Keywords: Diabetic Foot Syndrome, Diabetes mellitus, Microbiota, Purulent-necrotic lesions, Diabetic foot ulcer, Osteomyelitis, Bacteria, Comparative analysis of a microflora.

1 Introduction

According to the International Diabetic Federation (IDF) number of people affected by DFS in 2015 accounted for more than 415 million worldwide, by 2040 this number will increase up to 642 million. In Kazakhstan, 715500 people are affected by DFS (The diabetic Atlas of IDF, 2015) and more than 1 million people are glucose-intolerant. Amongst the diabetes mellitus diseases with possible late complications, DFS is on top of the list resulting in early disability and lethality. The frequency of DFS accounts from 4.6 to 25% (1-2): The secondary infection of DFS is the leading reason for amputation of legs. Every hour 55 patients undergo amputation due to DFS. (3-4) The purulent infection in patients is usually very heavy, and often it develops into septic character. (5-7) The main causative agent of a wound infection in DFS is S. aureus – 52%, in the second place in regards to occurrence are gram-negative microorganisms (18.4%). (8) However, use of traditional methods of cultivation does not allow to carry out full identification of the bacteria which are present at the lesion centers in DFS. Development and deployment of new molecular tools made a significant contribution to understanding the role of microbiota at DFS. Scientific progress in the sequencing of bacterial genomes, in combination with the development of new molecular approaches, allowed to obtain new information on a microflora in the center of a purulent infection, and also the development of a wound fever. (9-10)

Aim: to study a range of the microorganisms present at the centers of infection in purulent - necrotic lesions in diabetic foot syndrome by identifying the primary nucleotide sequence of DNA fragments of 16S rRNA gene including V3 and V4 variable sequences. For this purpose, Next Generation Sequencing machinery MiSeq (Illumina) was used and comparison of the received sequences of fragments of DNA with the database was made in order to identify origins of microorganism species present.

Infections remain a serious hazard for the diabetic patient. Good metabolic control is a major factor in limiting the development and spread of infections and, most importantly, the development of diabetic complications which predispose to infections. In some patient's recurrent infections can pose a problem, particularly if there is evidence of secondary immunodeficiency. In these patient's adjuvant therapies, including Biological Responses Modifiers (BRMS) should be considered. Several factors could predispose diabetic patients to infections. These factors include genetic susceptibility to infection; altered cellular and humoral immune defense mechanisms; local factors including poor blood supply and nerve damage, and alterations in metabolism associated with diabetes. In the context of a diabetic patient, all or some of these factors may operate. The purpose of this review is to assess the relative contribution of these potential mechanisms in leading to infection in patients with diabetes. (11)

The foot of patients with diabetes mellitus is affected by several processes which not only contribute to the development and progression of infection but on occasion alter the appearance of the foot in ways, which may obscure the clinical features of local infection. Neuropathy involving the motor fibers supplying muscles of the foot causes asymmetric muscle weakness, atrophy, and paresis which in turn result in foot deformities and maldistribution of weight (or pressure) on the foot surface. Dysfunction of the sensory fibers supplying the skin and deeper structural elements of the foot allows minor and major injury to these tissues to proceed without appreciation by the patient. As a result of neuropathy, the foot may be dramatically deformed, ulcerate in areas of unperceived trauma (mal perforans), and on occasion be warm and hyperemic in response to deep structural injury (acute Charcot's disease). This warmth and hyperemia may be misinterpreted as cellulitis and ulceration, whereas a major portal of entry for infection may be uninfected. In the patient with diabetes, peripheral neuropathy may develop in isolation or commonly in parallel with atherosclerotic peripheral vascular disease. The latter involves major inflow vessels to the lower extremity but commonly is associated with occlusive lesions of the tibial and peroneal arteries between the knee and ankle. The resulting arterial insufficiency can alter the appearance of the foot and obscure infection. Rubor may reflect vascular insufficiency rather than inflammation and conversely, pallor may mute the erythema of acute infection. Gangrene and necrosis may be primarily ischemic or may reflect accelerated ischemia in the setting of infection. In sum, the diagnosis of infection involving the foot in patients with diabetes requires a careful detailed examination of the lower extremity and its blood supply. (12)

