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Rep-PCR genotyping of infectious *Acinetobacter* spp. strains from patients treated in Intensive Care Unit of Emergency Department (ICU of ED) - preliminary report

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ABSTRACT

Purpose: Strains of *Acinetobacter* spp. are responsible for a considerable percentage of hospital infections. These pathogens have colonized hospital environment and developed resistance to many currently available antibiotics. The aim of this study was one year-long analysis of the occurrence of multiresistant strains of *Acinetobacter* spp. in population of patients hospitalized in ICU of ED and determination of their genetic similarity.
Material/Methods: Subject of research was the population of patients admitted to ED of University Hospital in Bialystok in the period from 01.08.2010 to 01.08.2011. In the analysed group of patients, infections were identified on the basis of the guidelines of CDC. Identification and drug susceptibility of strains was specified using the automatic methods with the analyzer Vitek 2XL. Genotyping using Rep-PCR method in DiversiLab system was performed on strains of *Acinetobacter* spp. to determinate their genetic similarity.
Results: During analyzed period 405 patients were hospitalized, from 14 of them multiresistant strains of *Acinetobacter* spp. were isolated. Conducted genetic research allowed to detect 5 clones. Rep-PCR method in DiversiLab system enabled to learn that different clone of multiresistant strain of *Acinetobacter* spp. is responsible for variable forms of infection.
Conclusions: Results of conducted research suggest that genotyping with rep-PCR method in DiversiLab system is useful tool in diagnostics of clones of multiresistant pathogens isolated from patients requiring intensive care, hospitalized in ED. Genotyping with rep-PCR method combined with epidemiological investigation enables to establish ways of spreading of multiresistant strains of *Acinetobacter* spp. in ED.

Key words: genotyping, rep – PCR, *Acinetobacter* spp., infections, emergency department

INTRODUCTION

Strains of *Acinetobacter* spp. are responsible for a considerable percentage of hospital infections [1-5]. These pathogens have colonized hospital environment and developed resistance to many currently available antibiotics [5,6]. Multidrug - resistant *Acinetobacter baumannii* are characterized by different mechanisms of resistance [6,7]. They can cause different forms of infections, of which the most common are: respiratory tract infections, urinary tract infections, surgical wounds infections, central nervous system infections and bacteraemia [8-10]. Eradication of *Acinetobacter* spp. strains from a human organism as well as from a hospital environment is unusually difficult. As a result of the above, microbiological monitoring of wards as well as strict following the procedures which reduce spreading these

microorganisms in a health care facilities environment is recommended [5,11].

The aim of this work was one year-long analysis of the occurrence of multiresistant strains of *Acinetobacter* spp. in population of patients hospitalized in Intensive Care Unit of Emergency Department and determination of their genetic similarity, using the rep-PCR method.

MATERIAL AND METHODS

The object of the study

Subject of research was the population of patients admitted to Emergency Department of University Hospital in Bialystok in the period from 01.08.2010 to 01.08.2011. In the analysed group of patients, infections were identified on the basis of the guidelines of Centers for Disease Control and Prevention in Atlanta (CDC) [12]. Biological material collected for microbiological tests was transferred to Department of Microbiological Diagnostics and Infectious Immunology of Medical University of Bialystok.

Antibiotic resistance

Standard procedures of microbiological diagnostics were used in isolation of *Acinetobacter* spp. (inoculation on standard set of microbiological media and culture in standard conditions). Isolated and identified strains of *Acinetobacter* spp. were transferred from MacConkey medium to Columbia – Agar medium in order to conduct genotyping using rep – PCR method.

Identification and drug susceptibility of strains was specified using the automatic methods with the analyzer VITEK 2 (bioMérieux) with the use of identification cards GN and drug susceptibility cards AST 93 in compliance with manufacturer's requirements. Used GN card contains 47 biochemical tests, allowing for identification of the most clinically significant fermenting and non-fermenting Gram negative bacilli. Antibiotics susceptibility was determined according to EUCAST norm (The European Committee on Antimicrobial Susceptibility Testing – www.eucast.org).

Genotypic characterization

Genotyping using rep-PCR method in DiversiLab system was performed on strains of *Acinetobacter* spp. which were obtained using culturing methods, on standard set of microbiological media used in Department of Microbiological Diagnostics and Infectious Immunology of Medical University of Bialystok. For genetic tests, 24-hour culture of strains on Columbia Agar (CA; bioMérieux) was used. All stages of the survey were performed with the use of ready-made, complete sets of reagents compatible with the DiversiLab system.

Extraction of genetic material was performed using ULTRA CLEAN™ Microbial DNA Isolation Kit in compliance with manufacturer's (MO BIO Laboratories, Inc.) requirements.

Density of extracted DNA was measured with the use of NanoDrop 2000C Spectrophotometer (Thermo Scientific), obtaining ranges recommended by the manufacturer, between 25ng/µl and 50ng/µl. Afterwards, tested samples of DNA were subjected to reaction rep-PCR using Acinetobacter Kit (bioMérieux) and Tag Polymerase by Applied Biosystems in accordance with the protocol course of parameters of reaction (Rep-PCR Worksheet), according to the manufacturer's recommendations. Obtained amplicons were brought to microchip (DNA Chips; bioMérieux) in accordance with the procedure Chip Worksheet; next they underwent electrophoretic separation with the Agilent 2100 Bioanalyzer. Using software of Agilent 2100 Bioanalyzer DiversiLab v 3.4 PC, an image of virtual gel, dendrogram and diagrams of strips for every examined strain were obtained.

