Diplomarbeit

Correlation of microbiological findings in patients with suspected clostridial myonecrosis

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Graz, 11.November.2014 Martina Wernik

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Graz, am 11.November.2014 *Martina Wernik eh.*

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Zusammenfassung

Hintergrund. Infektionen durch Clostridien sind ein seltenes aber sehr komplexes Krankheitsbild. Die Erkrankung ist aggressiv progredient und stellt sowohl an den Patienten, der um sein Überleben kämpft, als auch an die behandelnden Ärzte in Diagnosefindung und Therapie sehr hohe Ansprüche.

Methoden. Durchgeführt wurde eine retrospektive Studie, um die Methoden der Diagnosefindung dieses aggressiven Krankheitsbildes näher zu untersuchen. Dabei wurden zwei Methoden genauer betrachtet und die Ergebnisse miteinander verglichen: Zum einen die Gramfärbung, welche im Labor zuallererst durchgeführt wird und schon einen Hinweis auf das Erregerspektrum in der Probe liefern kann. Als nächstes wurden die jeweiligen Ergebnisse der Erregerkulturen analysiert. Anschließend haben wir die Kulturergebnisse mit den Ergebnissen der dazugehörigen Gramfärbung verglichen. Zusätzlich wurde die Auslastung des 24h Telefon-Notdienstes, ein Service des Institutes für Hygiene, Mikrobiologie und Umweltmedizin, der für Kliniker des Landeskrankenhauses Graz (LKH Graz) zur Verfügung gestellt wird, in Hinblick auf dieses Krankheitsbild untersucht.

Resultate. In den Jahren 2009 bis 2013 wurden 132 Patienten (90 männliche and 42 weibliche), durchschnittliches Alter 57 Jahre, mit Verdacht auf Gasbrand oder nekrotisierende Fasziitis am LKH Graz behandelt. 23 von 132 Patienten (17%) zeigten in der Kultur einen Nachweis von Clostridien. 12 von 132 Patienten zeigten in der Gramfärbung gram-positive Stäbchen. Zwei der 12 Gramfärbungen zeigten als Endergebnis in der Kultur keine Clostridien, sondern *Actinomyces turicensis,* ebenfalls ein gram-positives Stäbchen.

In den 5 Jahren des beobachteten Zeitraumes wurden insgesamt 61 Anrufe von Mitarbeitern des Institutes für Hygiene, Mikrobiologie und Umweltmedizin gemacht, bezüglich des Verdachtes auf Gasbrand. Von diesen 61 Anrufen wurden 45 außerhalb der normalen Arbeitszeiten (8 Uhr - 16 Uhr) getätigt.

Schlussfolgerung. Die Studie hat gezeigt, dass, wenn in der Gramfärbung gram-positive Stäbchen zu sehen sind, in 83% der Fälle auch tatsächlich Clostridien wachsen. Wenn allerdings die Gramfärbung negativ war, kann aber nicht ausgeschlossen werden, dass später in der Kultur Clostridien wachsen.

Abstract

Background. A clostridial infection is a rare disease with a complex, mostly severe and aggressive progressing course, with enormous demands not just for the patient and his or her survival but also for clinicians in diagnosis and treatment.

Methods. We performed a retrospective analysis to determine correlation between two microbiological methods, gram-staining and culture, with regard to clostridial myonecrosis. In addition, we assessed capacity utilisation of a 24 hours emergency system provided by the Institute of Hygiene, Microbiology and Environmental Medicine.

Results. From 2009 to 2013 a total of 132 patients (90 males and 42 females), average age 57 years, with suspected gas gangrene that were recorded were included in the analysis. 23 patients (17%) had laboratory evidence of an infection with a *Clostridium* species. 12 of 132 samples showed evidence of gram-positive rods in the gram-staining. Whereas in ten cases growth of *Clostridium* spp. was observed in two cases the gram-positive rods turned out to be *Actinomyces turicensis.* 61 calls regarding suspected gas gangrene were made during the observed time period. 45 were made outside the routine working hours and therefore carried out by the emergency service.

Conclusion. Our study indicates, regarding to samples with clinical suspicion of gas gangrene, that the reliability of a gram-staining result with gram-positive rods is acceptable good and in our case in 83% leads to the result of a cultured-proof of clostridia. In contrast, a negative result for gram-positive rods in gram-stainings can show clostridial growth after all.

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Glossary and Abbreviations

LKH	Landeskrankenhaus
gr.	Greek
spp.	Species
HBO	Hyperbaric oxygen
NSTI	Necrotizing soft tissue infection
MEDOCS	Communication and information network of styrian state hospitals
RKI	Robert Koch Institut
IDSA	Infectious Disease Society of America
SSTIs	Skin and soft tissue infections
VAP	Ventilator-associated pneumonia

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1 Introduction

1.1 History

Clostridium spp. include over 200 described species that have diversity in the meaning for humans. Clostridia (gr: >> klostér<< rachis) occur ubiquitous in soil and marine sediments. Therefore and because of their fulminant and often fatal course of disease, gas gangrene has been highly feared, especially in times of war. *Clostridium perfringens* is beside other *Clostridium* species and other species of bacteria the principal germ that causes the clinical picture of necrotising fasciitis, vernacular better known as gas gangrene (clostridial myonecrosis). Other species, than gas gangrene causing clostridia, that generated interest due to their severity and often fatal nature are *Clostridium botulinum* and *Clostridium tetani*, whose clinical features were already described by one of the earliest medical writers, Hippocrates (1). *C. perfringens* (former name: *Welch-Fraenkel-bacillus* or *Clostridium welchii*) has been first characterized by W.H. Welch and G.H.F. Nutall in 1892. Earlier Pirogoff, a Russian army doctor did the first description of the clinical features during the Crimean War. The first microbiological characterization was conducted by Veillon and Zuber in 1898. Heaped appearance was mainly seen and feared during wars due to the number of traumatic injuries: in World War I approximately 100.000 soldiers died of gas gangrene. Enteritis necroticans mainly caused by *C.perfringens* Typ C was first described in Germany after World War II and later in the 1960's in New Guinea (2).

1.2 Aetiology

1.2.1 Morphology

Clostridia are putrefactive, anaerobe, gram-positive rod-shaped bacteria that are spore forming, and except for some species (*C. perfringens*) flagellate. Spores are relatively resistant as far as environmental surrounding is concerned, especially to heat and dehydration, whereby a survival beyond anaerobic conditions is possible. Among many different species that have been isolated, there are only some that are regularly associated with human diseases of any severity. Under human medical aspects there are four species of clostridia that are of special interest: *C. tetani* (tetanus), *C. botulinum* (botulism), *C. perfringens* (gas gangrene) and *C. difficile* (antibiotic associated colitis).

With a light optical microscope, one can usually see gram-positively stained, cloddy and often pleomorphic rods (Fig.1). In working stocks and patient material the main difference between *C. perfringens* and other *Clostridium* species of the gas gangrene group is that *C. perfringens* is unmovable and spores are invisible.

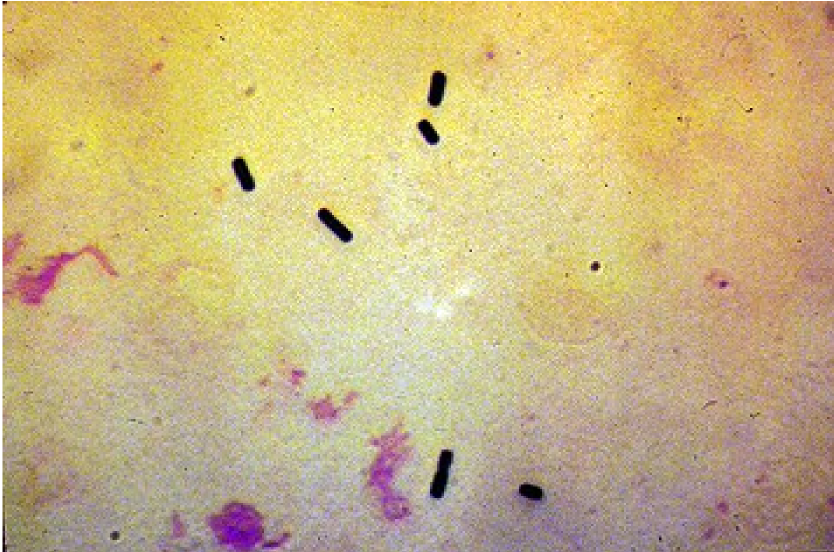

Figure 1: C. perfringens gram-stain
Reproduced from: (3)

According to present knowledge gas gangrene is usually caused by *C. perfringens*, less commonly clostridial myonecrosis is due to *C. septicum*, *C. histolyticum*, *C. novyi*, *C. sordellii*, and *C. bifermentans* (1, 2, 4). These clostridial species are also called clostridia of the gas gangrene group.

1.2.2 Pathogenicity

Pathogenicity and virulencefactors of gas gangrene agents are the exotoxins and exoencymes (Tab. 1). They cause cell and tissue damage, muscle decomposition, oedema and gas production in tissue as well as destruction of erythrocytes and leukocytes. Beside the main exotoxin Phospholipase C (Lecithinase , alpha-Toxin), the vegetative forms of the gas gangrene group clostridia also generate collagenases, hyaluronidases, haemolysines and proteases. Typ A of *C. perfringens* is mainly responsible for gas gangrene and food poisoning if it is producing enterotoxines. Typ C causes Enterocolitis necroticans by beta-toxins (2).

Toxins produced by *Clostridium perfringens*

Toxin	Strain types	Biologic Activity
α	All strains	Lecithinase
β	B and C	Necrotoxin, necrosis of the bowel
ε	B and D	Lethal, hemorrhagic
ι	E	ADP ribosylating; lethal
cpe enterotoxin	A,C and D	Cytopathic
Neuraminidase	All strains	Hydrolyses N-acetylneuraminic acid
δ	B and C	Hemolysins
κ	All strains	Collagenase
λ	B, D, and E	Protease
μ	All strains	Hyaluronidase
ν	All strains	DNAase

Table 1: Toxins produced by *C. perfringens*
Reproduced from (1)

The fact that different strains of *C. perfringens* vary in their ability of toxin production allows differentiation into strain types.

Mainly alpha-toxin and theta-toxin have been implicated to the high pathogenicity of *C. perfringens*. Alpha-toxin promotes the widespread of myonecrosis due to cease of perfusion as well as the absence of tissue inflammatory response due to restraint of leukocyte entry (3). It splits membrane-bound lecithin into phosphorylcholin and diazylglycerol and is thereby membrane damaging leading to hemolysis (Fig. 2) (5). The full range and the complexity of the tissue response are still unknown.