Infection represents the presence of an inflammatory response and tissue injury due to the interaction of the host with multiplying bacteria. The disease spectrum is a consequence of the variability in these interactions. Diabetes, because of its effects on the vascular, neurological, and immune systems, can compromise the local and systemic response to infection, potentially masking the typical clinical features and hindering diagnosis. The early recognition of infection, particularly osteomyelitis, is paramount in the management of diabetic foot disease. Careful clinical appraisal remains the cornerstone of the assessment. Hematologic, biochemical, and radiological investigations are important aids in assessing the severity of the infection. Microbiological assessment, particularly in more severe infection, requires good-quality samples, combined with rapid transport in an appropriate medium and effective communication with the laboratory. A focused, systematic approach to the accurate diagnosis and treatment of infection, combined with careful monitoring, ensures the maintenance of optimal management. (13)

2 Materials and Methods

Diabetic foot ulcerations have been extensively reported as vascular complications of diabetes mellitus associated with a high degree of morbidity and mortality. Diabetic foot syndrome (DFS), as defined by the World Health Organization, is an "ulceration of the foot (distally from the ankle and including the ankle) associated with neuropathy and different grades of ischemia and infection". Pathogenic events able to cause diabetic foot ulcers are multi-factorial. Among the commonest causes of this pathogenic pathway, it's possible to consider peripheral neuropathy, foot deformity, abnormal foot pressures, abnormal joint mobility, trauma, peripheral artery disease. Several studies reported how diabetic patients show a higher mortality rate compared to patients without diabetes and in particular these studies under filled how cardiovascular mortality and morbidity is 2-4 times higher among patients affected by type 2 diabetes mellitus. This higher degree of cardiovascular morbidity has been explained as due to the observed higher prevalence of major cardiovascular risk factor, of asymptomatic findings of cardiovascular diseases, and of prevalence and incidence of cardiovascular and cerebrovascular events in diabetic patients with foot complications. In diabetes, a fundamental pathogenic pathway of most of the vascular complications has been reported as linked to a complex interplay of inflammatory, metabolic and procoagulant variables. These pathogenetic aspects have a direct interplay with an insulin resistance, subsequent obesity, diabetes, hypertension, prothrombotic state, and blood lipid disorder.

Foot infection in diabetic patients can accelerate dramatically with devastating consequences if appropriate treatment is not given promptly. The role of the health professional caring for these individuals is to identify and treat the infection as early as possible, along with preventing further episodes. However, diagnosing infection in an ulcerated diabetic foot is not always straightforward. In diabetics, the host inflammatory response to injury or infection may be reduced because of impaired leukocyte function, vascular disease, and neuropathy. Thus, the classical signs of dolor, rubor, calor, and tumor associated with infection may be absent. Further confusing the issue are the effects of diabetic peripheral neuropathy, which can mimic some of these findings. When clinical signs are misleading, we rely on laboratory tests to help us diagnose infection. However, blood tests whose results can suggest infection (i.e., elevations in leukocyte count and erythrocyte sedimentation rate) often yield falsely normal results. Also, in the presence of chronic wounds, microbiological results may be difficult to interpret. Herein we examine definitions related to infection and describe, from our clinical experience, how we diagnose infection in the ulcerated diabetic foot.

There are many definitions of infection. It is most frequently described as a disease caused by a microbial pathogen that occurs when the presence of replicating organisms is associated with tissue damage. The American College of Surgeons (14) defined infection as the product of the entrance, growth, metabolic activities, and resultant pathophysiological effects of microorganisms in the tissues of the patient. More specifically, White, Cooper, Kingsley, et al. (15) defined infection as the presence of multiplying bacteria in body tissues, resulting in spreading cellular injury due to competitive metabolism, toxins, intracellular replication, or antigen-antibody response (host reaction).

