The objective of the study was to perform a comparative analysis of genetic similarity, with the use of pulsed field gel electrophoresis (PFGE), of Clostridium perfringens isolates originating from patients with gas gangrene and from the hospital environment. The study encompassed two patients with a clinical and microbiological diagnosis of gas gangrene, who were hospitalized in one of the hospitals of the Małopolska province in the time period between 31st March 2012 and 18th May 2012. Clostridium perfringens isolates genotyping indicated that the isolates originating from the two studied patients did not display genetic similarity and represented two different PFGE types, which corresponded to two different clones (clone A and B). Whereas the strains isolated from the hospital environment were genetically identical with the strain coming from the second patient and represented one PFGE type, which corresponded to one clone (clone A). As a result of the study, it is possible to conclude that both patients developed endogenous infection. Even so, the examination of the hospital environment indicates the possibility of the appearance of exogenous infections. It prompts recommending and following the exact regulations of sanitary regime in the ward and the operating theater if a patient is diagnosed with gas gangrene.

**Key words:** Clostridium perfringens, gas gangrene, pulsed field gel electrophoresis – PFGE, epidemiological investigation

Gas gangrene, also known as Clostridial myonecrosis (Lat. gangrenea gaseosa) (1), is a bacterial infection with severe clinical course caused by anaerobic Gram-positive bacilli of the genus Clostridium. The most commonly isolated etiological factor of gas gangrene is Clostridium perfringens species (up to 90% of cases) (2, 3).

Bacteria of the genus Clostridium are widespread in nature and can be found in, among others, soil, dust, as well as the intestinal and reproductive tracts of humans and animals (3). Among healthy individuals, the percentage of C. perfringens carriage ranges from 6% to 31% but it applies primarily to elderly patients (4). The frequency of occurrence of gas gangrene, according to literature in the English language, is assessed to be from 0.1 to 1.0 incidences per one million residents per year and is mainly associated with traumas (5). In the 1970s, there were 70-80 cases reported in Great Britain per year, 200-400 in the USA, while Polish estimates talk about 100 cases of gas gangrene per year (3).

C. perfringens pathogenesis is determined by a range of extracellular toxins, which were...
designated by consecutive letters of the Greek alphabet. Alpha-toxin (α), that belongs to the group of major toxins, as well as theta-toxin (θ) and kappa-toxin (κ), are responsible for local and systemic symptoms characteristic of gas gangrene (6).

For reasons of high toxicity, which results in invasiveness, the species *C. perfringens* is recognized as alert microorganism in accordance with the Minister Regulation of 23rd December 2011 concerning the list of alert factors, hospital infections and alert factors records as well as reports on current epidemiological situation of the hospital (7).

Depending on the clinical course, 3 types of soft tissue infections caused by bacteria of the genus *Clostridium* are distinguished. The first type is constituted by a simple contamination of the wound with bacteria, which is not characterized by inflammatory reaction at the site of injury. A more serious degree of infection is second type, which includes skin and subcutaneous tissue inflammation without affecting the muscles. It may, however, affect the fascia and result in necrotizing fasciitis. In this case, a seropurulent discharge from the wound is present, sometimes with a slight toxic reaction. The third, and most severe, infection type is extensive muscle necrosis with severe toxemia. In this case, the symptoms of gas gangrene may develop as soon as 6 hours from infection, in many cases leading to shock and multiple organ dysfunction syndrome (50% of the cases) and mortality reaching 40% (5).

The most common symptoms of gas gangrene are sudden, very severe pain, which is poorly responsive to painkillers, edema and erythema of the skin and soft tissues, epidermoid cysts filled with blood and serum or sanies, or a discharge of similar fluid from the wound, crepitus to the touch and a characteristic fetid smell. Moreover, general symptoms dependent on the stage of toxemia appear, there are manifestations of the toxic shock syndrome and of the multiple organ dysfunction syndrome (MODS) (5,8).

The basis for diagnosing gas gangrene is, apart from clinical picture, microbiological diagnostic analysis. In the said analysis, it is prerequisite to properly take material for testing, which is constituted by a tissue fragment or sanies from the border of healthy tissues, and in the situation of inability to take biopsy material – a swab. A direct smear is prepared from biological material and it should be stained using Gram stain technique and tested under the microscope for Gram-positive cylindrical forms. It is very characteristic of direct smears not to contain leucocytes, which results from lytic activity of lecithinase and theta-toxin. Simultaneously, biological material should be used in order to prepare a culture for aerobic and anaerobic bacteria, that enables a conclusive confirmation of occurrence – isolation of *Clostridium perfringens* species. A histopathological test of tissue biopsy material taken from the site affected by gas gangrene enables, in a way similar to direct smear, quick preliminary confirmation of the diagnosis. In order to make a diagnosis of gas gangrene and implement the treatment, it is sufficient to have a clinical picture of the patient and observe the presence of Gram-positive cylindrical forms in the direct smear, while the *C. perfringens* culture is of confirmatory significance (5, 8).

