POLISH HYPERBARIC RESEARCH 3(48)2014
Journal of Polish Hyperbaric Medicine and Technology Society

THE DETECTION OF ACINETOBACTER BAUMANNII BACTERIA IN MEDICAL AND ENVIRONMENTAL SAMPLES

Małgorzata Dąbrowiecka [1)], Iwona Piątek [2)], Michał Obuchowski [3)], Zbigniew Dąbrowiecki [1)], Romuald Olszański [1)]

1) Maritime and Hyperbaric Medicine Department, Military Institute of Medicine in Gdynia, Poland
2) Intercollegiate Faculty of Biotechnology, University of Gdańsk, Poland
3) Intercollegiate Faculty of Biotechnology, Medical University of Gdańsk, Poland

ABSTRACT

Acinetobacter microogranisms belong to the genus of Gram-negative rod-shaped bacteria. These free living organisms are observed in the soil and water reservoirs with some of the representatives of the *Acinetobacter* genus being a component of the microflora of the human skin, which causes them to be of great clinical significance due to their presence in the hospital environment and causing opportunistic infections occurring particularly in immuno-compromised patients. The objective of the project was to prepare a fast and credible method for detection of *Acinobacter baumannii* bacteria in medical and environmental samples.
Key words: hospital infections, Acinetobacter baumannii, molecular diagnostics.

ARTICLE INFO

PolHypRes 2014 Vol. 48 Issue 3 pp. 19 – 32
ISSN: 1734-7009 **eISSN:** 2084-0535
DOI: HTTP://DX.DOI.ORG/10.13006/PHR.48.2
Pages: 14, figures: 4, tables: 0.
page www of the periodical: www.phr.net.pl

Publisher
Polish Hyperbaric Medicine and Technology Society

Original article

Delivery date: 08.06.2014 r
Date of approval for print: 13.08.2014 r

INTRODUCTION

The epidemics resulting from hospital infections still constitute a great challenge, they are the reason for prolonged treatment and recuperation, which is usually very costly, or may even lead to a patient's death. The main threat still involves the methicillin resistant staphylococcus aureus (MRSA) and vancomycin-resistant enterococcus (VRE). However, we also must not disregard such common Gram-negative pathogens as: *Escherichia coli*, *Pseudomonas aeruginosa*, *Enterobacter* sp., *Klebsiella pneumoniae* or *Acinetobacter baumannii*. [1,2,3]

The latter is known to quickly gain multi-drug resistance and raise its clinical meaning due to the limited possibilities of treating the infections that it induces. This bacterium is capable of surviving on abiotic surfaces, and in consequence, of long-term existence in hospital. [4]

An effective mechanism of horizontal gene transfer allows this pathogen to acquire resistance to a large spectrum of antibiotics and bacteriostatics over a short period of time. What is noted is a spread of multi-drug resistant traits among hospital patients across the world [5]. Another particularly critical problem involves infections among wounded soldiers taking part in military missions. There have been numerous reports on serious infections induced by *Acinetobacter* microbes in American soldiers returning from Iraq and Afghanistan [6].

What is helpful in restricting hospital infections is the implementation of control programmes aimed at examining the routes of infection and potential pathogen reservoirs. To accomplish this, microbiological tests on patients and aseptic tests on medical equipment are carried out. Moreover, the requirements regarding work hygiene by hospital staff are made more restrictive [2].

Acinetobacter microorganisms do not exhibit much motility and belong to Gram-negative bacilli, and seldom to spherical-rods. They are prototrophs with small growth requirements. Their metabolism is strictly anaerobic, hence they are categorised as the so-called non-fermentative bacilli.

They are free-living micro-organisms found in the soil and water reservoirs. However, some representatives of the *Acinetobacter* genus may also constitute a component of human skin. *Acinetobacter baumannii* and *Acinetobacter lwoffii*, and, to a lower extent, *Acinetobacter calcoaceticus* and *Acinetobacter haemolyticus*, are clinically significant as they often populate hospital environments and are the cause of hospital-related opportunistic infections, mainly in patients with reduced immunity.

They are isolated from different clinical materials – urine, blood, cerebrospinal fluid or material from postoperative wounds [2,7].

Acinetobacter bacteria were first recognised as significant hospital pathogens in 1970. Initially, *in vitro* tests revealed that the majority of clinical isolates were sensitive to commonly used antibiotics such as ampicillin (60-70%), gentamicin (92.5%), chloramphenical (57%) and nalidixic acid (97.8%), thus the infections induced by those organisms could be treated relatively easily.

However, the last two decades showed an increase in the quantities of *Acinetobacter sp.* multi-drug resistant isolates. This is due to the common and not always justified use of strong broad-spectrum antibiotics in hospitals worldwide [3,5,7].