In some situations, such as when established pathogens are isolated from properly obtained specimens of normally sterile fluid or tissues, diagnosing infection is easy. The presence of microorganisms in a wound, however, does not in itself define a clinical infection. All wounds are exposed to skin commensals, and their microflora will represent the surrounding environment. These contaminating microbes can quickly become established within a wound, reaching a state of colonization. Colonization is defined as the presence of multiplying bacteria with no overt host immunologic reaction Ayton M. (16) Diabetic foot ulcers are commonly colonized with multiple species of organisms (17) that do not normally interfere with healing. Multiplication of bacteria within the wound can reach a stage of "critical colonization" (18), in which the host defenses are unable to maintain a balance, thus resulting in delayed healing. Infection results when the invading organisms overwhelm the host defenses, either by their sheer numbers or by impairing the host's immunity.

Infection confined to an ulcer bed can be described as a local infection. This is typically manifest as purulent secretions, often accompanied by inflammatory signs. Untreated, the local infection can progress to involve the surrounding and deeper tissues. Superficial soft tissue infection may be accompanied by painful spreading erythema, known as cellulitis. Superficial infections involve the skin but do not extend to the fascia, muscle, tendon, bone, or joint, as defined by the International Consensus on the Diabetic Foot. Deep infections are those with evidence of abscess, septic arthritis, osteomyelitis, or septic tenosynovitis. The International Consensus on the Diabetic Foot distinguishes bone infections as osteitis, infection of the cortical bone only, and osteomyelitis, in which the bone marrow is involved.

2.1 Mechanisms of Infection

Although microorganisms are responsible for the infection, there is debate as to the exact mechanisms by which they cause their adverse consequences and their effect on a non-healing chronic wound. Several factors are thought to be involved, including the bacterial burden, or load, within a wound. Many authors have reported healing to be delayed in a variety of wounds by an excessive bacterial burden, and the likelihood of infection rises as the bacterial burden increases. (19) Controversy persists over whether the mere presence of a high bacterial bioburden warrants antimicrobial therapy. (20) Some have proposed that a burden of >105 cfu of bacteria per gram of tissue is required to cause wound infection. (19) However, particularly virulent organisms, such as β-hemolytic streptococci, secrete toxins that allow rapid spread through the host's tissue planes and are capable of producing clinical infection at a lower burden.

As demonstrated by β-hemolytic streptococci, the virulence of the colonizing microorganism correlates with the likelihood of infection. The significance of other individual species of bacteria in a wound is not yet known. In uninfected diabetic foot ulcers, the microflora is likely to be polymicrobial. (17) Staphylococcus species are the most frequently isolated organisms, along with Streptococcus species, Pseudomonas aeruginosa, and various coliform bacteria. (21) When infection ensues, especially in patients who have not recently received antibiotics, aerobic gram-positive cocci are the dominant pathogens. (22) With careful sampling and culturing techniques, some anaerobic bacteria can also be recovered in 74%–95% of more severe diabetic foot infections. (23-24) A culture with polymicrobial flora from a diabetic foot ulcer does not reveal which microorganisms are pathogens. In fact, bacteria are thought to be synergistic and form biofilms on the surface of chronic wounds. This allows anaerobes to survive on wound surfaces and supports the growth of bacteria not normally considered pathogenic. (25)

The final factor potentially influencing the manifestation of clinical infection is the host response. In diabetic patients, hyperglycemia reduces the activity of neutrophils and macrophages, the cells responsible for the phagocytosis of bacteria and foreign material in the initial inflammatory phase of healing. (26) Ischemia, edema, and neuropathy reduce the capillary vasodilation response to injury, further impairing the host's response to infection. Thus, the interaction between the bacteria present within the wound and the host response determines whether a wound will progress from colonization to infection and how infection will manifest.