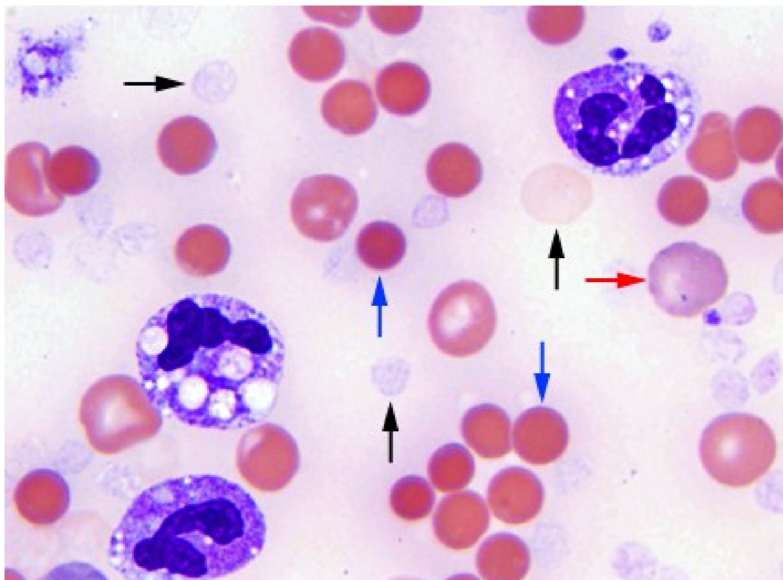

Figure 2: Intravascular hemolysis due to *C. perfringens* sepsis
Peripheral blood smear from a patient with severe intravascular hemolysis due to sepsis with *C. perfringens*.
Neutrophils show toxic changes, including toxic granulation and vacuoles. There is an increased number of spherocytes (blue arrows) and polychromatophilic red cells (ie, reticulocytes, red arrow). The major finding on this slide is the large number of red blood cell ghosts (black arrows), due to the intravascular lysis of red cells from the phospholipase and other lytic enzymes elaborated by the Clostridial organisms.
Reproduced from: (6)

1.3 Epidemiology

1.3.1 Clostridial Infections

In literature we find a differentiation between three major categories of clostridial soft tissue infections: wound contamination, anaerobic cellulitis, and myonecrosis (gas gangrene).

- Wound contamination is due to spores or vegetative organisms found in soil. This is a frequent type of contamination, but in the absence of damaged tissue and a vessel impairment, it does not inevitably lead to infection.
- Anaerobic cellulitis is the next step found in patients with clostridial contamination in open wounds and devitalized tissue. The infection remains locally. Bacteremia and invasion of healthy tissue is usually not seen. Prompt and adequate management maintain a good outcome.
- Clostridial gas gangrene is comparatively fulminant and life-threatening, due to the extensive tissue damage and invasion of muscle tissue. This article is designed to present the two types of clostridial myonecrosis: traumatic and spontaneous gas gangrene.

1.3.2 Occurrence

Clostridia spores exist in the soil and marine sediments as well as they are a part of the gastrointestinal microflora of 70 percent of humans and also animals (1). From the remaining species only *C. septicum* occurs in two percent of humans. Thus it is not considered a normal bowel inhabitant. Spores of *C. perfringens* were found numerously in soils compared to other species that were rarely found (2).

Most gas gangrene causing clostridia need obligate anaerobic conditions to increase and convert from the spore to the vegetative form. However, a few species such as *C. histolyticum*, *C. tertium*, *C. innocuum* and some strains of *C. perfringens* are able to develop in aerotolerant terms as well (1).

1.3.3 Incidence

There is no explicit data in literature available about the incidence of clostridial myonecrosis in Austria. In 1997 122 cases have been reported in Germany (5). It is assumable that the number of cases between these two countries is at a comparable count.

1.4 Pathogenesis

1.4.1 Risk Factors

Clostridial myonecrosis is most commonly associated with a traumatic injury that leads to a lowered tissue oxygen saturation. This environment allows spores to convert into their vegetative form. The injuries are mostly crushing or penetrating war or surgical wounds, compound fractures or septic abortions. The presence of foreign bodies, including soil or the penetrating object as well as mixed infections with organisms that are capable of lowering the oxygen level at the infection site promote a clostridial infection. Myonecrosis frequently occurs also in body sites that have been compromised by ischemia, malignancy, surgery or injection of illicit drugs. On account of the autochthon local bacterial flora of the intestinal tract surgeries of the bowel or biliary tract as well as structures that are exposed to fecal or oral contamination are particularly at risk. Vascular impairment such as vascular insufficiency and hereon diabetes ulcera or tissue damage on ground of burns may also be a predisposing factor to the occurrence of gas gangrene (7, 8).

1.4.2 Traumatic vs. Nontraumatic or "Spontaneous" Gas Gangrene

In the urban populations mainly traumatic injury with introduction of soil accounts for cases of gas gangrene with *C. perfringens* due to their anaerobic conditions. Devitalized tissue, especially in deep wounds with vascular leaking, is ideal for the growth of clostridial organisms. The produced extracellular toxins are responsible for further texture demise and systemic manifestation. The other major presentation of clostridial gas gangrene, spontaneous gangrene is most commonly caused by *C. septicum* (3).
70 percent of gas gangrene is due to a traumatic injury in which about 80 percent the germ is *C. perfringens*. Other pathogens found in traumatic infection sites are *C. septicum, C. novyi, C. histolyticum, C. bifermentans, C. tertium,* and *C. fallax* (3).
After traumatic injury organisms are directly drawn into deep tissue with almost always compromised blood supply. This forms an anaerobic environment with low oxygen saturation and acid pH. In such conditions necrosis progresses within 24 to 36 hours.

Spontaneous clostridial infection appears via hematogenous seeding of muscle from a gastrointestinal tract infection path way. The most frequent origin of these mucosal ulcerations that facilitate entry of germs into the circulation or other tissue is a known or unknown adenocarcinoma of the colon. Unlike *C. perfringens, C. septicum* does not

require strict anaerobic conditions, a coinfection with *C. perfringens* and *C. septicum* is common. The diagnosis of a spontaneous gas gangrene is even more challenging because it is not entertained and therefore often delayed or missed and as a result more often fatal than wound-related clostridial infections (3). Spontaneous clostridial infection is remarkably more often associated with an underlying disease such as hematologic and gastrointestinal malignancies, in particular with colorectal cancer, diabetes mellitus, peripheral vascular disease and other conditions associated with immunodeficiency (9–12). Both bacteria, *C. perfringens* and *C. septicum* show a rapid and severe reduction in microvasculature correlating with the fatal course of the disease. Hickey et al. demonstrated a remarkable decline of blood flow in capillaries with consequential expected impact on the necrosis based on the importance of oxygen and nutrient supply through capillaries (13).

1.5 Clinical Picture

1.5.1 Traumatic Gas Gangrene

Usually traumatic gas gangrene starts with a sudden onset of severe pain at the side of the wounded area. Pain can be excruciating and beyond severity of the obvious wound respectively physical findings. Clinical presentation has a high variety due to the infected location, the bacterial inoculum and the extent of vascular damage. The incubation period ranges from a few hours to several days, on average less than 24 hours. The upcoming pain is likely assigned to toxin-mediated ischemia (3). For clinicians the initial nonspecific clinical features are often difficult to interpret.

The skin over the effected site may appear pale, later turning into a brown-bronze and then purple discoloration. Within hours, the skin gets tensens due to the edematous swelling and turns extremely damageable. The multiplication of clostridia produces carbon dioxide and hydrogen gases. In a later stage, a palpable but not preeminent emphyseme can be found. Soft tissue gas is occasionally seen in radiography before it is palpable, but is not pathognomonic for clostridial gas gangrene. Secondary to necrosis there may appear overlying bullae that drain peculiar foul-smelling exudat after pressure. Ultimately, a green-black cutaneous necrosis develops.

Due to the pro-ischemic ability of alpha-toxin, *C. perfringens* and *C. septicum* show a similar extent of deep ischemia. Also theta-toxin, which is found in *C. perfringens* decreases microvascular perfusion. Platelet aggregation and fibrin attachment lead to

obstruction of small vessels reducing blood supply unlike other soft tissue infections with inflammation increasing blood flow. Studies show that vascular leukostasis and the lack of neutrophils can be assessed as clinical characteristics of clostridial myonecrosis.
The patient soon looks severely ill and the course is usually rapidly deteriorating. Signs of a systemic intoxication including tachycardia and low-grade fever develop fast. Later anemia, jaundice and delirious changes often lead to profound shock and multiorgan failure. Crucial for the outcome is the period between the first symptom and the beginning of the treatment (14).

1.5.2 Nontraumatic Gas Gangrene

Occasionally, gas gangrene appears without an obvious external entry path. In this case *C. septicum* is the primary contributor of *Clostridium* spp. found in samples. It is quite aerotolerant and spreads by bacteremia and can grow in normal undamaged tissue. The typical clinical findings of traumatic gas gangrene are more difficult to diagnose in spontaneous myonecrosis, as there is a multifocal involvement observed with even more fulminant progression than that of *C. perfringens* myonecrosis. Sometimes only heaviness or numbness is named as symptom. Inflammatory cells are also rare as with *C. perfringens* infections. There is very few knowledge in existing work on the impact to microcirculation and the spectrum of its alpha-toxin. However, the virulence of infections with *C. septicum* is imputable to its alpha-toxin.

1.5.3 Mortality

C. septicum, the main causative agent in spontaneous clostridial infections beside *C. perfringens*, shows a higher rate of mortality in adults and children. The mortality rate in adults with *C. septicum* myonecrosis is about 79 percent in comparison to 32 percent mortality rate in adults with *C. perfringens* myonecrosis (15).

1.6 Laboratory and Microbiological Findings

Laboratory diagnosis is based on two steps: a) Gram-stain and b) microbiological culture on agar plates. Gram-staining is a fast and well performable method to search for bacteria in clinical samples. In case of suspected gas gangrene material for the gram-stain can be extracted from ichor, or muscle biopsy. This provides a direct proof of the presumptive

diagnosis and counts as a microbiological emergency, while laboratories have an alert system to inform the clinicians as soon as the gram-staining is ready.
In an infection with *C. perfringens* the gram-stain usually shows the typical boxcar-shaped rods. Inflammation cells, especially leukocytes are often missing due to the fatal nature of alpha-toxin. Therefore an absence of frank pus is also often seen. Few polymorphonuclear cells and gram-positive rods in fluids or exudates be among earliest laboratory signs (7). In the histological assessment sweeping myonecrosis with edematous tissue and thrombosis is predominant beside a lack of perivascular leukocyte infiltration. Clinical samples usually do not show any spores. If blood cultures are obtained, bacteremia is only found in about 15 percent of the patients and may be accompanied by intravascular hemolysis (3). Microbiological cultures are performed on different nutrient agars, such as blood agar or Schädler agar, with an incubation of the plates under anaerobic conditions at 37°C. Incubation period is a minimum of 24 hours up to five days (Fig. 3) (16).

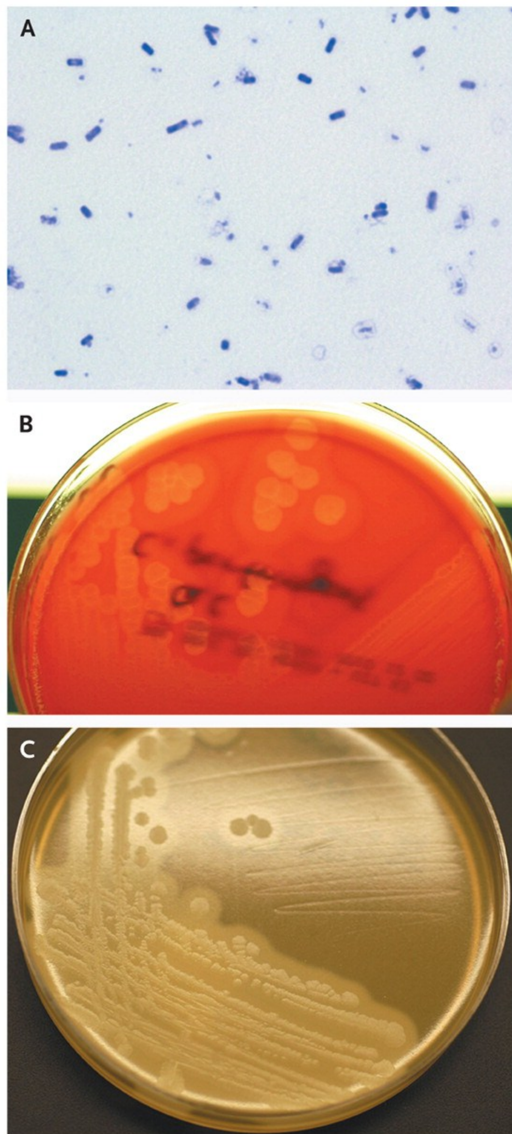

Figure 3: Morphologic features of *C. perfringens* from a reference culture.
Gram's staining (Panel A) shows typical gram-positive, boxcar-shaped *C. perfringens*. A blood agar plate (Panel B) shows a double zone of hemolysis surrounding the *C. perfringens* colonies. A McClung Toabe agar plate (Panel C), which contains egg yolk, shows a halo of precipitate around the colonies, which is characteristic of the lecithinase activity of *C. perfringens*.
Reproduced from: (17)

1.7 Clinical Diagnosis

The main finding in gas gangrene is pain at the site of traumatic injury combined with signs of systemic toxicity and crepitus in soft tissue. Palpable gas is the most specific as well as sensitive symptom for clinicians but also a late sign of gas gangrene. A radiographic examination with X-ray, computerized tomographic (CT) scan, or magnetic resonance imaging (MRI) can give an idea of the extent and depth of muscle and soft tissue damage. It is also useful in detecting gas in the first place, and for determining the exact localization and if the infection has already spread along fascial planes. Before a surgical exploration takes place, a needle aspiration or a punch biopsy are less aggressive ways of

getting samples for gram-stain and culture. If there is a reasonable suspicion for gas gangrene a surgical exploration is unavoidable. As soon as possible patients have to be transported to a Center for Hyperbaric oxygenation to receive additional therapy.
During surgical procedures a lack of bleeding or contraction after stimulation is a typical sign for infected muscle tissue.