The greatest interest among researchers is observed in relation to *A. baumannii*, as it is the most common human pathogen amidst all the species of the *Acinetobacter* genus.

It is responsible for inducing urinary tract infections, dangerous wound infections and vast burns, as well as pneumonia and secondary meningitis. In fact, severe pneumonia has in recent years been the most frequently reported disease caused by *A.baumannii*.

What is particularly disturbing is the fact that hospital-induced pneumonia is second with regard to incidence in US hospitals. The mortality rate for this type of infection is maintained at the level of 30-50%, with the highest percentage of those induced by *A. baumannii* and *P. aeruginosa*. Additionally, *A. baumannii* belongs to the group of 8 pathogens causing the majority of hospital pneumonia cases in Europe and Asia [3,7,8].

The high pathogenicity of this micro-organism is related to a number of characteristic features of this species, including its capability to populate and survive over a long period of time on abiotic surfaces, insensitivity to numerous antibacterial agents used in disinfection, or the high genetic competence enabling it to quickly acquire drug-resistance. It is estimated that ca. 10-30% of hospital isolates belong to the multi-drug resistant category.

Even the sensitivity to antibiotics from the carbapenem group has begun to significantly decrease over the last decade, which suggests that the antibiotic era in relation to *A. baumannii* may end much faster than it was predicted for methicillin-resistant traits of *S. aureus* (MRSA) to date considered as the greatest threat [5,7,9].

For this reason it is crucial to develop epidemiological studies examining various factors influencing the occurrence and spread of pathogenic micro-organisms, as well as defining the measures for infection prevention and control.

The adaptation of molecular methods for investigating the spread of *A. baumannii* bacteria, the role of medical personnel in transferring infections and the environmental impact on infection development may reduce the risk, or at least help to control it. Reports on epidemic outbreaks caused by multi-drug resistant *A. baumannii* traits are becoming more and more frequent.

The world epidemiological data base contains over 130 records concerning outbreaks of hospital epidemics with the participation of *A. baumannii* published between 1977 and 2006 [10].

It is suggested that in-hospital and countrywide epidemics are induced by only several traits. These are single clones capable of spreading throughout hospital departments, mainly the intensive care units. This resembles the epidemiology of methicillin-resistant traits of *S. aureus*, and for this very reason the *A. baumanni* bacteria are often referred to as the "Gram-negative MRSA".

There have been numerous reports from different places in the world on hospital epidemics caused by *A. baumannii*. An example is an intensive care unit of a hospital in Greece where the epidemic lasted over three months, or an

epidemic induced by two different *A. baumannii* clones in a neonatal intensive care unit in a hospital in Brazil, as well as two subsequent outbreaks in a surgical ward in one of Italy's hospitals.

Hospital epidemics may spread dynamically or occur endemically over a longer period of time, as it was the case in a hospital in Nottingham, where the presence of a trait belonging to the discussed genus was noted for 11 years.

Data in the available literature focus on two main infection transmission routes: (i) infections originating in the environment and (ii) infections transferred from patient to patient, directly and indirectly, with the participation of medical personnel.

Another place commonly populated by *A. baumannii* is medical equipment, a source of multiple infections. Usually these are elements of devices connected with the treatment of respiratory illnesses and respiratory support, such as respirometers, mouthpieces, ventilation tubes, temperature and oxygen sensors, etc. It is the capability of this species to remain on abiotic surfaces (metals, plastic, etc.) over a relatively long period of time (up to several weeks) without any loss in vitality and infectivity that creates the greatest problem in the context of spread and eradication of hospital infections.

Tests conducted in one of the hospitals in New York revealed presence of *A. baumannii* bacteria in 11% of samples collected from abiotic surfaces (beds, tables, screens) and in as many as 29% of samples taken from the hands of medical personnel.

What is more, there are data proving that the main source of infection are in fact medical care centres, as it was observed that ca. 60% of hospital isolates are closely related as compared to merely 17% of environmental isolates. And the source of transfer and spread of epidemics are the patients wandering between hospital units, and the medical personnel [2,7,8].

UNO and NATO military levies, including soldiers of the Armed Forces of the Republic of Poland, are usually stationed in countries with a different climate, with difficult sanitary-hygienic conditions.

Moreover, the low level of economic development, lack of proper medical care ensuring prevention and treatment of diseases in native people produced an additional threat to the health of soldiers who are staying there. Therefore, it is of vital importance to ensure the presence of military medical personnel in mission locations, capable of providing medical protection of national levies and, in consequence, of the entire mission.