In diabetic foot disease, we should aim to diagnose infection at an early stage before it progresses toward deep infection and damage to underlying tissue. Obtaining a rapid and accurate diagnosis is, however, compounded by several factors. Because

the clinical signs of infection and microbiological analysis may be misleading, it is important to combine all information available and not rely on any single laboratory report. Sometimes subtle findings, such as failure of a wound to heal within the expected time frame, may suggest infection.

2.2 Microbiological Sampling

Traditional methods of sampling to determine the causative agents of a wound infection include rubbing the wound surface with a cotton swab, aspirating purulent secretions, and obtaining tissue by curettage or biopsy. Surface swabbing will collect skin contaminants, which may or may not be pathogenic. Furthermore, routine processing of swabs in clinical microbiology laboratories is rarely sufficient to isolate anaerobic or fastidious bacteria; this results both from the inadequate collection and/or transport method and variations in laboratory processing and incubation. The culture of aspirated fluid or pus is more likely to reveal the pathogenic organism, especially if taken from a deep pocket within the wound. The culture of debrided infected tissue is an excellent method for diagnosis in diabetic foot ulcers. Removing superficial debris before sampling will eliminate surface contaminants and provide more specific results. Tissue biopsy is generally regarded as the reference standard for diagnosing infection. Quantitative analysis of the deep tissue can identify heavily inoculated wounds (>105 cfu/g of tissue), but the clinical significance of this finding is unclear because it requires expertise in obtaining the sample and specialist laboratory processing. If osteomyelitis is suspected, a specimen of bone obtained at surgery or by percutaneous biopsy is the most useful sample for culture. Although culture and histological examination of a specimen is the most accurate method for diagnosing infection, it is not always easily obtainable. The technique used to obtain a microbiological sample is crucial. Although some methods are clearly superior, those selected sometimes depend on local clinical and laboratory expertise.

2.3 Hematologic and Biochemical Markers

Blood tests, such as WBC count, erythrocyte sedimentation rate, and C-reactive protein level, are commonly requested to aid diagnosis. However, they are neither sensitive nor specific and are unlikely to be elevated in local or superficial infection. Up to 50% of patients with a deep foot infection will not have leukocytosis; therefore, normal results do not preclude infection. Inflammatory blood markers are simple and relatively inexpensive to detect and may help guide the clinician in assessing treatment responses in severe infection when used in combination with other factors. The erythrocyte sedimentation rate is frequently used to monitor the response to treatment for osteomyelitis. C-reactive protein levels have been demonstrated to be elevated in diabetic foot ulceration, and other acute-phase proteins, such as ferritin, α_1-antitrypsin, and haptoglobulins, are currently under investigation. Blood glucose and hemoglobin A_{1c} levels may rise in infection.

2.4 Radiological Diagnosis of Osteomyelitis

Many imaging techniques have been used to confirm or refute the presence of bone infection. Plain radiographs are useful as an initial evaluation and can be used as comparisons for later assessments. Radiography can also detect gas in soft tissues, which may represent severe soft tissue infection by anaerobic organisms and possible abscess formation. Osteolytic bone changes or periosteal elevation are suggestive of osteomyelitis. However, these changes may not be present in the first few weeks of infection, and their absence does not exclude osteomyelitis. Follow-up radiography is usually done 2–6 weeks later, although there is no agreed best interval. If the diagnosis remains in doubt, further investigations may include an isotope bone scan or labeled WBC scan, infrared thermography, ultrasound, or MRI. Among these, MRI has been found to be more sensitive and far more specific than bone scans for the diagnosis of osteomyelitis in diabetic feet.

2.5 Clinical Diagnosis of Infection

The most important diagnostic tool for infection is bedside clinical evaluation. The patient should be asked about an increase in pain, odor, or exudate. Local infection of an ulcer can be difficult for inexperienced clinicians to recognize. Cutting and Harding described signs of infection in a granulating wound: delayed healing, friable tissue, offensive odor, secretion of pus, increase in lesion size, pain or discomfort, and prolonged exudate production. Although symptoms may be absent in the neuropathic foot, the clinical signs of abnormal granulation tissue, such as a change in color from bright red to dark red, brown, or gray and increased fragility and contact bleeding, should alert the clinician to the possibility of infection. Spreading superficial infection, usually represented by warmth, erythema, and edema may be less obvious in the diabetic foot. Systemic signs, such as pyrexia, chills, and lymphadenopathy, are usually absent. Even if the infection is present, it can be difficult to differentiate from acute neuro-osteoarthropathy (Charcot's foot). Radiological and clinical assessments, together with laboratory tests, should aid the differentiation of infectious from noninfectious bone lesions.