1.8 Differential Diagnosis

A number of clinical entities can mimic a similar clinical presentation as found in clostridial myonecrosis (Tab. 3). Diseases under the umbrella term necrotizing soft tissue infection (NSTI) contain different entities such as types of necrotizing cellulitis, myositis, and fasciitis. The great diversity of described types of NSTI in the literature should be remembered, due to their potential of entailing useful pointers in diagnosis and the time of initiating measures if necessary.The major causatives leading to myonecrosis are *Clostrium spp.*, *Group A Streptococci (Streptococcus pyogenes)*, or other ß-hemolytic *Streptococci* (18). Presumably the biggest challenge lies in differentiating the entities of this clinical presentation. Early signs include mainly subtle changes and seem to be entirely indistinguishable (Tab. 2). Progressive changes and typical symptoms indicate an advanced disease which is why an early diagnosis is the most crucial part of a successful management.

Differential diagnosis of necrotizing myositis and fasciitis

Clinical findings	Type I*	Type II*	Gas gangrene	Pyomyositis	Myositis viral/ parasitic
Fever	++	++++	+++	++	++
Diffuse pain	+	+	+	+	++++(1)
Local pain	++	++++(2)	++++	++	++
Systemic toxicity	++	++++	++++	+	+
Gas in tissue	++	-	++++	-	-
Obvious portal of entry	++++	±(3)	++++(4)	-	-
Diabetes mellitus	++++	±	-	-	-

Table 2: Differential diagnosis of necrotizing myositis and fasciitis
* Type I and type II refer to the forms of necrotizing fasciitis; spontaneous gangrenous myositis is type II.
1. Pain with influenza consists of diffuse myalgia; pleurodynia may be associated with severe, localized pain (eg devil's grip): pain with trichinosis may be severe and localized.
2. Severe pain is associated with necrotizing fasciitis due to *Group A streptococcal* infection; the pain may not be severe in type I necrotizing fasciitis because is commonly associated with diabetes with neuropathy.
3. 50 percent of patients with necrotizing fasciitis due to *Group A streptococcal* infection do not have an obvious portal of entry.
4. Gas gangrene associated with trauma may be caused by *Clostridium perfringens*, *C. septicum*, or *C. histolyticum* which always have an obvious portal of entry; in comparison, spontaneous gas gangrene caused by *C. septicum* usually does not have an obvious portal of entry – organisms lodge in tissue as a result of bacteremia originating from a bowel portal of entry.

Reproduced from: (18)

As in clostridial myonecrosis, diabetes counts as a predisposing or associated condition. Also, there seems to be an assuming connection between diabetes and the occurence of particular bacteria (19).

Group A streptococcal necrotizing myositis is also described as NSTI type II. The infection is seen in young and healthy people, often to a recent trauma and leads to an aggressive form of myositis, with intensive pain, fever, and swelling. Generally it is a monomicrobial infection with *Group A ß-hemolytic Streptococcus*, occasionally a coinfection with other species is possible, most commonly with *Staphylococcus aureus* (20). The skin over the infected area may be uninvolved at first, or discolored with sometimes petechiae and bullae. Type II NSTI is characterized by an aggressive course with vascular impairment and signs of systemic toxicity. Unlike clostridial myonecrosis in streptococcal myonecrosis bacteremia and toxemia are seen frequently and are attributed to the high mortality of this entity. Also a typical finding is the compartment syndrome, due to the rapid spread of infection and the increasing pressure (7).

Type I necrotizing soft tissue infection includes a poylmicrobial spectrum of causative agents. A varying combination of anaerobes plus one or more facultative anaerobic streptococci and Enterobacteriaceae can be isolated (18). The patient is comparatively older with more than one medical condition and an overall state of poor health. Normally there is no clear history of trauma. Diabetes, peripheral vascular disease, obesity, chronic renal insufficiency, alcohol abuse, existing abscesses, blunt trauma, chicken pox, HIV or AIDS, and i.v. drug abuse are predisposing conditions (20). The infected site is usually the trunk or perineum, for example *Fournier's gangrene* (infection of the male perineum). In case of *Fournier's gangrene* the spectrum varies from facultative organisms, *E. coli*. *Klebsiella sp.*, and *Enterococci* to a combination with anaerobes, such as *Bacteroides sp.*, *Fusobacterium sp.*, *Clostridium sp.* and *Streptococci sp.* (21). Due to the location of the infection, the spectrum of bacilli can be further distinguished by the resident flora.

Fournier's gangrene is often found in patients at the age between 50 and 60 years in both sexes, with older men particularly affected. As with necrotizing fasciitis and gas gangrene underlying diseases, like diabetes mellitus, are common (22). The disease starts apruptly with intense pain and a similar picture as described above, and can have a progressive and fatal course. Women can also be affected by infections of the vulvar or perineal

involvement, often in combination with predisposing diabetes and obesity (23). As in other necrotizing diseases the treatment should consist of a prompt surgical evaluation and debridement or drainage, together with an appropiate antimicrobial regime. To quote Laucks: '*Empiric broad-spectrum antibiotic therapy should be instituted, regardless of Gram's stain and culture results. The antibiotic regimen chosen must have a high degree of effectiveness against staphylococcal and streptococcal bacteria, gram-negative coliforms, pseudomonas, bacteroides and clostridia*'(24).

A muscle infection with *Staphylococcus aureus* leads to pyomyositis (primary muscle abscess) of the skeletal muscle. It is also characterized by fever, pain, edema, and tenderness. Unanalogous most bacterial infections of the muscle, pyomyositis occurs without a predisposing area of infection (7).

Viral infections, especially with *Influenze A* and *B*, can also lead to symptoms of myositis (3).

Differential Diagnosis of Crepitant Soft Tissue Wounds*

Factor	Clostridial Cellulitis	Nonclostridial Anaerobic Cellulitis	Clostridial Myonecrosis (gas gangrene)	Anaerobic Streptococcal Myositis	Necrotizing Fasciitis (Type I)°	Infected Vascular Gangrene	Synergistic necrotizing cellulitisˆ	Noninfectious Causes of Gas in Tissues
Predisposing conditions	Local trauma or surgery	Diabetes mellitus, preexisting localized infection	Local trauma or surgery	Local trauma	Diabetes mellitus, abdominal surgery, perineal infection	Peripheral arterial insufficiency	Diabetes mellitus, cardirenal disease, obesity, perirectal infection	Mechanical effects of penetrating trauma, injuries involving the use of compressed air, entrapment of air under loosely sutured wounds or under ulcers, irrigation of wounds with hydrogen peroxide, intravenous catheter placement, dissection of air from tracheostomy or spontaneous mediastinal emphysema
Incubation period	Usually >3 days	Several days	1-2 days	3-4 days	1-4 days	>5 days	3-14 days	less than 1hr
Onset	Gradual	Gradual or rapid	Acute	Not as rapid as gas gangrene	Acute	Gradual	Acute	Usually present immediately after trauma or manipulation; may not be recognized until examination sveral hours later
Pain	Mild	Mild	Marked	Occurs late, marked	Moderate or svere	Variable	Severe	Mild
Swelling	Moderate	Moderate	Marked	Moderate	Marked	Moderate or marked	Moderate or marked	Slight or absent
Skin appearance	Minimal discoloration	Minimal discoloration	Yellow-bronze, dark bullae, green-black patches of necrosis	Erythema	Erythematous cellulitis, areas of skin necrosis	Discolored or black	Scattered areas of skin necrosis	Only those resulting from initiating trauma
Exudate	Thin, dark	Dark pus	Serosanguineous	Abundant, seropurulent	Seropurulent	None	"Dishwater" pus	None
Gas	++++	++++	++	±	++	+++	++	Variable but present; does not extend
Odor	Sometimes foul	Foul	Variable, slightly foul or peculiarly sweet	Slight, "sour"	Foul	Foul	Foul	None
Systemic toxicity	Minimal	Moderate	Marked	Only late in course	Moderate or marked	Minimal	Marked	None
Muscle involvement	None	None	++++	+++	None	Dead	++	None

Table 3: Differential diagnosis of crepitant soft tissue wounds

±: rarely present, ++: present to mild extend, +++: present to moderate extent, ++++extensive.

*In addition to the causes of crepitant infections listed in this table, *Aeromonas hydrophila* myositis may be associated with gas in soft tissues.

°The term *necrotizing fasciitis* is used here to designate forms of this syndrome other than streptococcal gangrene.

ˆSynergistic necrotizing cellulitis is essentially the same process as type I necrotizing fasciitis. Because the former occasionally tends to involve muscle, it is given a separate designation here; however, the two processes are clinically indistinguishable in most cases.

Reproduced from (7)

1.9 Therapy

The mainstays of treatment are a combination of parenteral antibiotics and prompt surgical intervention. The best outcome is achieved the earlier an aggressive and precise surgical evaluation is performed (Fig. 4). Extensive debridement of necrotic tissue with amply resection margins is as important as in time amputation of an extremity if necessary. The overriding aim is the complete removal of necrotic tissue and to relieve and decompress the swollen surrounding compartments to prevent further destruction. Pending results of microbiological findings an empiric antibiotic therapy should be started. With regard to possible differential diagnosis of myonecrotic infections the initial antibiotics treatment should cover *Clostridium spp.*, *Group A Streptococci,* facultative anaerobic streptococci, and Enterobacteriaceae. Penicillin is the first choice for infections with *C. perfringens* since most strains are susceptible (1). Other potential antibiotics that showed superior activity in animal models are clindamycin, tetracycline, chloramphenicol, metronidazole and a couple of cephalosporins (3). Experimental gas gangrene models comparing single versus combinational use of antimicrobial agents indicated that clindamycin and metronidazole used singly are more effective than penicillin. Since some strains of clostridia may show resistances advisement of combinating penicillin and clindamycin appears rational. The demands on optimal antimicrobial therapy are complex and struggle with tissue penetration, ineffectiveness in high inocula, bacteria colony killing effects and supposing toxin supression problems (25). The Infectious Disease Society of America (IDSA) published this summer an update of their recommendations about the treatment of skin and soft tissue infections (SSTIs), with a section about necrotizing fasciitis and Fournier's gangrene as well as clostridial gas gangrene and myonecrosis. Table 4 gives an overview about the latest guidelines from the IDSA.