The treatment of injured soldiers often entails complications which extend the recovery period. These may include wound infections caused by missile or mine shrapnel, contaminated fragments of clothing, but also infections caused by contact with inadequately disinfected equipment. What is particularly dangerous are injuries related to burns.

The conducted tests showed that in this respect hospital infections are responsible for 50% of mortalities in severe burns victims. Micro-organism isolation and identification indicated the presence of *Pseudomonas aeruginosa* (37.5%), *Staphylococcus aureus* (20.2%) and *Acinetobacter baumannii* (10.4%). However, the analysis of complications in the injury treatment in soldiers evacuated from the mission in Iraq to the USS Comfort hospital revealed predominance of *A. baumannii*, species responsible for severe infections, extremely difficult to cure due to the resistance of isolates to numerous antibiotics [11,12,13].

An important role in reducing hospital and other types of infections is played by microbiological diagnosis allowing for the isolation of an aetiologic factor and determination of its drug-sensitivity.

The currently dominant classical methods of micro-organism typing consist in the analysis of their phenotypic properties, i.e. observation of external effects of the expression of genetic information.

Nonetheless, sometimes there are problems with providing unambiguous identification of a micro-organism, which significantly extends the time of reaching accurate diagnosis. This is related to the common phenomenon of phenotypic variation. For this reason, attempts are being made to find new diagnostic methods allowing quick verification of specious affiliation of pathogens. Such possibility is provided by genotypic methods based on molecular biology techniques of molecular biology.

They are becoming more and more popular in micro-organism identification and differentiation, in particular in epidemiological investigations. They are based on an analysis of genetic material, which is unique and unchangeable for each micro-organism (as opposed to phenotypic properties).

Their advantage is that they are not limited to a strictly defined number of micro-organisms but, quite to the contrary, allow testing of those micro-organisms, whose laboratory growth is extremely difficult or even impossible.

Moreover, as compared with classical methods, the time of performed analyses is definitely shorter, which entails less work and reduced expenses. And, in addition, due to the versatility of the equipment and reagents it is possible to compare the obtained results with other laboratories [1,14,15,16,17,18].

Despite many advantages of molecular biology, current standard diagnostic procedures with regard to the *Acinetobacter* strains still encompass classical methods based on phenotype analysis.

Micro-organisms are cultured with the use of selective and differential media and subjected to a number of biochemical and enzymatic tests. For the purpose of procedure facilitation the tests are carried out with commercially available kits such as API 200NE (bioMerieux) or automated bacteria identification systems, e.g. VITEK (bioMerieux).

However, it is often found that high phenotypic variability causes great difficulties in the classification of those micro-organisms to a particular species, necessitating analysis time elongation and the need of extension with additional tests. Unfortunately, thus far there has not been a description of a reaction, whose result would expressly indicate that the tested micro-organism belongs to a particular species.

Biochemical and enzymatic tests are based on the principle of exclusion (the positive result eliminates affiliation to *A. baumannii*, whereas the negative result of all isolates requires consecutive tests), hence they necessitate further analyses on a molecular level (e.g. 16S rDNA amplification and sequencing) [19, 20].

The above problems cause routine diagnostics of infectious diseases and epidemiological investigations to rely more on methods based on nucleic acid amplification with the PCR technique. Their sensitivity, specificity and ease of use cause them to slowly become standard tools of medical diagnostics [17, 21, 22, 23].

For this reason this project makes an attempt to work out a diagnostic test with the use of a PCR method variation, i.e. the real-time PCR technique. This method allows for a quick detection of *Acinetobacter baumannii* bacteria cells in the tested samples.

What is more, it also enables determination of their quantity. Such a test requires high specificity, which made the selection of genome fragments to be replicated in Real-Time PCR reaction not an easy task.

It was required that they were common for all the strains across a species and, at the same time, specific enough to prevent the product from occurring in the matrix of other micro-organisms of different species.

The objective of the project was to work out a fast
and reliable method of detection of bacteria of the *Acinetobacter baumannii* species in medical and environmental samples, as well as epidemiological preparation of the Polish Navy health care facilities and places adjusted for a long-term stay for soldiers, such as submersible vessels, minesweepers, etc. for the purpose of detection potential health hazards.

METHODOLOGY

Sample collection

Collect swabs from the examined surfaces with sterile swabs soaked in physiological saline (0.85% NaCl) or LB culture medium. Next, place the swabs in sterile test tubes. Store the labelled test tubes at a temperature of 4°C until the samples are moved to the laboratory.