If a bone is visibly exposed within the wound or can be detected on gentle probing with a sterile instrument, osteomyelitis is likely. In a study of 75 patients with 76 ulcers, osteomyelitis was confirmed in 50 ulcers (66%). Thirty-three of these ulcers had bone detectable on probing, whereas 4 with underlying osteomyelitis did not, giving a sensitivity of 66%, a specificity of 85%, and a positive predictive value of 89%. Other deep structures exposed within the wound, such as tendon or joint capsule, also signify deep infection. Probing a wound can also detect foreign bodies and sinus tracts. It is essential that a wound is carefully probed with a narrow, blunt instrument able to convey to the user the presence of hard material within the wound. It is among the quickest and easiest procedures to do when evaluating a diabetic foot ulcer and among the most important. (13)

To accurately diagnose infection, a combination of clinical, laboratory and imaging investigations must be used. Various studies have defined the proper techniques for obtaining and the values of various tests. Determining which diagnostic procedures to order depends somewhat on local expertise and availability. Among the simplest and most important of tests is probing the debrided wound at the base of an ulcer; this should be done on every wound to evaluate its depth and exclude osteomyelitis. If in doubt, it is better to treat potential infection empirically while waiting for a definitive diagnosis than to delay treatment.

34 patients with DFS participated in the study; all of them were undergoing hospital treatment in the department of bone and purulent surgery No. 4,7,12, HEMS (Hospital of Emergency Medical Service) in Almaty.

All patients were informed on the main provisions of the research and signed informed consent to participate in the study. The study was approved to proceed by the local Ethics committee of the Center for Life Sciences of Nazarbayev University (Protocol No 14 of 30 June 2014).

The majority of patients with DFS were men (64.6±6.0%), a proportion of women was 35.4%. Type 2 diabetes was prevalent in 96.9% of the patients, whereas type 1 was observed in 3.1±6.0% of the patients. The average age of the patients was 63.8 years old, with duration of diabetes 14.6 years, and duration of DFS 2.0 months.

88.2% of patients were registered with angiopathy, 76.5% of infected people had polyneuropathy which allowed to allocate them in the group of major factors of risk of development of DFS. The retinopathy was registered in 35.3%, nephropathy 50.0%, encephalopathy – at 11.8%. The depth of distribution it is purulent - necrotic defeats it was estimated as 4-5 degree according to Wagner classification in 52.9% of patients.

Study material of a range of the microorganisms present at the centers of purulent - necrotic defeats in a diabetic foot syndrome, 10-20 mg of biomass was taken from the deep center of the affected area and placed in 1 ml of a solution of 50mmol EDTA in 1,5 ml to a micro centrifuge test tube.

DNA from the samples was extracted using bacterial Easy Pure Bacteria Genomic DNA Kit DNA (Transgenbiotech, China).

16S rRNA gene amplification for the sequencing library preparation:

All samples were used in PCR amplification for the 16S rRNA gene for further library preparation of sequencing. Each PCR reaction included: 7 µl of genomic DNA, 1,5 µl of 10pM pair of primers, 12.5 µl of KAPA HiFi Hot Start ReadyMix PCR Kit (Kapa Biosystems Ltd.) which included an appropriate concentration of buffer, MgCl2, dNTPs, and polymerase. A total volume of each reaction was 25µl. Amplification was done using IQ5 thermocycler (BioRad, USA) with the following regime: Initial denaturation at 94° C for 3 min; Cycling (30 cycles): denaturation at 94° C for 45 sec, annealing at 50° C for 1 min, elongation at 72° C for 1 min 30 sec, additional elongation at 72° C for 10 min, hold at 4° C.