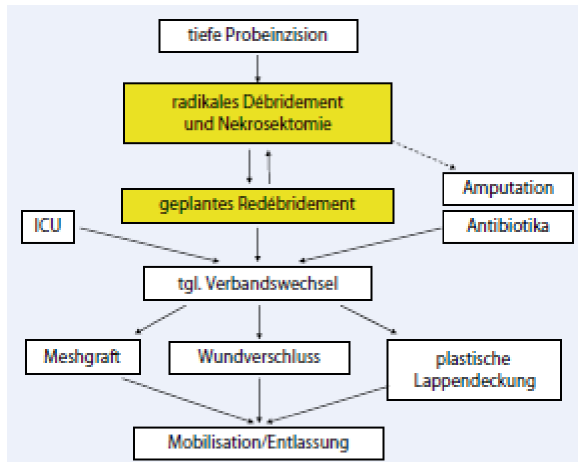

Figure 4: Algorithm for the treatment of patients with suspected necrotizing SSTI
Adapted from: (26)

Treatment of necrotizing infections of the skin, fascia and muscle

Type of Infection	1st-line Antimicrobial Agent	Adult Dosage
Mixed infections	Piperacillin-Tazobactam plus Vancomycin Imipenem-Cilastatin Meropenem Ertapenem Cefotaxime plus Metronidazole or Clindamycin	3.37g every 6-8h IV 30mg/kg/d in 2 divided doses 1g every 6-8h IV 1g every 8h IV 1g daily IV 2g ervery 6h IV 500mg every 6h IV 600-900mg every 8h IV
Streptococcus	Penicillin plus Clindamycin	2-4 milion units every 4-6h IV 600-900mg every 8h IV
Staphylococcus aureus	Nafcillin Oxacillin Cefazolin Vancomycin (for resistant strains) Clindamycin	1-2g every 4h IV 1-2g every 4h IV 1g every 8h IV 30mg/kg/d in 2 divided doses IV 600-900mg every 8h IV
Clostridium species	Clindamycin plus Penicillin	600-900mg every 8h IV 2-4 milion units every 4-6h IV
Aeromonas hydrophila	Doxycycline plus Ciprofloxacin or Ceftriaxone	100mg every 12h IV 500mg every 12h IV 1-2g every 24h IV
Vibrio vulnificus	Doxycycline plus Ceftriaxone or Cefotaxime	100mg every 12h IV 1g qid IV 2g tid IV

Table 4: Treatment strategies for NSTI, gas gangrene and Fournier's gangrene
IV: intravenous; qid: 4 times daily, tid: 3 times daily
Reproduced from (22)

In addition to these two main concepts there are several supportive measures.

The adjunct treatment with hyperbaric oxygen (HBO) is potentially useful but still debatable, largely to a shortcoming of sufficient knowledge and deviate results in studies.

Studies in animals showed that HBO treatment alone does not improve the outcome. The combination of HBO and antibiotics enhances the response rate of antibiotic-treated bacteria to HBO and has an reductive effect on the bacteria population at the infected site. As with surgical debridement, HBO is most beneficial if applicated as soon as possible. The positive effects of HBO are attributed to the antimicrobial effect of high oxygen concentrations, amelioration of tissue oxygenation and the bacteriostatic effect due to rise of oxidation-reduction potential in and around the infected area and creation of an aerobe, less favorable milieu for anaerobic growth. Murine models showed a direct in-vivo inactivation of *C. perfringens* by HBO. Inhibition of alpha-toxin production was seen but could not be confirmed and alters from trail to trail depending on used gas pressure and exposure regime. The best outcome was observed with intense exposures to HBO in the initial phase of the infection which leads to the supposition that a practical approach would be the highest dose and frequence of HBO exposure a patient can tolerate safely (27).
There are no randomized, controlled trials in humans confirming that HBO solitary used or in combination with antibiotics and surgery increases the survival. The major problems are the small number of clostridial myonecrosis cases in normal population and no area-wide availability of clinical centers with hyperbaric chambers. Additionally, the therapy regimes used and the clinical techniques for exposure vary strongly. However, due to the positive effects on infected tissue in several animal models there is rationale assumption for profitable contribution of HBO in the treatment of gas gangrene. Besides, HBO is at large safe, quite well tolerated and it's only absolute contraindication is an untreated pneumothorax.

Other adjuvant options but less often performed than HBO, include G-CSF (Granulocyte-Colony Stimulation Factor) to impel hematopoietic growth and approaches direct against toxin effect, such as vaccines (1). In one series, mice were protected against *C. perfringens*, after active immunization with a subunit vaccine. The inoculant was a direct derivative from the alpha-toxine (28). Another study confirmed these results and demonstrated prime efficacy of immunization against the C-domain of *C.perfringens* alpha-toxin. They also elucidated that alpha-toxin-induced activation of leukocytes, platelets, and endothelial cells leads to occlusive intravascular aggregation, such that hypoxic impairment extends and *C. perfringens* thrives under optimal anaerobic conditions. Furthermore, symptoms of immunized mice showed to be more localized and

transient than in the control group (29). Antitoxin for a passive immunization or active immunization in humans is not yet available.

1.10 Prognosis

Clostridial myonecrosis has an overall poor outcome. Though the affection of an limb has a better prognosis than myonecrosis of the trunk or visceral organs. Patients with clostridial bacteremia have a great probability of progression towards shock.

1.11 Prevention

A precise combined therapy approach with radical debridement of damaged tissue and correction of blood circulation impairment is crucial and attributed to a better outcome.

2 Material and Methods

A clostridial infection is a disease with a complex, mostly severe and fatal course that confronts clinicians with a difficult challenge in diagnosis and therapy. In Austria only the Section of Thoracic Surgery and Hyperbaric Surgery of the Universitiy Hospital Graz offers a hyperbaric facility with hyperbaric patient treatment chambers.

The aim of this study was to retrospectively analyse the correlations between different findings of microbiological methods, as well as the reviewing of the epidemiology of clostridial infections among the Graz conurbation in the last 5 years. Additional we took a look in detail at the 24 hour emergency service for clinicians, which is provided by employees of the Institute of Hygiene, Microbiology and Environmental Medicine. This service mainly ensures an immediate alert and information exchange between clinicians and microbiologists for the medical emergency of gas gangrene or necrotizing fasciitis. Communication in both directions is facilitated in terms of clinicians call if they have a sample with suspicion of gas gangrene or necrotizing fasciitis and microbiologists call back after performing a gram-staining.

2.1 Study Setting

The period of time included in the analysis was from 2009 to 2013. Data was raised in a cooperation between the Institute of Hygiene, Microbiology and Environmental Medicine and the Section of Thoracic Surgery and Hyperbaric Surgery at the Medical University of Graz.

2.2 Patients

Several concepts of terminology were recorded to determine and to limit a suitable patient population. A search process with targeted search words was performed in the electronic patients database (MEDOCS). In-patients from the 1st of January 2009 to the 31st of December 2013 were included.

2.3 Inclusion Criteria

- male and female of any age
- lab conducted by the Institute of Hygiene, Microbiology and Environmental Medicine
- clinical suspicion of gas gangrene

search terms in MEDOCS:

- Wie “*gasbrand*“ Oder (Wie “*nekrotis*“ Und Wie “*fa[sz]ciit*“) Oder (Wie “*fournier*“ Und Wie “*gangr*“)

2.4 Exclusion Criteria

- data that shows to be classified incorrect in accordance with the search terms (e.g. sterile osteonecrosis)
- patients with no microbiological data at the Institute of Hygiene, Microbiology and Environmental Medicine (samples were sent to an external microbiological laboratory)
- infections with *C. difficile*

2.5 Data

All data regarding the diagnosis and classification of the infection were collected through the electronic patient database (MEDOCS). Due to the availability of a hyperbaric chamber in Graz, gas gangrene or any necrotizing disease is usually treated in cooperation with this facility. To limit the patient data we contrasted the list of patients treated in the hyperbaric chamber with those who matched the search terms.

The remaining information concerning age, sex, laboratory results and if an alert call was made were gathered via the electronic and analog archieves of the Institute of Hygiene, Microbiology and Environmental Medicine. Data from 2012 and later was electronically available. Earlier data had to be collected manually for the most part.

In lab findings we differentiated any causing agent from *Clostridium* spp.. Furthermore all emergency calls made from employees from the Institute of Hygiene, Microbiology and Environmental Medicine to a clinician at the Medical University of Graz were listed and sorted in order of outgoing time. The outgoing time is interesting from a economical point of view since calls outside routine working hours are charged seperate.

2.6 Statistical Analysis

The study was performed retrospectively on medical records of the LKH Graz. The data was accomplished via a computer-generated approach and completed through the analogous archive. The analysis was undertaken with Microsoft Excel.

From the clinical side we counted the cases with proven infection with *C. perfringens* and other *Clostrium* spp. itemized for each year and for the total period of the analysis. Furthermore specific data of the patients (concerning age, gender, etc.) were investigated. From the side of microbiologic laboratory we analyzed the frequency of emergency calls outside of the routine working hours and the correlation between the results of the gram-staining and the culture was also ascertained.

3 Results

3.1 Referral Diagnosis

The search was first based on specimens with the referral diagnosis gas gangrene or necrotizing fasciitis. We found that differentiation between these two disease entities is difficult, because a consistent description of the clinical presentation is not found and also the nomenclature is used variously and ambiguously. The search terms were reconsidered and extended to a list of diagnosis related to these entities. We identified a total of 157 patients that matched the terms at first search. Next we had to sort out patients with for example sterile osteonecrosis and some other kinds of necrosis that did not fit to the referral diagnosis. A further inconsistency we observed was that nomenclature did not always meet the official ICD-10 nomenclature. Therefore, a ranking based on obtained referral diagnosis, as we had hypothesized - on the one hand gas gangrene and on the other hand necrotizing fasciitis - was unsatisfactory and unusable for evaluation.

3.2 Annual distribution

In total we abstracted 132 eligible patients treated from 2009-2013 at the Section of Thoracic Surgery and Hyperbaric Surgery, University Hospital Graz that matched all the inclusion criteria of the referral diagnosis of suspected gas gangrene or necrotizing fasciitis and were subsequently analyzed in this study. 90 (68%) patients were male, 42 (32%) were female. The review showed an average of 26.4 cases per year over the last 5 years with suspected gas gangrene. Two peaks occured in 2011 and 2013 with each 32 cases. In 2009 the lowest number with only 20 cases was recorded, followed by 21 cases in 2012, and a total of 27 cases in 2010 (Fig. 5).