Preparation of samples for analysis

Flush the swab with 500 µl of LB culture medium for ca. 30 seconds. The obtained suspension is to be centrifuged and the supernatant carefully drained. Approx. 150 µl of the material should remain in the tube (sediment + LB medium). After precise pipetting divide the bacterial emulsion into 3 parts. Add to one of them the same quantity of 50% glycerol (to obtain ca. 1:1 proportion) and freeze at at -70°C. Use the remaining quantity for *real-time* PCR and plating tests with LAM and MacConkey agars.

Real-time PCR reaction

The matrix consists of properly prepared medical and environmental samples. To the reaction we add (per 20 µl):
- oligonucleotides and optimal concentration probes (experimentally determined)
- 10 µl SuperMix (Bio-Rad, USA)
- water up to 20 µl

The reaction is carried out in CFX96™ thermocycler (Bio-Rad, USA) followed by result interpretation. Positive samples are those with an amplification reaction combined with light emission of the probes that have joined into the DNA matrix.

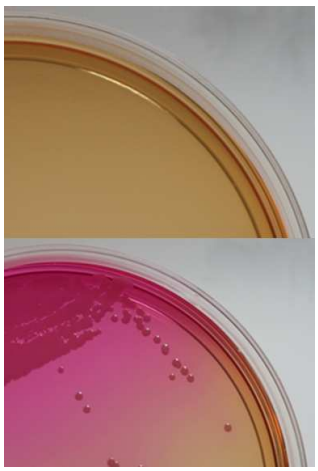

Fig. 1 **LAM Medium**. Medium's colour change induced byAcinetobacter spp. colonies.

Methods of phenotypic diversification of tested isolates

- Inoculation on LAM agar (Leeds Acinetobacter Medium)

Prepare the place in accordance with the instruction included in the publication. From each of the earlier prepared serial dilutions of the tested sample inoculate 100µl onto the agar.

Place into an incubator (37°C) for 24 hours. If bacteria growth is noted, select a dilution which allows the distinguishing of individual colonies. After a 24-incubation period A. baumannii colonies have a pink tint with the surrounding area changing the colour into pink-violet, they are round, convex, smooth and matt with the diameter of 1-2 mm. [24].

- Isolation procedure on MacConkey agar

Prepare the plate in accordance with manufacturer's recommendations. From each of the earlier prepared serial dilutions of the tested sample, inoculate 100µl onto the agar.

Place into an incubator (37°C) for 24 hours. If bacteria growth is noted, select such dilution which allows the distinguishing individual colonies. Select several morphologically diversified bacteria colonies and describe their characteristic features, including the colour, shape, size, surface and protrusion above the surface.

- LA plate inoculation

Inoculate selected isolates and describe colonies from the MacConkey or LAM agar with the use of sterile inoculation loops onto an LA medium by drawing a "line" with a length of ca. 3-4 cm. The obtained material will serve as a starting point for further tests.

- Cytochrom oxidase activity test

Take a fragment of the colony from an LA plate, sampled with the use of a sterile tip transfer to the reactive zone of the test strip and distribute evenly. After ca. 30 seconds, compare colours of the test strips with the colour scale provided on the packaging.

- DNase activity check

Test for DNase presence in the pure culture from the examined strain sampled from the LA plate inoculate on the surface of the previously prepared agar (acc. to manufacturer's recommendations).

Inoculate each of the plates also with the reference strains – Staphylococcus aureus (DNase-positive) and Escherichia coli (DNase-negative). Incubate for 24 hours at a temp. of 37°C.

Next, pour 400µl of 1N hydrochloric acid and observe plate turbidity and occurrence of a brighter area around the DNase-positive colonies.

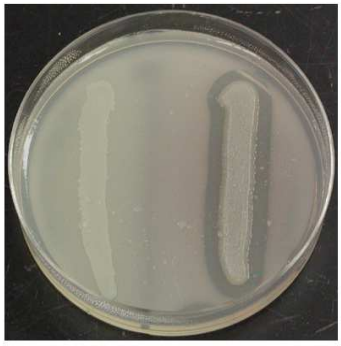

Fig. 2 DNase activity marking.

- Sugar oxidation and fermentation – Hugh-Leifson medium

The medium should be prepared according to the manufacturer's recommendations in 3 separate flasks.

Add to each of them 100ml/litre of 10% carbohydrate solution sterilised through filtration and glucose, lactose and xylose respectively and carefully mix. Pour the prepared medium into test tubes to the level of ca. 5 cm. In half of the test tubes cover the medium with ca. 1 cm layer of sterile paraffin immediately after cooling. Inoculate the pure strain culture by injection to the bottom of the test tube with each carbohydrate (one tube with paraffin and one without).

Leave for 48 hours at a temp. of 37°C. After this time, evaluate the colour change in the test tubes. Yellow tint in the tube without paraffin will indicate oxidative decomposition, whereas colour change in both test tubes – with and without paraffin – will indicate fermentation decomposition of a tested hydrocarbon.