The PCR products were cleaned up using magnetic beads AMPure XP Beads (Agencourt AMPure XP) according to the Illumina protocol for sequencing library preparation.

The research of the variety of microorganisms present in the center of purulent - necrotic defeats in diabetic foot syndrome was carried out by the analysis of variable sites of V3 and V4 of a gene 16S of ribosomal RNA. Variable sites 16S of rRNA were used for phylogenetic classification of the non-uniform microbial population according to the origin of species.

For obtaining DNA fragments of 16S rRNA a gene which includes V3 and V4 sites, highly specific primers covering the region were synthesized. These fragments of DNA were analyzed using MiSeq, which allowed to identify the primary nucleotide sequence of DNA. The primary sequence of DNA was compared with data from the database which allowed to identify types of studied microorganisms, and also their quantity in a percentage ratio present in the material. This allowed a comparison and ratio analysis of samples by the species difference in the studied population of microorganisms.

3 Results

The DFS microbiota contained 34 different types of species, 26,5% of which were aerobes and 73,5% were anaerobes. A high proportion was mixed-infections was containing both anaerobes and aerobes was present (81,5%), only 11,1% of samples were purely aerobes, and 7,4% - anaerobes (Fig.1).

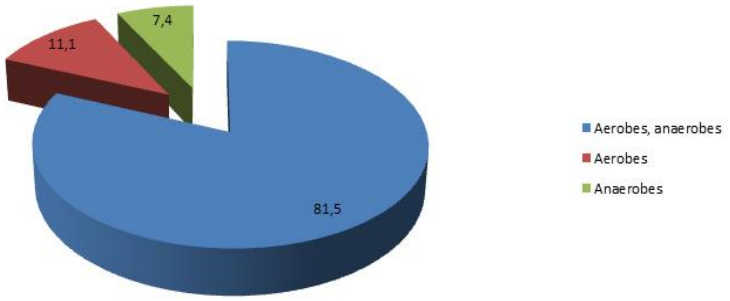

Figure 1. Specific Weight of Aerobes, Anaerobe Bacteria's and Mixed-infections

Aerobes were represented in the following 3 types of classes: Staphylococcus, Streptococcus, Pseudomonas, Campylobacter, Acinetobacter, Corynebacterium, Macrococcus, Achromobacter, Stenotrophomanas (Figure 2).

From aerobes representatives of Streptococcus spp appeared most often (44,4%), Pseudomonas spp. (37,0%), Achromobacter spp. (26,0%), Corynebacterium spp. (22,2%). As seen from the represented data, representatives of Staphylococcus were registered only in 14,8% and include the following species: S. aureus, S. intermedius, S. chromogenes, S. pseudolugdunensis, S. agnetis, S. lugdunensis.

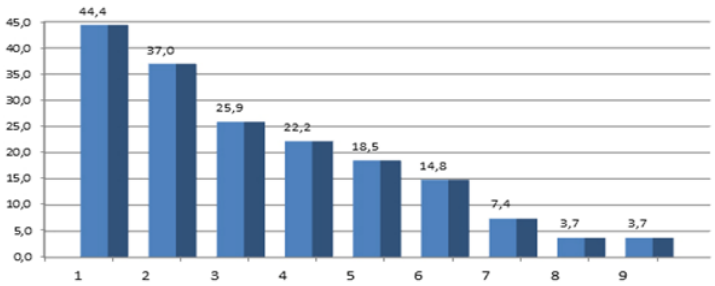

Figure 2. Aerobes. Distribution of Aerobes According to Genus: 1- Streptococcus; 2- Pseudomonas; 3- Achromobacter; 4- Corynebacterium; 5- Acinetobacter; 6- Staphylococcus; 7- Stenotrophomanas; 8- Campylobacter; 9- Macrococcus

Overall, bacteria representing 25 different genus groups were identified. Figure 3 shows the most frequently observed genus types of microorganisms, amongst which Anaerococcus spp., which belongs to the Clostridia family, accounted for 44.4% of frequency appearance, which was followed by Peptoniphilus spp. and Fusobacterium spp. representatives of Bulleidia spp., Enterobacter spp., Helcococcus spp. were identified in 14,8%. The most rarely occurring bacteria belong to the following types of the genus': Peptostreptococcus, Morganella, Citrobacter, Clostridium, Moryella, Negativicoccus, Tolumonas, Granulicatella, Oxalobacter.