Owing to the use of molecular methods employed for bacterial isolates typing, it is possible to determine the genetic similarity of species originating from infections and from hospital environment (9). Clinical isolates typing is particularly important in epidemiological investigation testing, which aims at detecting causes, sources and mechanisms of microorganisms spreading among patients, e.g. in hospital wards (10). One of the most widespread method for *C. perfringens* isolates typing is pulsed field gel electrophoresis (PFGE). In this method, upon the application of a restriction enzyme that cuts bacterial DNA, we obtain patterns of genetic profiles. A comparative analysis of these, conducted with the use of specialist software, enables the determination of genetic similarities and of affiliation of individual isolates to clonal types (9).

The objective of the study was to perform a comparative analysis of genetic similarity, with the use of pulsed field gel electrophoresis (PFGE), of *Clostridium perfringens* isolates originating from two patients with gas gangrene and from the hospital environment.

The study encompassed two patients with a clinical and microbiological diagnosis of gas gangrene, who were hospitalized in one of the hospitals of the Małopolska province in the time period between 31st March 2012 and 18th May 2012.
CASE REPORTS

Patient 1. A man aged 60, was admitted to the General Surgery Ward of the district hospital on 31st March 2012 due to right lower extremity ischemia with the diagnosis of gas gangrene of the right foot. After taking the patient’s medical history, it became known that he underwent a left thigh amputation in 2006. On 12th April 2012, first symptoms of right extremity inflammation became visible and it was amputated that day. In the first 24 hours after the amputation of the right extremity, the patient’s general condition was serious. A mixture of blood and serum was discharged from the drains. In the night from 12th April to 13th April, the patient’s condition deteriorated and at 4:50 the contact with him was lost. Blood pressure decrease was recorded to be BP 90/60. After consultation, the patient was intubated and transferred to the Anesthesia and Intensive Care Ward. On 13th April, in the morning, a surgical consultation was conducted, stitches removed, the wound opened, integuments incised and H₂O₂ washing was performed and oxygen was administered to the wound. That day, empiric antimicrobial therapy was introduced: metronidazole IV 3 x 0.5 g/24 h. On 13th April 2012 at 11:50, the patient was pronounced dead. Final diagnosis: underlying cause – atherosclerosis, intermediate cause – right thigh gangrene, immediate cause – multiple organ dysfunction syndrome.

Microbiological testing

In order to perform a microbiological diagnostic analysis of etiological factors of infection, different types of material were taken from the studied patients and from the hospital environment. These were, among others, blood, peritoneal fluid, a smear from the patient’s bed mattress, a smear from the aspirator in the operating room, and a smear from the table in the morgue (tab. 1). Portagerm Amies Agar (bioMerieux) was used as transport medium. Directly upon the delivery to the lab, the materials were cultured into Columbia Blood Agar (Oxoid) medium and incubated at 37°C for 24 h under aerobic conditions and into Schaedler Anaerobe Agar (Oxoid) incubated at 37°C for 48 h under anaerobic conditions. *Clostridium perfringens* species identification was conducted with the use of API 20A (Oxoid) tests. *C. perfringens* isolates were secured for further testing on VIABANK (MW&E) at -70°C.

* C. perfringens isolates typing with PFGE

The pulsed field gel electrophoresis (PFGE) method was employed for the analysis of genetic similarity of *C. perfringens* isolates taken from the following clinical materials: from patient number 1 – isolate 1/1 characterized by morphotype 1 and isolate 1/2 characterized by morphotype 2; from patient number 2 – isolate 2; from the hospital environment – isolates 3, 4, and 5 (tab. 1). The chosen *C. perfringens* isolates underwent molecular typing using the pulsed field gel electrophoresis (PFGE) method according to the methodology of Maslanka et al. (11). *C. perfringens* ATCC 12915 (The American Type Culture Collection) standard was used as reference strain. Chromosomal DNA of bacterial strains was isolated in agarose blocks and then digested with the use of restriction enzyme *Smal* (MBI Fermentas). Electrophoretic separation was performed on CHEF-DR III (Bio-Rad) machine, while restriction analysis
was carried out using GelCompar II (Applied Maths) software with the application of UPGMA clustering method and Jaccard index. The obtained genetic profiles were interpreted according to the guidelines given by van Belkum et al. (9).

RESULTS

The diagnosis of gas gangrene made on the basis of clinical picture in the two patients hospitalized in the General Surgery Ward of the district hospital was confirmed with microbiological testing, in which *C. perfringens* strains were isolated from the taken clinical materials (patient 1 – blood, patient 2 – peritoneal fluid). In the case of the first patient, the isolated strain number 1 was present in the form of two morphotypes, which were designated 1/1 and 1/2 for further research.

Strains belonging to *C. perfringens* species were also isolated from the materials taken from the hospital environment. In this case, a positive culture was obtained from the smear from the bed number 5 mattress in the room of the patient number 2, as well as from the smear from the aspirator in the operating theater, and from the smear from the table in the morgue (tab. 1).