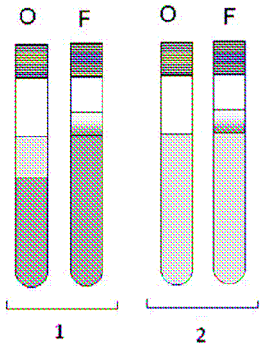

Fig. 3. Interpretation of results of sugar oxidation/fermentation test (Hugh-Leifson medium) O – oxidation; F – fermentation.

DNA fragment amplification with the PCF method

The matrix is chromosomal DNA. Add to the reaction: primers to the final concentration of 1μM, deoxynucleotides to the final concentration of 0.2 mM, buffer for Taq polymerase, Taq polymerase in the quantity of 0.2 units per reaction, water to the final concentration of

10 or 50 μl. Reaction parameters, such as DNA anealing and elongation time, temperature of starter hybridisation to the matrix or the number of cycles, should be adopted depending on the type of oligonucleotides and product length.
The reaction is performed in thermocycler Mastercycler Personal (Eppendorf, Germany).

Electrophoretic separation in agarose gel

Electrophoretic separation is carried out in horizontal electrophoresis apparatus with the use of agarose gel with the concentration adjusted to the product length (from 1 to 2%) in TAE buffer. Mix a DNA sample with a loading dye in 5:1 proportion and provide gel into the wells.

The separation takes place at the voltage of 100V/cm of the gel's length. Next, the gel is stained in ethidium bromide solution for approximately 5-10 minutes and observed in UV light with the wavelength of λ 354 nm with a photograph taken with the use of *Gel DocTM XR System* imager (Bio-Rad, USA).

Sequencing of selected and replicated DNA segments

For the purpose of the final confirmation of specific affiliation of selected and examined isolates, it was decided to carry out sequencing of 16S rDNA and recA. These are highly conservative regions with gen recA being commonly used in the identification of *Acinetobacter* bacteria. DNA fragment replication with the PCR method is conducted with purified chromosomal DNA of particular isolates.

This is followed by electrophoretic separation of the obtained PCR products in order to verify whether the selected isolates belong to the *Acinetobacter baumannii* species. Should the result be positive, the amplification is repeated and the fragments 16S rDNA and recA are purified.

Use the Clean-up kit by A&A Biotechnology (see the manufacturer's recommendations) to clean and sequence the DNA fragments. The thus prepared material is transferred to an external company for the purpose of sequencing.

The obtained results are verified with the use of FinchTV programme, compared with the database and evaluated whether the selected isolates belong to the *Acinetobacter baumannii* species.

Examination of isolates with pulsed-field gel electrophoresis

Modify the protocol REA-PFGE to type Acinetobacter baumannii [25, 26].

- Preparation of agarose blocks

1) Inoculate ca. 50 ml of sterile LB medium with selected isolate – an overnight culture: 18 hours at 37ºC or inoculate onto LA medium.
2) Select a single bacterial colony and transfer it with the use of a sterile loop into 1 ml of CBS buffer, gently mix in order to obtain a more or less homogenous suspension or swirl 1 ml of . overnight culture with OD – 1.0 - 1.3 (1000g;
   5 MIN; 4ºC), rinse the sediment with 1 ml of CSB,
   centrifuge and prepare a suspension in 1 ml of CSB. The prepared suspension containing bacterial cells may be kept at room temperature until its mixing with agarose solution, however not longer than 5 minutes.
3) Before preparing the bacterial suspension, prepare a 2% solution of low temperature melting agarose in CBS buffer or ddH_2O, mix the agarose solution with 10% SDS solution to obtain the final SDS concentration of 1%, keep the dissolved agarose in a water bath at the temp. of between 50-60ºC until use.
4) Prepare 1 block at a time (to avoid contamination). Mix 165 μl of agarose/SDS solution and 135 μl of bacterial suspension, gently pipette and quickly transfer ca. 200 μl of the mix into a sterile block matrix. Place the matrix in a temp. of 4ºC until hardening – ca. 20 min.
5) For the purpose of lysis of bacterial cells suspended in the agarose block it is required to transfer the hardened block into sterile test tubes, add 2 – 3 ml of CSL-1 buffer (to completely cover the block) and place in a water bath at a temp. of 37ºC for 2 hours. Next, move the blocks again into sterile test tubes and add 2 – 3 ml of CSL-2 buffer and place in a water bath at 55ºC for the night.
6) On the following day transfer the blocks to sterile test tubes and rinse 3 times with 4 ml of ddH_2O (30 min; 50ºC) and another 3 times with 4 ml of TE buffer (30 min; 50ºC). The prepared blocks, covered with 3 ml of TE buffer, may be stored at 4ºC for a period of up to 4 weeks.