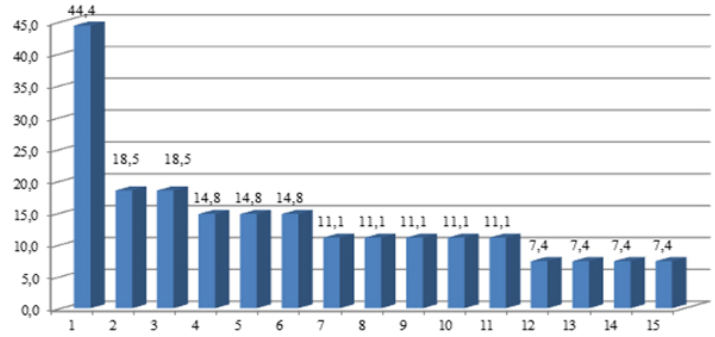

Figure 3. Anaerobes. Distribution of Anaerobes According to Genus: 1- anaerococcus; 2- Peptoniphilus; 3- Fusobacterium; 4- Bulleidia; 5- Enterobacter; 6- Helcococcus; 7- Finegoldia; 8- Escherichia; 9- Providencia; 10- Prevotella; 11- Porphyromonas; 12- Klebsiella; 13- Bacteroides; 14- Enterococcus; 15- Actinomyces

4 Discussion

T Our results indicate that mainly microflora of the infected sites with DFS is represented by a polymicrobial association of aerobes and anaerobes with the high degree of colonization 81.5%. As stated above, anaerobes belong to 25 types of the genus, which exceeds the number of aerobe microorganisms (Pseudomonas aeruginosa, Acinetobacter spp. etc.). Bacterial diversity in chronic wounds is represented by less than 8 specie origins in each type.

As seen in the given results of molecular-genetic results, the bacterial profile of purulent - necrotic defeats in DFS is characterized by the presence of anaerobic infectious organisms. The most frequently registered types of the Anaerococcus which was extraction from almost 44.4% of the patients. Three types were registered: A. vaginalis, A. lactolyticus, A. tetradius. This data is consequent with results which determined the dominating role of A. lactolyticus and A. vaginalis using 16S rRNA sequencing (27), in which these organisms are involved in biofilm formation in diabetes, the frequency of their appearance is 55%. Resistance to antibiotics of certain types of Anaerococcusspp is established. (28)

The polymicrobial etiology of microbiota at the centers of infection in DFS was characterized by the presence of representatives of a normal microflora: with an identical frequency we defined species of Peptoniphilus genus (18.5%), Fusobacterium genus (18.5%); 14.8% types of Bulleidia origin species, the Enterobacter genus, the Helcococcus genus. The Peptoniphilus genus is presented by 5 types of species: P. asaccharolyticus, R. gorbachii, P tyrrelliae, R. ivorii, R. methioninivorax. These types represent normal vaginal and intestinal microflora. (29-30) However, bacteria of these types were registered at a diabetic infection of the skin and soft tissues, an infection of bones and joints, surgical infections, a chorioamnionitis and infections of a blood-groove. (30) Usually, bacteria of this type are present as a part of polymicrobial associations, the sequence of which is difficult to be determined by routine cultural methods but can be revealed using the microbiome analysis using 16S rRNA sequencing and MALDI-TOF method of identification. (31) Fusobacterium spp. included F. gonidiaformans and F.naviforme. These types were identified in 5 patients, 4 of which were classified by 4-5 degree of infection on Wagner scale. There is evidence that elderly people with associated diseases have of F. nucleatum present, these people are at the stage of dialysis or with malignancies. (32-33)

Bulleidia genus included: Bulleidiaextructa and Bulleidiamoorei, identified in 4 samples from patients and classified at the degree of 4-5 according to Wagner scale. Presence of B. moorei in infections was described in the paradontitis patients, including those exhibiting dentoalveolar abscess. (34) This research (35) allowed revealing V. moorei at a wound fever as a part of the mixed microflora of aerobic and anaerobic bacteria.