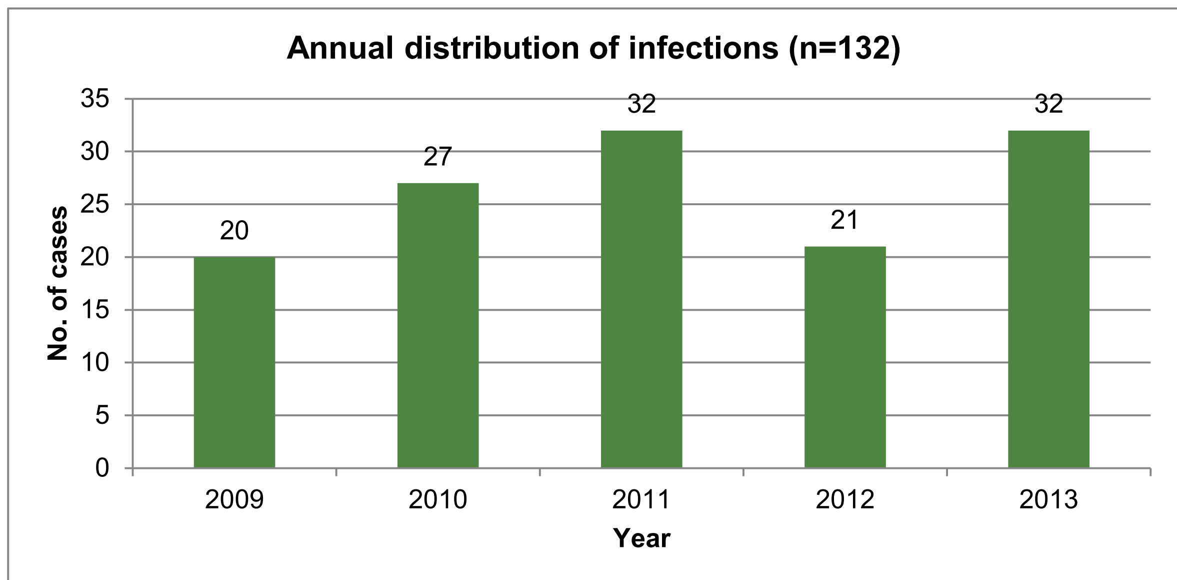

Figure 5: Annual spread of infections suspected as gas gangrene or necrotizing fasciitis

3.3 Age and Gender

All age groups were included, the median age of all 132 patients was 57 years. The youngest patient was an 11-year-old female, the oldest a 94-year-old female. The highest number occurred between the ages of 40 - 49 years with 26 cases. The age groups older than that deviated insignificantly between 21 cases between 50 - 59, 24 cases between 60 - 69 years, and 23 cases between the ages of 70 - 79 years. Older people were less affected. Only 15 cases occurred with an age older than 80 years, and only one woman was older than 90 years. The age groups 0 - 19 and 20 - 29 showed each only 3% of the cases. 15 patients (11%) were between 30 - 39 years, which created an abrupt increase in the case number with rising age.

The distribution between men and women was in favour of men, 90 (68%) patients were male, 42 (32%) were female. Between the median age of male patients, 57 years, and female median age, 56 years, was no distinct difference. The highest peak for men was between the ages of 40 - 49 years, for women over 80 years.

The following figures show the age distribution of the observed population and the age distribution subdivided into males and females (Fig. 6, Fig. 7).

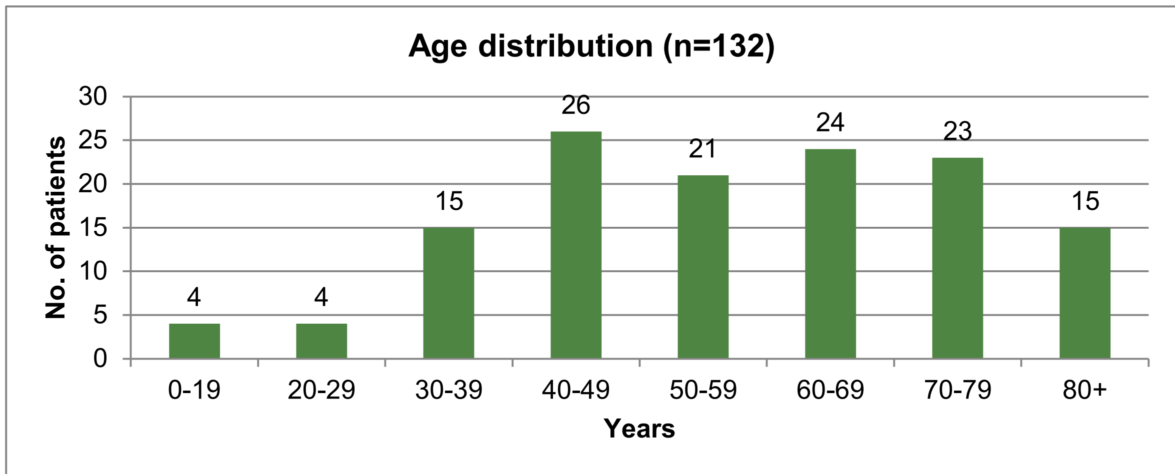

Figure 6: Age distribution of patients with diagnosis of gas gangrene or necrotizing fasciitis treated at Medical University Graz from 2009 to 2013

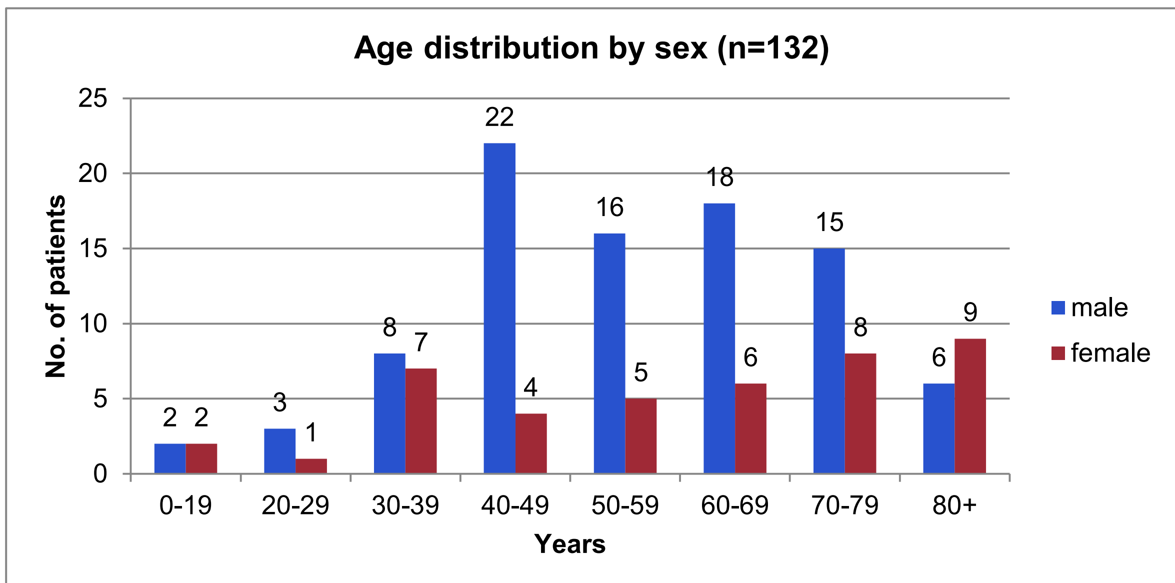

Figure 7: Age distribution by sex of patients with diagnosis of gas gangrene or necrotizing fasciitis treated at Medical University Graz from 2009 to 2013

Later, analyzing the differences in the occurrence of the different *Clostridium* sp., two peaks could been seen when using a subdivision into age distribution. One for *C. perfringens* in the age groups over 80 years and one for different *Clostridium* spp. in the age between 70 - 79 years (Fig. 8). In men infections with *C. perfringens* or other kind of clostridia were balanced at 7 cases each. One man had a double-infection with *C. perfringens* and *C. paraputrificum*. Women more often had infections with *C. spp.* other than *C. perfringens*.

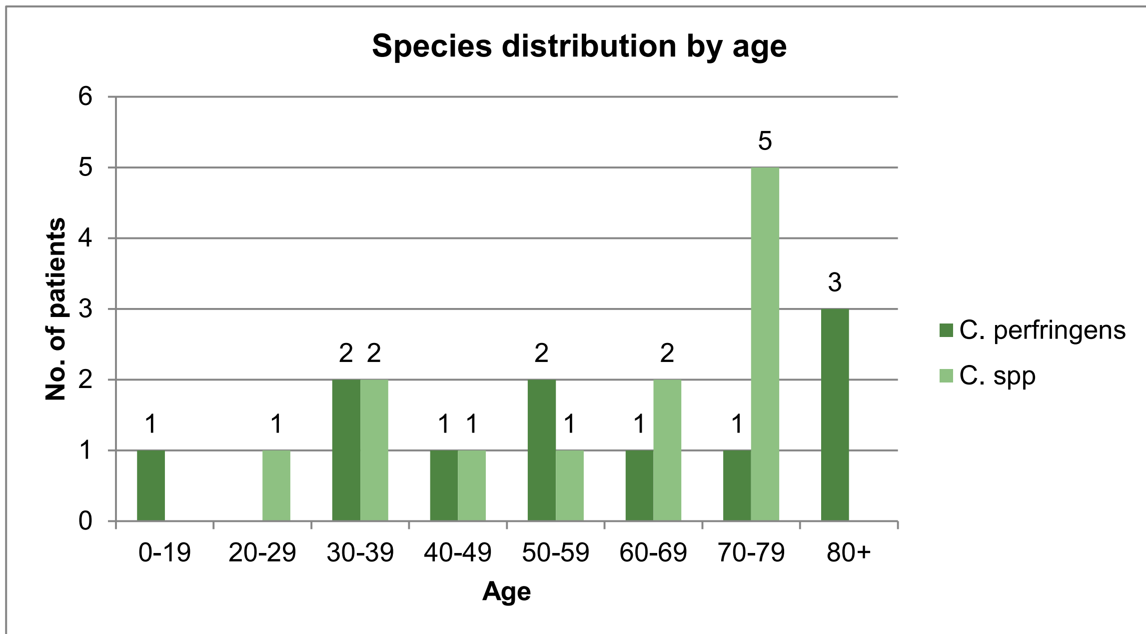

Figure 8: Connection between *Clostridium* sp. and age of the patients
*C.*spp: *Clostridium* species (such as *C. septicum*, *C. bifermentans*, etc.)

3.4 Distribution of specimens according to origin

40% (53 of 132) of the culture-positive specimen were from the lower extremity, including the lower leg or lower leg stump followed by the thigh. Second most frequently the infectious source was the abdomen (15%), 11% were from the upper extremity, especially from the upper arm. Five percent were from the thorax, four percent were from the inguinal area, and two percent were from the gluteal area. Eight percent were from the genital area, the part most affected here was the scrotum. Due to a lack of complete entries 15% of the origins of the specimen remained unclear and were defined miscellaneous. These are specimens, which were not specifically designated, such as wound swab, puncture from a blister, or sample or swab from a drainage.

The distributions of specimen is depicted in Figure 9.