- Restriction digestion of the DNA suspended in the blocks

7) Cut the prepared blocks into 6 parts, place one of them in a 1.5 ml Eppendorf tube and pour in 200 μl of restriction buffer containing bovine albumin in the quantity of 100 μg/ml and incubate at 37ºC in a water bath for 10 min. Next, remove the buffer and add 200μl of fresh restriction buffer containing rare cutting restriction enzyme ApaI in the quantity of 30 U/block, incubate at 30ºC in a water bath for 2 hours.

- Agarose gel preparation

8) Prepare 110 ml of 1% high temperature melting agarose in borate buffer (0.5x TBE) and leave in a water bath at 50ºC until gel release.
9) Take the digested agarose blocks out of the buffer and place on the teeth of the electrophoretic comb, remove excess buffer with blotting paper. Wait 5 minutes until the blocks are glued to the comb and place the comb in gel matrix

and slowly pour 100 ml of agarose solution to prevent bubble formation. Gently remove the comb after ca. 30 min and cover the produced holes with the remaining portion of agarose solution.

10) In gel preparation use the DNA of the standard A. baumannii strain suspended in the block and digested with ApaI enzyme as well as mass standards.

- Electrophoresis

11) Carefully transfer the prepared agarose gel into an electrophoretic chamber and slowly pour 2000 ml of 0.5xTBE buffer until it is completely covered.

12) Adjust separation parameters and begin the electrophoresis.

- time – 22 hours
- voltage – 200V
- impulse duration – initial 5 sec/final 30 sec
- temperature – 14ºC

13) After the electrophoresis is completed, place the gel in a container with ethidium bromide solution, possessing a concentration of 5μg/ml, for 20 minutes, in order to stain the gel for photographing under a UV light.

RESULTS

Epidemiological tests aimed at the detection of *Acinetobacter baumannii* were carried out on samples collected in various healthcare facilities, environmental samples obtained from military mission areas and samples collected in submersible vessels (places of prolonged stay for soldiers).

The examined samples were inoculated in several dilutions onto a Leeds Acinetobacter Medium (LAM), i.e. a selective and differential medium. The *Acinetobacter baumannii* colonies isolated with the use of this medium are round, pink and convex, with the diameter of ca. 1-2 mm after a 24-hour incubation. Colonies which grew on LAM agar and met the above criteria were subject to further testing.

Selected isolates were examined for the presence of cytochrome oxidase with the use of colour test strips. Cytochrome oxidase is an enzyme belonging to iron porphyrins that oxidise reduced cytochrom c and turns into a reduced inactive form. It is again activated through electron transportation to molecular oxygen.

The pattern of cytochrom oxidase – cytochrom c in the presence of molecular oxygen may transport electrons from numerous organic compounds. Isolates which produced negative results for the presence of cytochrome oxidase (lack of staining) were inoculated onto an agar testing for the presence of DNase. Colonies producing DNase hydrolyse the deoxyribonucleic acid in the immediate vicinity to their culture.

The test was performed with hydrochloric acid which induced DNA precipitation allowing observation of a brighter zone around the inoculation area. When the result was negative, the isolate was subject to final testing on oxidation and fermentation capacities.

The test was performed on a Hugh-Leifson agar with an addition of proper sugars: glucose, lactose and xylose respectively. The *Acinetobacter baumannii* bacteria are capable of aerobic decomposition of these sugars, however, being obligatory aerobes, they are incapable of inducing fermentation.

The results of the above biochemical tests allowed the initial determination of the affiliation of particular isolates to the *Acinetobacter baumannii* species.

Simultaneously, a real-time PCR test was conducted with the use of three previously tested pairs of oligonucleotides and probes allowing detection of the presence of the *Acinetobacter baumannii* genetic material.

The obtained results were compared with phenotypic and biochemical measurements of the tested environmental samples. If the performed tests were compatible, further testing of the obtained isolates was
conducted by inoculation onto GEN III agar produced by BIOLOG inc., which enable a percentage identification of a selected micro-organism.

Also for the purpose of verification of the received results, the genetic material of the tested isolates was cleansed and PCR of recA and 16SrDNA was carried out, the results being compared with the use of electrophoresis with model micro-organisms, i.e. *Acinetobacter baumannii* ATCC17978.