In the studied samples Enterobacter nickellidurans, Enterobacter soli, Enterobacteraceae were also identified, which role in the development of intrahospital infections is undoubtable. Now enterobacterium causes up to 15% of all intrahospital infections, this is up to 0% of all bacteremia's. It is known that among intrahospital infections 5 to 10% of all cases contain pneumonia caused by enterobacterium.

Our comparative analysis of a microflora of ulcer and necrotic defeats in DFS depending on the extent of the defeated area allowed to identify the specific structure of the microflora. The frequency of staphilococci occurrence was 6,5%, unlike streptococci which in colonizing the defeated center 1,7 times more at 4-5 degree of classification on Wagner. There is a 4 times increase in presence of Fusobacteriumspp species. (13.0% in comparison to 3.2%), 3 times increase in Porphyromonas spp. (9.7% in comparison to 3.2%), double increase in Achromobacter spp. (13.0% in comparison to 6.5%), 1.2 times increase in Anaerococcus spp. (22.6% in comparison to 19.3% (fig. 4). According to this 4-5 degree of classification on Wagner scale, the number of representatives of normal flora decreases Corynebacterium (3.2% in comparison to 13.0%).

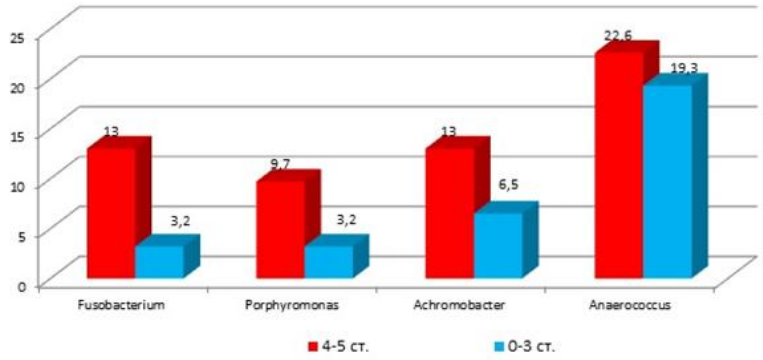

Figure 4. Frequency of Occurrence of Normal Microflora Representatives According to Wagner Scale of Classification

5 Conclusion

Therefore, the data obtained from our study using molecular and genetic methods of identification of microorganisms in DFS, allowed to generate a number of important conclusions:

- Development of purulent - necrotic complications are linked to long developing processes and irrespective of the degree of defeated are characterized by polymicrobial association, which supports the concept of "pathogroups";
- The consortium of genotypically different bacteria varies in accordance to origin of species depending on the degree of the infected area: at high degree on Wagner scale the frequency of the anaerobe bacteria increases which are generally responsible for development of intrahospital infectious diseases (Fusobacterium spp., Porphyromonas spp., Achromobacter spp., Anaerococcus spp.); the synergetic effect of "pathogroups" is provided with the functional equivalence at a co-aggregation of all terms of a microflora irrespective of pathogenicity degree;
- Correlation between the degree of violation of a microbiota and development of DFS attempts to find the solution in search of potential bacterial targets for medicines. These methods allow choosing the most adequate antibacterial therapy more effectively.

The molecular and genetic research of a microbiota in case of DFS has broad perspectives for the selection of genes as functional biomarkers for prevention or increase in risk in purulent-necrotic complications in case of DFS and to create methods of management of these mechanisms.

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