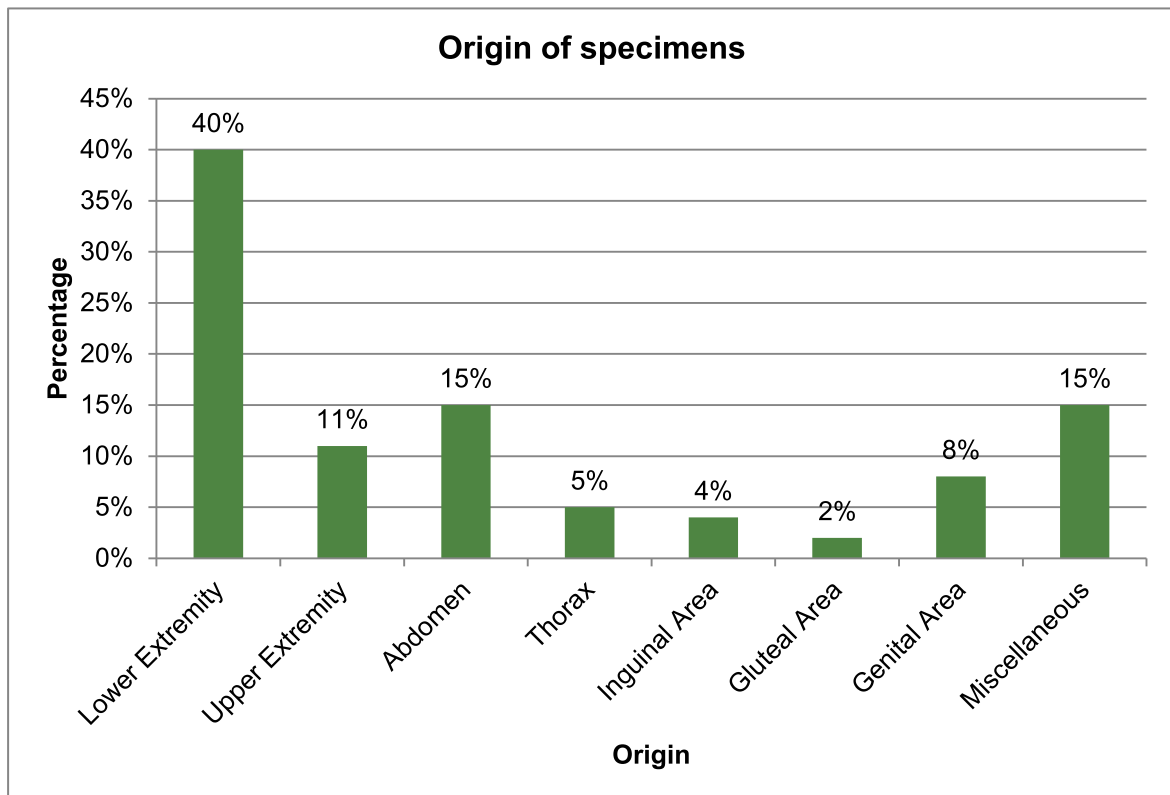

Figure 9: Percentage distribution of specimen taking location

3.5 Microbiological Results

3.5.1 Results of Gram-staining

In the microbiological laboratory each routine sample is normally gram-stained at the beginning of the culturing process and after that cultivated on different agar plates. To define, routine samples are samples with no urgent call from the clinician, mainly transported by a transport service.

If clinicians declare the sample to be emergent (emergency call, suspicious diagnosis of gas gangrene or necrotizing fasciitis) a priority processing in the laboratory will be performed with an immediate gram-staining.

On result of the gram-staining the microbiologist calls the clinician to share the details of the gram-staining, such as cells and formation and gram-behavior of included bacteria.

For this study the results of each gram-staining of the included patients were analyzed and compared to the results of the microbiological culture.

In total 132 gram-stainings and the respective results of the microbiological cultures could be analyzed. The gram-stainings showed various components in a different incidence (Fig. 10). Gram-positive rods were found in 12 gram-stainings (9%). Apart of *Clostridium* spp.

in ten cases, in two cases *Actinomyces turicensis* were found in definite microbiological culture results.

Nearly half (46%) of the samples contained inflammation cells (Leukocytes). Erythrocytes were found in 20% of the gram-stainings. In 69% the microscopy showed a species of bacteria. 36% were gram-positive cocci, later identified as i.e. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pyogenes*, *Streptococcus agalactiae*, and different *Enterococci*. Gram-negative rods were found in 23% of the samples. Illustrative are *Escherichia coli*, *Prevotella denticola,* and different *Klebsiella* spp. found in microbiological culture. 0.8% showed a sign of fungus. 26% of the gram-stainings showed a negative result.

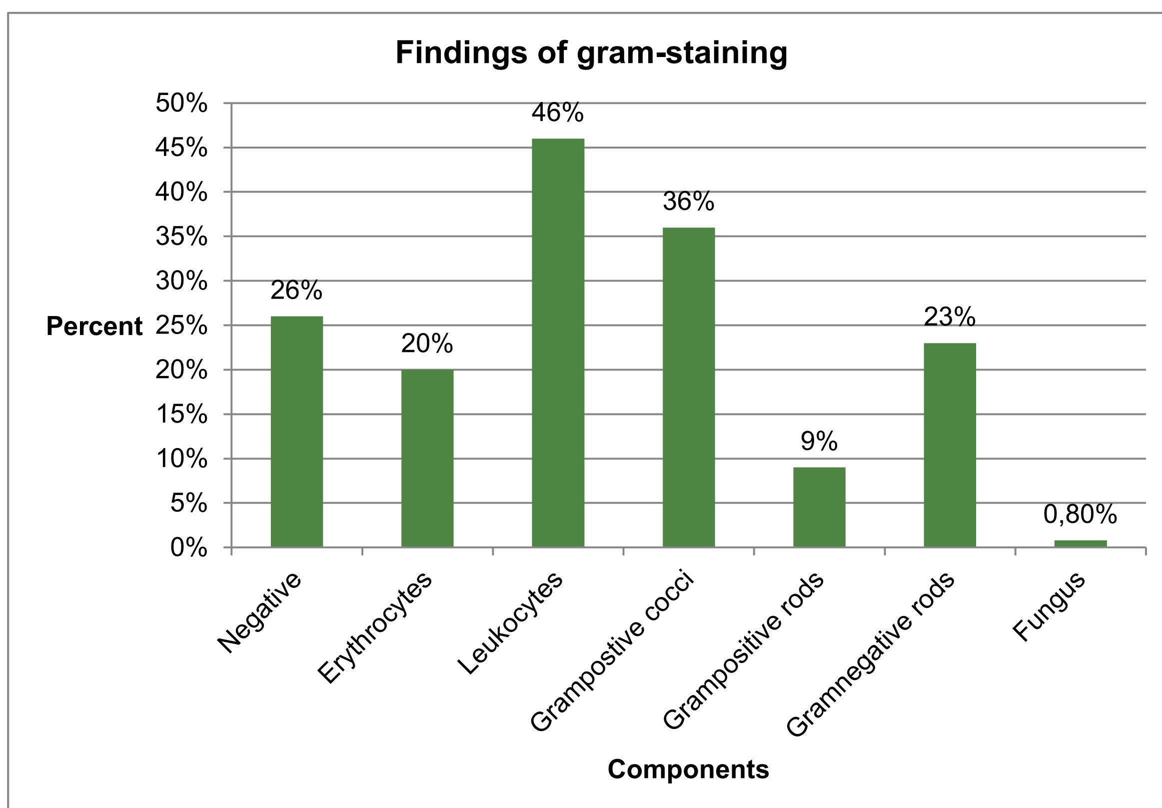

Figure 10: Percentage of components in the gram-stainings

Regarding the behaviour of demand of the clinicians, in 63 cases (48%) clinicians sent the samples with the diagnosis of suspected gas gangrene or necrotizing fasciitis. In 69 cases (52%) clinicians sent samples with a standard request for microbiological examination.

18 (14%) of the 63 samples with suspected gas gangrene or necrotizing fasciitis showed in the microbiological culture growth of clostridia. In samples with standard request only five of 69 samples (four percent) showed clostridia, none of these had gram-positive rods in the gram-staining.

Regarding the total 23 samples with cultured clostridia, the result of the respective gram-stainings showed gram-positive rods in 10 cases.
When comparing the negative gram-stainings and those with no evidence of gram-positive rods, but other bacteria, in 13 out of 120 (11%) cultures, growth of a *Clostridium* spp. was found.

To analyze the accordance between the results of the gram-staining and the results of the cultures, we defined the accordance as complete accordance between the two methods, partly accordance, for example if only one kind of bacterium was seen in the gram-staining but different kinds were cultivated afterwards, and as no accordance, for those samples with a negative gram-staining but bacterial growth in culture.
To note: gram-staining has an operational limitation, as it can only show bacteria with a concentration of 10^5/ml or higher. Therefore it is possible that a negative result in the gram-staining can lead to a growth of bacteria anyway.

Total accordance of the gram-staining and the respective microbiological culture was found in 82 (62%) of all cases, 12 cases (nine percent) were partly according. In 38 cases (29%) no accordance was found (Fig. 11). With regard to our main interest, clostridial infections, the distributions of the 23 cases is as follows (Fig. 12): nine cases (39%) accordance, five cases (22%) partly accordance, and nine cases (39%) no accordance.

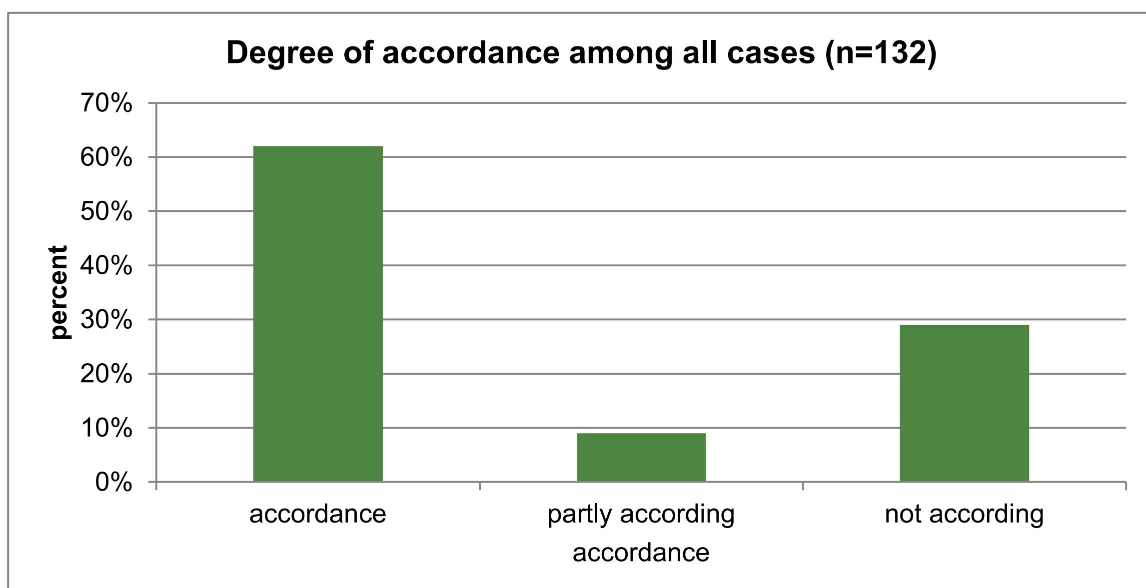

Figure 11: Accordance between gram-staining and the final results of the culture in all cases

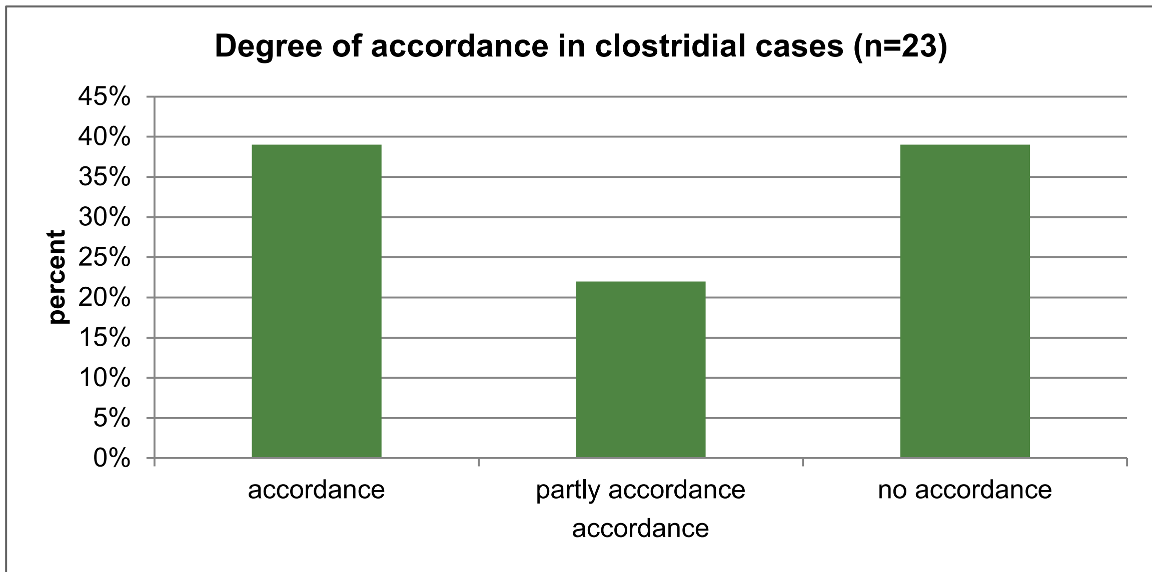

Figure 12: Accordance between gram-staining and the final results of the culture in cases with clostridia

To elucidate the operational limitation of gram-staining, bacterial count and microbiological culture, we took a look in detail if there was an explanation for the number of cases in which the gram-staining and the culture results came to no consens. This analysis was only done for samples with clostridial growth, as again this was the main interest of our study. On the one hand a positive result in the gram-staining could only be seen with a bacterial count of 10^5 or higer. On the other hand microbiological laboratories use various agar plates, therefore bacterial growth can be estimated in very low concentrations. Routinly bacterial concentration on agar plates will be defined as 'bacterial count' and is divided in several categories, such as very sporadic, sporadic, moderate and massive bacterial count, estimated by the number of colonies counted on the agar plates.
In 21 samples the bacterial count could be assessed (Fig. 13).