SUMMARY RESULTS OF REAL-TIME PCR IN RELATION TO THE DETECTION ACINETOBACTER BAUMANNII

Environmental samples from military mission territory I – 2009

84 environmental samples; 8 A. baumannii isolates confirmed with phenotypic methods, PCR sections 16S and recA and sequencing of section 16S

Samples from healthcare facility I – 2010

30 samples; *real-time* PCR S1 and S10 3 positive samples ; the same samples confirmed with phenotypic tests , PCR 16S and rec A and sequencing

Samples from healthcare facility II – 2011

samples collected from 26 places x 2 = 52 test tubes; from 7 places - *real-time* PCR S1 and S10 positive ; isolates confirming the real-time PCR test obtained for 1 place of sample collection

Samples from healthcare facility III – 2011

20 samples; *real-time* PCR S1 and S10 6 positive samples; the same samples confirmed with phenotypic tests, PCR 16S and rec A

Environmental samples from vessels – 2011

20 samples; *real-time* PCR S1 and S10 all samples negative

Samples from healthcare facility IV – 2011

20 samples; *real-time* PCR S1, S10, S3 6 positive samples; only 2 test tubes produced isolates that were confirmed with phenotypic tests, PCR 16S and rec A

Samples from healthcare facility V – 2011

20 samples; *real-time* PCR S1, S10, S3 all samples negative, no growth of colonies typical of *A. baumannii* on LAM agar

Environmental samples from military mission territory II – 2012

117 samples; *real-time* PCR S1 and S10 negative, no growth of colonies typical of *A. baumannii* on LAM agar

Samples from healthcare facility VI – 2012

20 samples (collected following disinfection of a ward which was an epidemic centre of *A.baumannii* in spring 2012); *real-time* PCR S1, S10 – 1 positive sample , LAM – isolate typical of *A.baumannii*

Samples from healthcare facility VII – 2012

72 samples; *real-time* PCR S1 and S10 negative, no growth of colonies typical of *A. baumannii* on LAM agar

Samples from healthcare facility VIII – 2012

31 samples; *real-time* PCR S1, S10 – 1 positive sample, LAM – isolate typical of *A.baumannii*

Samples from healthcare facility IX – 2012

30 samples; *real-time* PCR S1, S10 – 2 positive sample, LAM – isolates typical of *A.baumannii*

Nearly 500 samples from different environments have been examined. Not all of the positive results of real-time PCR were confirmed by obtaining an isolate from the tested sample, which makes us further convinced that this bacteria species is not easy to diagnose with classical methods.

PFGE Tests – results

During the first phase of testing the PFGE protocol was modified for *Acinetobacter baumannii*.

This was followed by electrophoretic separation of digested genetic materials originating from healthcare and environmental facilities.

The results confirmed the presumption that three samples from one of the health care facilities, S12, S13 and S14 were closely related isolates.

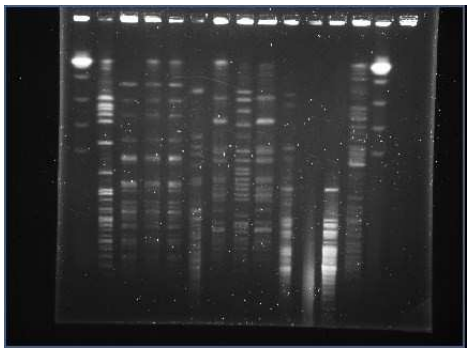

Fig. 4. Acinetobacter baumannii isolates, hospital samples S12, S13, S14 and other environmental isolates.