*Clostridium perfringens* isolates genotyping with the use of pulsed field gel electrophoresis (PFGE) indicated that the isolates originating from the two studied patients (isolates 1/1 and 1/2 taken from patient number 1 and isolate 2 isolated from patient number 2) did not display genetic similarity and represented two different PFGE types, which corresponded to two

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Patient/hospital environment</th>
<th>Type of clinical material</th>
<th>Material collection date</th>
<th>Species identification</th>
<th>PFGE type / clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/1</td>
<td>patient 1 blood</td>
<td>13.04.2012</td>
<td><em>C. perfringens</em></td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>1/2</td>
<td>patient 1 blood</td>
<td>13.04.2012</td>
<td><em>C. perfringens</em></td>
<td>B</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>patient 2 peritoneal fluid</td>
<td>18.05.2012</td>
<td><em>C. perfringens</em></td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>hospital environment mattress smear, bed no. 5, patient room no. 2</td>
<td>18.05.2012</td>
<td><em>C. perfringens</em></td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>hospital environment aspirator smear (before disinfection) from operating theater</td>
<td>18.05.2012</td>
<td><em>C. perfringens</em></td>
<td>A</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>hospital environment table smear from the morgue</td>
<td>19.05.2012</td>
<td><em>C. perfringens</em></td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Analysis of genetic profiles of *Clostridium perfringens* isolates, subjected to DNA digestion by restriction enzyme Smal, carried out with the use of pulsed field gel electrophoresis (PFGE) method and GelCompare II software

Key:
REF – *C. perfringens* model strain (12915 ATCC)
1/1 – *C. perfringens* isolate (morphotype 1) isolated from patient 1 – clone B
1/2 – *C. perfringens* isolate (morphotype 2) isolated from patient 1 – clone B
2 – *C. perfringens* isolate isolated from patient 2 – clone A
3 – *C. perfringens* isolate isolated from mattress smear from bed no. 5 from patient 2’s room – clone A
4 – *C. perfringens* isolate isolated from aspirator smear from the operating theater – clone A
5 – *C. perfringens* isolate isolated from the table smear from the morgue – clone A
Genetic similarities between Clostridium perfringens in gas gangrene patients and from hospital environment

Different clones (clone A and B). Whereas the strains isolated from the hospital environment (isolates numbers 3, 4, and 5) were genetically identical with the strain number 2 coming from the second patient and represented one PFGE type, which corresponded to one clone (clone A) (fig. 1).

DISCUSSION

Gas gangrene may develop following planned surgical procedures and is then called postoperative gas gangrene (35% of cases). Operations predisposing patients to the development of gas gangrene are orthopedic surgeries including amputations of extremities due to ischemia, alimentary canal procedures especially the ones involving intestines and gall-bladder, and surgeries on female reproductive organs. In such cases, it is usually the patient’s endogenous flora that is to blame for the infection. It colonizes the mucous membranes or the skin and, in the conditions of postoperative trauma, is introduced deeper into the tissues, where – coupled with coexistent atherosclerotic ischemia and a decrease in blood pressure – perfect conditions for the development of anaerobic bacteria infections arise (5, 8).

Owing to the very rapid progress of gas gangrene, which is combined with irreversible soft tissue changes, the success of therapy is highly dependent on the time of the diagnosis on the basis of the clinical picture, bacteriological study results and the introduction of treatment (5, 8).

Gas gangrene treatment is a complex process, which consists in a debridement, an application of empirical and then targeted anti-biotherapy as well as hyperbaric therapy. Hyperbaric oxygen therapy (HBO) effectively limits the number of toxins produced by C. perfringens that are responsible for tissue necrosis in gas gangrene, and in connection with antibiotherapy reduces the treatment time and significantly decreases mortality (5, 8, 12).

The antibiotic of choice in the treatment of gas gangrene is crystalline penicillin applied intravenously in high doses (e.g. 24 m units per 24 h), most often combined with metronidazole. Additional effective antibiotics are constituted by aminoglycosides, lincosamides e.g. clindamycin, semisynthetic penicillins e.g. amoxicillin, ticarcillin combined with bacterial β-lactamase inhibitors e.g. clavulanic acid, fluoroquinolones and tetracyclines combined with aminoglycosides (3, 8).

The two described clinical cases of gas gangrene, ending with patients’ death, confirm the fact that gas gangrene is a very serious surgical infection connected to high mortality. At present, gas gangrene is very rare, however, similar cases were previously described in Polish literature (2, 13). As a result of the epidemiological study of the two clinical cases of gas gangrene, it is possible to conclude that both patients developed endogenous infection. Even so, the examination of the hospital environment indicates the possibility of the appearance of exogenous infections since the C. perfringens isolates, which were isolated from the environment that the second patient stayed in, were genetically identical to the ones coming from clinical material from that patient. It prompts recommending and following the exact regulations of sanitary regime in the ward and the operating theater if a patient is diagnosed with gas gangrene (1).

CONCLUSIONS

Methods of molecular biology, especially the pulsed field gel electrophoresis (PFGE) method which is recognized as the gold standard in microbial typing, allow the comparison of genetic affinity of Clostridium perfringens isolates which permits selecting the bacterial clones that are genetically identical. This method is a very useful tool employed in epidemiological investigation of gas gangrene cases reported among patients hospitalized on the same hospital ward.

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