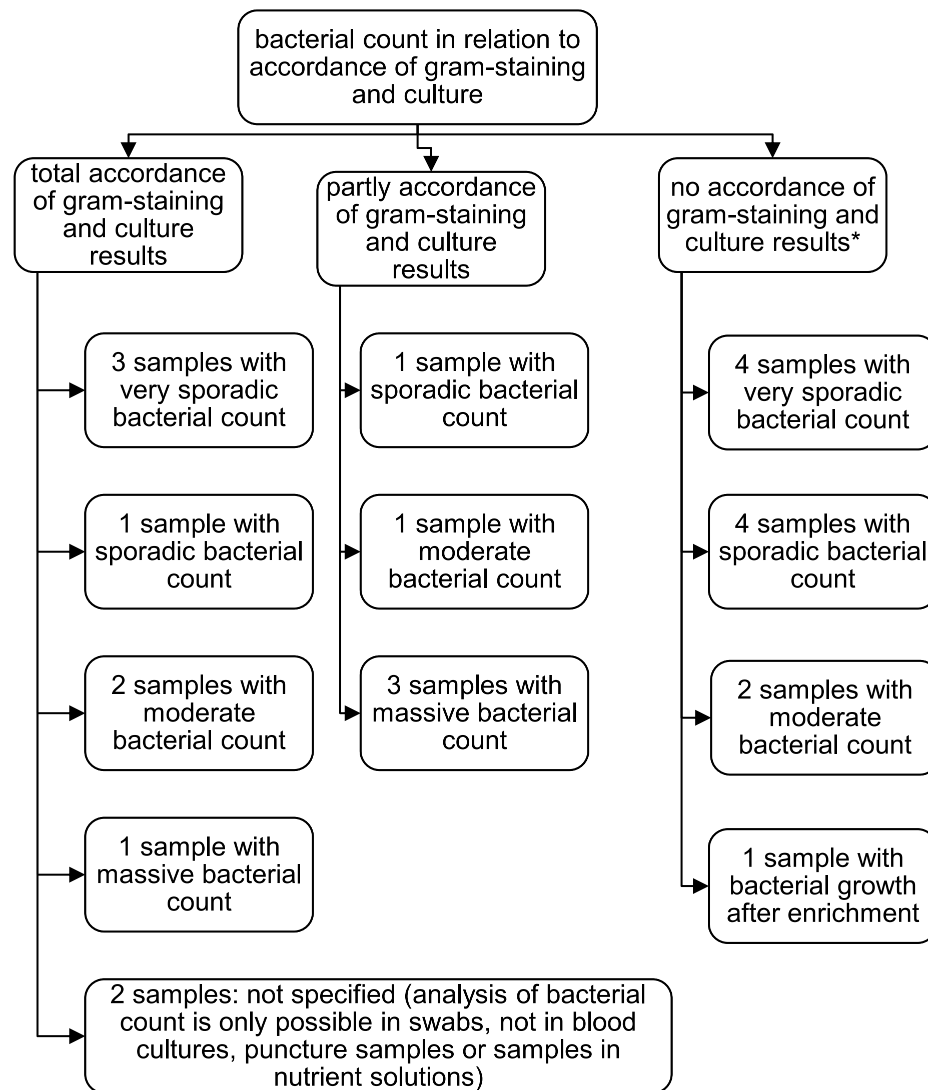

Figure 13: Flow diagram of bacterial count in relation to concordance of gram-staining and culture
*Values to not add to total because of double counting samples with multiple kinds of bacteria

In the group with total accordance between the two obtained results (gram-stain and culture) one sample showed a massive bacterial count, two samples showed a moderate bacterial count, one a sporadic bacterial count and after all three samples showed a very sporadic bacterial count. In the group with no accordance between the gram-staining and the microbiological culture eight of nine samples showed very sporadic or sporadic growth of clostridia. Only one sample showed a moderate bacterial count in microbiological culture. Two samples could not be analysed with this method, because it works only with swabs, but not with samples from blood cultures, punctures or samples in a nutrient solution. These samples were valued as ‘not specified’. One sample showed a bacterial growth only after enrichment of the medium, designated as ‘after enrichment’.

3.5.2 Comparison of gram-stainings with gram-positive rods and cultures with proof of clostridia

In this section two different points of the study were analyzed:

On the one hand all gram-stainings of the included 132 samples with proof of gram-positive rods were analyzed, and on the other hand gram-stainings with negative results or no proof of gram-positive rods. This anaylsis showed that in the group with gram-positive rods the results were according or at least partly according, while all disaccording results were in the group with no clue for gram-positive rods in the gram-staining at the beginning of microbiological laboratory.

10 of the 12 gram-stainings with gram-positive rods showed in the culture a growth of a *Clostridium* spp.. The two other samples showed *Actinomyces turicensis* as gram-positive rod as definite result.

Results of the microbiological laboratory used

Results	Culture incl. gas gangrene series*	Routine Culture	Total
No. of total examinations performed	63	69	132
Proof of *Clostridium* species	18	5	18
Gram-positive rods in gram-staining	11	1	12
Other bacteria than grampositive rods in gram-staining	29	49	**
Negative gram-staining	13	21	34

Table 5: Results of the different microbiological methods

* Culture with definitive referral diagnosis of gas gangrene or necrotizing fasciitis include a minimum of one anaerobic plate more than routine samples

** Values do not add to total because of double counting samples with multiple kinds of bacteria

3.5.3 Results regarding samples with clostridia

Beside *Clostridium perfringens* in 11 cases we found some other species from the gas gangrene causing clostridia in the remaining 12 cases, such as *C. bifermentans*, *C. septicum*, and *C. sordelli*. Furthermore we found *C. paraputrificum*, *C. clostridioforme*, *C. subterminale*, *C. ramosum*, *C. sporogenes*, and *C. tertium*. The number of infections with *C. perfringens* (11 infections) and infections with any other kind of *Clostridium* spp. (12 infections) was nearly evenly spread. The distribution for an infection with *C. perfringens* between men and women turned out in favor of men, as men were generally more affected. Infections with different *Clostridium* spp. (other than *C. perfringens*) turned out fairly equally distributed between both sexes. Eight infections processed as a monoinfection. In

three cases the patients had more than one *Clostridium* spp.. In total 15 patients presented with a mixed infection with mostly more than two different bacteria.

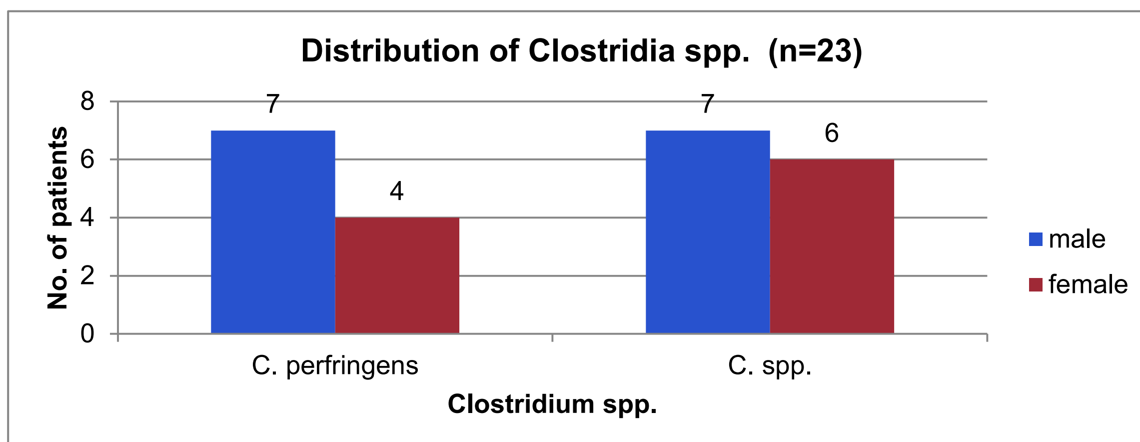

Figure 14: Distribution of *Clostridia* spp. between men and women
Values do not add to total because one patient had an infection with both, *C. perfringens* and *C. paraputrificum*

Concerning all, the highest peak of clostridial infections was in 2010 with seven infections out of 27. On average 4.6 cases per year of infections with secured evidence of clostridia occurred. The lowest incidences were noted in 2009 and 2011 with three cases each year, followed by 2012 and 2013 with five cases each.

Distribution of clostridia cases by year

Year	*Clostridium perfringens*	Other *Clostridia* species	Total
2009	2	1	3
2010	3	4	7
2011	1	2	3
2012	3	2	5
2013	2	3	5
mean value per year			4.6

Table 6: Distribution of clostridia cases by year, patients treated at the Section of Thoracic Surgery and Hyperbaric Surgery

3.6 Analysis of emergency calls made by the microbiologists

Within the observation period of five years in total 61 emergency return calls were documented by the microbiologists regarding suspicion of gas gangrene or necrotizing fasciitis aroused in the clinical setting (Fig. 15). Four of the calls were second calls, which followed the next day for further detail. Of all 61 calls, 45 were made outside the routine working hours from 8 am to 4 pm. It might be reasonably assumed that calls during working hours were not always documented in the case-files, therefore presumably we cannot consider these as absolute figures. Calls outside 8 am – 4 pm were made by the

microbiological emergency service provided by the Institute of Hygiene, Microbiology and Environmental Medicine.

Normally the calls take place on the day of entry of the samples after a gram-staining is performed, in our study these were 56 calls (Fig. 16). Included were also calls which were made on the second day or even later, due to new developments seen on the culture plates.

Our data showed three calls made on the 3rd day and two on the 5th day of incubation to inform the clinicians about a definitive growth of *C. perfringens* that could be observed. Four of these *C. perfringens* were foung in routine samples, only one *C. perfringens* came with a request for gas gangrene/ necrotizing fasciitis.

The annual number of emergency calls increased constantly from 2009 to 2013. On average 12.2 calls were made each year, at which 9 were outside 8 am – 4 pm, and therefore made by the emergency service.