BIBLIOGRAPHY

1. Krawczyk B: Molecular diagnostics in hospital infections. Post.Mikrobiol. 2007, No 46, 367-378
2. Maragakis L, Perl T: Acinetobacter baumannii: Epidemiology, Antimicrobial Resistance and Treatment Options. Clin. Infect. Dis. 2008, No 46, 1254-1263 , doi: 10.1086/529198
3. Meijie J, Zhijun Z, Shuping Z: Epidemiological Characteristics and Drug Resistance Analisis of Multidrug-Resistant Acinetobacter baumannii in China Hospital at Certain Time Polish Journal of Microbiology , 2014,Vol.63, No 3
4. Jawad A, Seifert H, Snelling A.M, Heritage J, Hawkey P.M: Survival of Acinetobacter baumannii on dry surfaces: comparison of outbreak and sporadic isolates. J. Clin. Microbiol. 1998, July, 1938-1941
5. Zmudziński M, Gospodarek E, Gierlotka K: Resistance mechanisms of Acinetobacer spp. to non-beta-lactam antibiotics. Post. Mikrobiol. 2007, No 46, 335-342
6. Zapor M.J, Moran K.A: Infectious diseases during war time. Current opinion in Infectious Diseases , 2005, No18, 395-399
7. Peleg A, Seifert H, Paterson D: Acinetobacter baumannii: Emergence of a Successful Pathogen. Clin. Microbiol. Rev. 2008, Jul, 538-582 , doi: 10.1128/CMR.00058-07
8. Falagas M.E, Karveli E.A, Siempos I.I, Vardakas K.Z: Acinetobacter infections: a growing threat for critically ill patients. Epidemiol. Infect. 2008, No136,1009-1019
9. Wendt C, Dietze B, Dietz E, Rüden H: Survival of Acinetobacter baumannii on Dry Surfaces. J. Clin. Microbiol.1997 June, 1394-1397
10. Villgas M.V, Hartstein A.I: Acinetobacter outbreaks. Infect. Control. Hosp. Epidemol. 2003, No 24, 284-295
11. Murray C.K, Roop S.A, Hospenthal D.R: Bacteriology of war wounds at the time of injury. Mil. Med. 2006, No 171,821-825
12. Griffith M.E, Ceremuga J.M, Ellis M.W, Guymon C.H, Hospenthal D.R, Murray C.K: Acinetobacter skin colonization of US army soldiers. Infect. Control Hosp. Epidemiol. 2006, No 27, 659-661
13. Griffith M.E, Lazarus D.R, Mann P.B, Boger J.A, Hospenthal D.R, Murray C.K: Acinetobacter skin carriage among US army soldiers deployed in Iraq. Infect. Control Hosp. Epidemiol. 2007, No 28, 720-722
14. Kur J, Krawczyk B: Comparison of DNA-based typing methods of microbial organisms for epidemiological studies. Adv. Agric. Sci. 2002, No 8, 7-20
15. Seifert H, Schulze A, Bagiński R, Pulverer G: Comparison of Four Different Methods for Epidemiologic Typing of Acinetobacter baumannii. J. Clin. Microbiol. 1994, July, 1816-1819
16. Riley L.W: Molecular Epidemiology of Infectious Diseases. ASM Press 2004
17. Clifford R.J, Milillo M, Prestwood J, Quintero R, Zurawski D.V, Kwak Y.I, Waterman P.E, Lesho E.P, McGann P: Detection of Bacterial 16S rRNA and Identification of Four Clinically Important Bacteria by Real-Time PCR. PLOS ONE 2012, November, Vol.7 Iss.11 , doi: 10.1371/journal.pone.0048558
18. Martin-Pena R, Dominguez-Herrera J, Pachon J, McConnell M.J: Rapid detection of antibiotic resistance in Acinetobacter baumannii using quantitative real-time PCR J. Antimicrob. Chemother. 2013, February 23
19. Scheinert P, Krausse R, Ullmann U: Molecular differentiation of bacteria by PCR amplification of the 16S- 23S rRNA spacer. J. Microbiol. Meth. 1996, No 26, 103-117
20. Krawczyk B, Lewandowski K, Kur J: Comparative studies of the Acinetobacter genus and the species identification method based on the recA sequences. Mol. Cell Probes, 2002, No16, 1-11
21. Krawczyk B, Kur J: Molecular diagnostic in microbiology. Publ. Polit. Gd. 2008
22. Mackay I.M: Real-Time PCR in Microbiology, from Diagnosis to Characterization. *Caister Academic Press* 2007
23. McConnell M.J, Perez-Ordonez A, Perez-Romero P, Valencia R, Lepe J.A, Vazquez-Barba I, Pachon J: Quantitative Real-Time PCR for Detection of *Acinetobacter baumannii* Colonization in the Hospital Environment. *J. Clin. Microbiol.* 2012, February, 1412-1414, doi: 10.1128/JCM.06566-11
24. Jawad A, Hawkey P.M, Heritage J, Snelling A.M: Description of Leeds Acinetobacter Medium, a New Selective and Differential Medium for Isolation of Clinically Important Acinetobacter spp. and Comparison with Herellea Agar and Holton's Agar, *J. Clin. Microbiol*. 1994, Oct, p. 2353-2358
25. Bannerman *et al.*: PFGE Typing Protocol Recommended by ARPAC for *Acinetobacter baumannii*, *J. Clin. Microbiol*. 1995, No 33, 551-555
26. Durmaz R, Otlu B, Koksal F, Hosoglu S, Ozturk R; The Optimization of a Rapid Pulsed Field Gel Electrophoresis Protocol for the Typing of *Acinetobacter baumannii*, *Escherichia coli* and *Klebsiella spp*. Jpn. J. Infect. Dis.2009, No 62,372-377

mgr biol. Małgorzata Dąbrowiecka
Zakład Medycyny Morskiej
i Hiperbarycznej
Wojskowy Instytut Medyczny
ul. Grudzińskiego 4 81-103 Gdynia 3
skr. poczt. 18
tel. 58/6264109