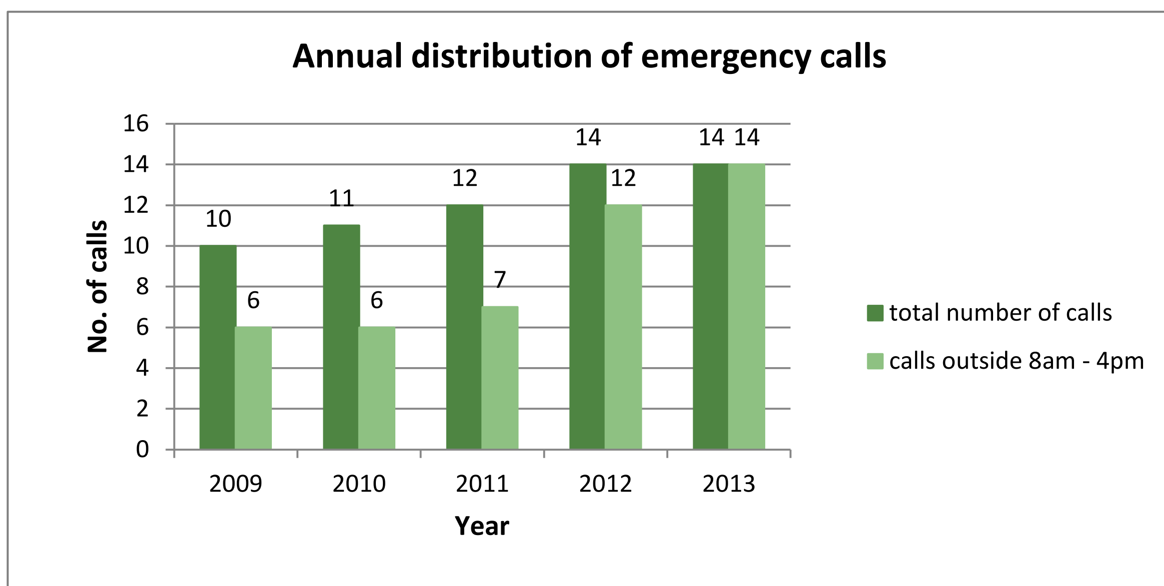

Figure 15: Annual distribution of emergency calls and calls made outside the routine working hours

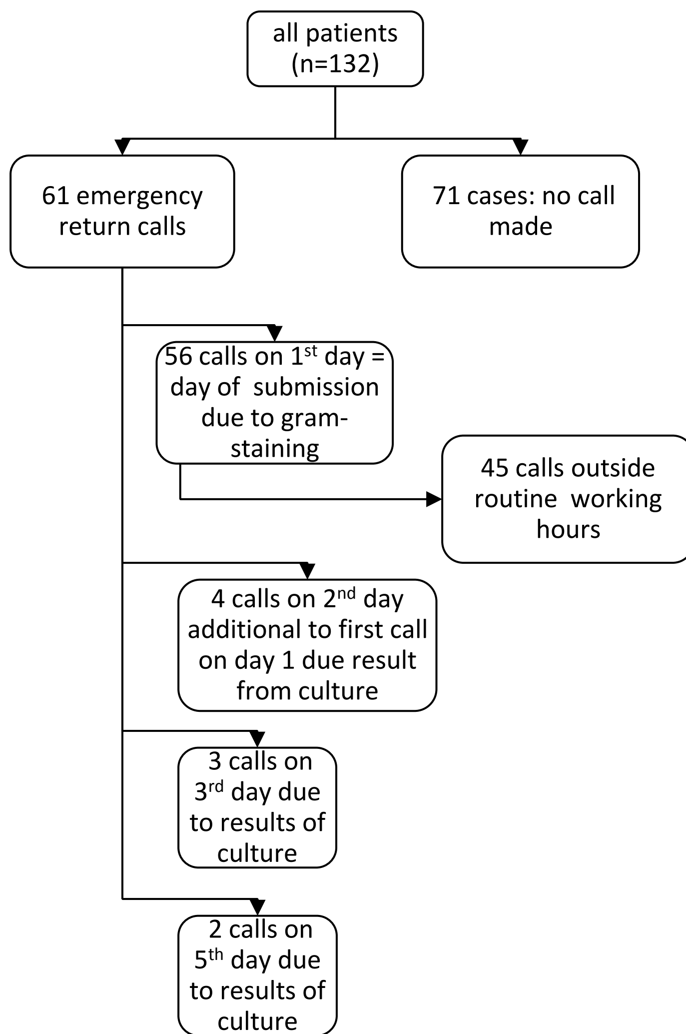

Figure 16: Flow diagram of distribution and temporal behavior of emergency calls mady by microbiologists

4 Discussion

Clostridial myonecrosis due to *Clostridium perfringens* is a rare and uncommon disease in civilian population. Optimization of fast and secure detection of infections with *Clostridium* species has to be investigated because of the aggressive progression and the high morbidity and mortality rate that are associated with this kind of disease. Gram staining is a method, characterized by simple practicability, high availability and low costs. Therefore, it would be an ideal supplement to facilitate diagnosis of clostridial myonecrosis. Gram-staining is already used to support clinicians in planning and selecting empiric clinical management for infections due to bacteria pending definite culture results. For a disease with a fatal nature like clostridial infections the results of such methods are crucial, and should be characterized by high reliability. Browsing literature about the use of gram-staining compared with the use of culture regarding clostridial infections remained unsatisfactory. However, there are studies on the importance of gram-staining regarding e.g. bacterial meningitis and pneumonia. In a study of 10 patients with *Klebsiella pneumoniae* meningitis Khan *et al.* were able to demonstrate, that '*the examination of Gram stain on admission can be negative or misleading; because of the small number of organisms, patients being partially treated, and culture time of more than one day may be required, thus delaying the final diagnosis of meningeal infection and representing a prognostic factor of mortality*' (30). This statement meets the challenges that come with a clostridial infection. Several other authors examinated the use of gram-staining in different kinds of pneumonia. Mimoz *et al.* compared the results of two observers, performing gram-staining on 10 fields and 50 fields, reporting that gram-staining of protected pulmonary specimens is able to predict ventilator-associated pneumonia (VAP) and additionally partly identified causing agents when they were growing at considerable concentrations in culture (31). In a meta-analysis examination by O'Horo *et al.* an admissible concordance between the negative result of gram-staining and probability of occurrence of VAP was evaluated. Despite a positive gram-staining was not as specific for VAP than previously assumed (32). Given all these unequal statements, these are subject to considerable uncertainty about approaching a reliable method for bacterial infections with the necessity of immediate action.

The aim of this retrospective study was to show how microbiological findings in relation to a suspected clostridial myonecrosis complete each other and if special circumstances of

these form group, warrant special measures, in view of the 24h emergency service for clinicians.

In a 5-year period, from 2009-2013, patient data was collected from the Institute of Hygiene, Microbiology and Environmental Medicine and the Section of Thoracic Surgery and Hyperbaric Surgery at the Medical University of Graz. The background of the cooperation with the Section of Thoracic Surgery and Hyperbaric Surgery is based on the fact, that for patients with this kind of disease a therapy session in a HBO facility is essential part of the therapy.

The study was conducted at the Medical University Graz, where a total of 132 patients was reviewed and analyzed. Our study was untertaken to see how reliable gram-staining is as a first-line method (also performed by the emergency service of the microbiological laboratory), compared to microbiological culture, for the detection of *Clostridia* spp. in the context of clinical suspected gas gangrene. The population consists of patients with clinical referral diagnosis of suspected gas gangrene or necrotizing fasciitis. 23 patients had culture-proven infections with a *Clostridium* species. The current incidence of gas gangrene is not well observed and literature about that is rare. Studies from the 1960-ties to the 1980-ties reported an incidence about 0,1/100.000 population per year (10,33). In Germany 100 - 140 cases of gas gangrene have been reported from the Robert Koch Institute during 1992 and 1996. In 1997 data was published by the RKI with 122 cases of gas gangrene, recorded in a register of notifiable infections/ contagious diseases (34). In our study 90 (68%) patients were male, 42 (32%) female, giving the male-to-female ratio of 2.2:1, men seem to be more often affected than women. Other studies have reported a comparable distribution between men and woman (33,35).

We first tried to sort the patients by their referral diagnosis. This presented as mentioned before as a big challenge that ultimately could not be solved. The classifications of NSTI and gas gangrene vary considerably and do not meet ICD-10 classification in our case. Even if the terminology is not uniform, the different definitions can give a clue to the disease and about its course.

Based on origin of the specimens we classified groups by bodypart affected by the infection. This showed that 40% of the infections were located in the lower extremity, most frequently in the lower leg. Our study shares this findings with similar findings for gas gangrene and necrotizing fasciitis in literature (36,37). It appeares that there might be a conection between the higher incidence for pre-existing conditions and underlying diseases in the lower extremity, such as diabetes, PAVK, foot ulcera and others mentioned above

(38). Subsequently we analysed the collected microbiological data. Therefore, we evaluated first all patients and further on distinguished between patients who showed an infection with clostridia and those who did not. All 132 patients had a gram-staining and a culture result. First, we searched gram-stainings for samples with gram-positive rods. 12 out of 132 revealed a positive result for gram-positive rods. Afterwards microbiological culture results were analyzed exclusively for clostridial growth. Clostridia were cultured in 23 out of 132 cases (17%). 11 of 23 patient samples showed *C. perfringens*, the remaining 12 samples belonged to different kinds of clostridia. We also noticed a broad spectrum of coinfecting or causing bacteria other than clostridia species, such as *Group A ß-hemolytic Streptococci*, and *Staphylococcus aureus*, responsible for the clinical appearance of gas gangrene or necrotizing fasciitis. Just as Elliott reported in his analysis, an extremly wide range of microbial pathogens could be found in patients with NSTI (39).
Gram-staining is an economical way of providing fast information which can point clinical findings in a distinct direction. This method provides clinicians with useful clues directed towars a fast diagnosis and supports their decision for empirical antibiotic treatment.
In this study in 12 gram-stainings gram-positive rods could be observed and were confirmed as *Clostridium* sp. in ten and as *Actinomyces turicensis* in two cases. Regarding the result of microbiological cultures, clostridia were found in 23 cases, wherease in these cases gram-positive rods were seen in only ten cases (8%). In all investigated 132 patients accordance of gram-stainings and the regarding microbiological cultures was found in 62% (total accordance) and 9% (partly accordance). To analyse why the accordance was not hundred percent we took a look at the bacterial count of the culture results. We hypothesize that a lower bacterial count in the samples attributes to a negative result in the gram-stainig. This may have affected the ability of detecting a match between gram-staining and culture result. Our data showed that eight of nine samples with no accordance between gram-staining and microbiological culture contained only very sporadic or sporadic bacterial count.

At the Institute of Hygiene, Microbiology and Environmental Medicine a 24h emergency call service is offered for patients with suspected gas gangrene or necrotizing fasciitis. This allows a rapid exchange of information between clinicians and microbiologists. While routine laboratory work is normally from 8 am to 4 pm, in case of patients with suspected gas gangrene emergency return calls are provided 24 hours a day. Our analysis showed 61

emergency return calls in the evaluated time period. 45 of these calls were made outside the routine working hours (8 am – 4 pm).
As mentionened above the study has some limitations that should be assessed. There are limits that come with methods or a statistically approach as disscussed above. Apart from the bacterial count necessary for a proper gram-staining, the evaluation of the documented return calls is prone to error, since calls during routine working time might not be documentent. This is leading to a false positive result in the number of emergency calls outside the given time period.

5 Conclusion

Our study indicates, regarding to samples with clinical suspicion of gas gangrene, that the reliability of a gram-staining result with gram-positive rods is acceptable good and in our case in 83% lead to the result of a cultured-proof of clostridia. In contrast, a negative result for gram-positive rods in gram-stainings can show clostridial growth after all. Also other kinds of bacteria could be seen in the primarily gram-stain with an accordance (total or partly) of more than 70% (gram-staining at the beginning and microbiological culture result) This could be used as an argument to continue the emergency service provided by the Instiute of Hygiene, Microbiology and Environmental Medicine. As well as the service is not only for suspected gas gangrene but also provided for cases with suspected meningitis.

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