Statistical analysis

The results are descriptive, therefore the statistical analysis is not applicable.

RESULTS

During the period from 01.08.2010 to 01.08.2011, at the Emergency Department of University Hospital in Bialystok

Table 1. **Values of markers inflammation in tested group of patients on a day of collecting biological samples.**

Parameter of inflammation	C-Reactive Protein (CRP)	Procalcitonin (PCT)	Leukocytosis
Normal range	0.0 – 10.0 mg/L	< 0.5 ng/ml	4000 – 10000/ul
Minimal value	5	0.3	6000
Maximal value	435.3	32.1	122100*
Mean value	140.3	7.1	23.7
Standard deviation	127.1	9.4	1.9

** patient with acute myeloid leukemia*

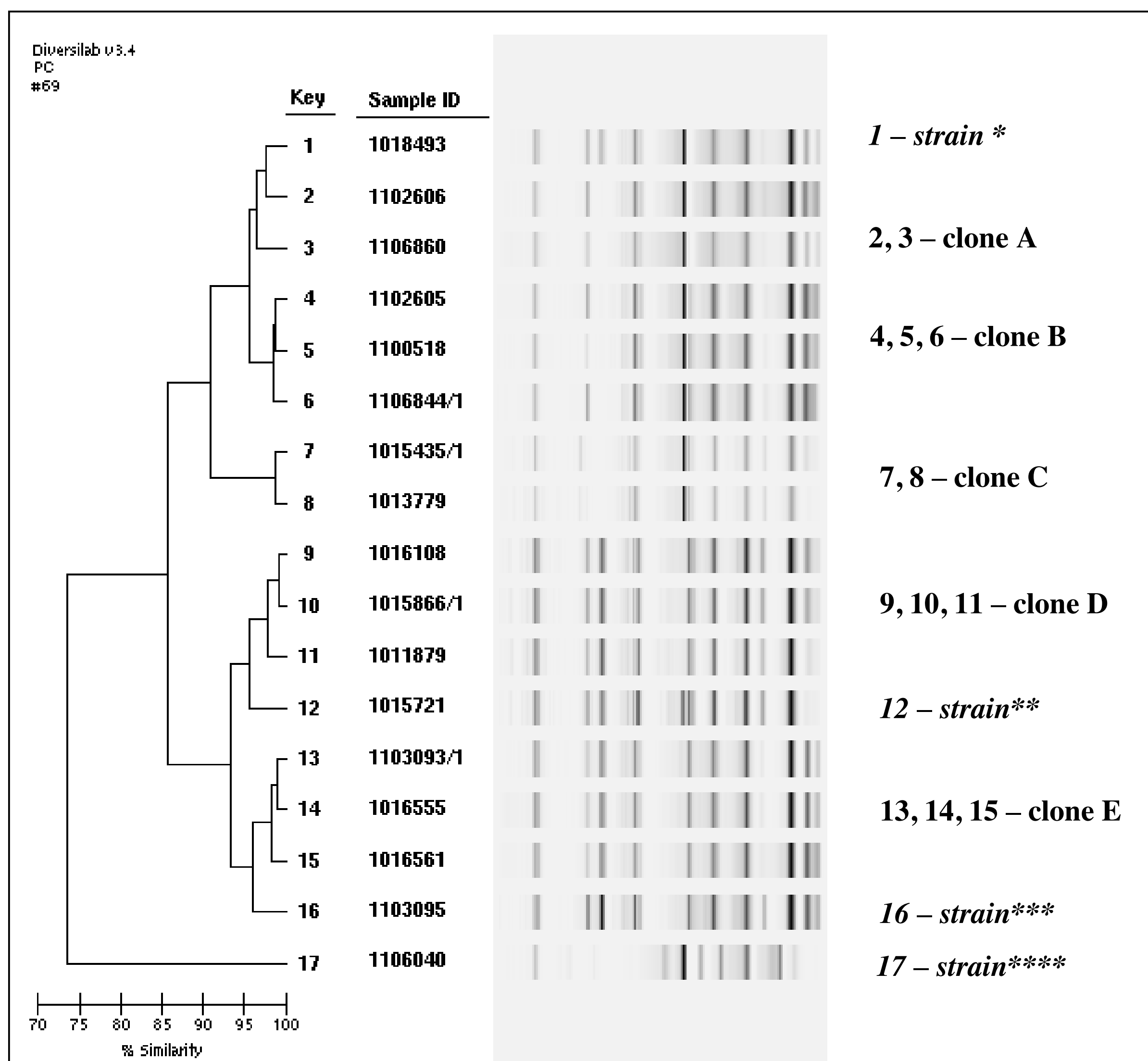
405 patients were hospitalized, from 14 of them multiresistant strains of *Acinetobacter* spp. were isolated. In the tested group 35.7% (n=5) were women, and 64.3% (n=9) were men. Among tested, 4 patients originated from rural environment and 9- from urban environment. Age of tested patients ranged from 21 to 78 years old, average 58 years old (SD ± 15.9). All of the patients were in severe general condition - APACHE II score – average 24.5 points (SD ± 7.9). Values of markers of inflammation in the tested group of patients are presented in the *Table 1*. Seventeen multiresistant strains of *Acinetobacter* spp. were isolated from 14 examined patients. In two cases (14.3%) colonization of respiratory tract by multiresistant strains of *Acinetobacter* spp. was diagnosed, and in 12 cases (85.7%) infection caused by this pathogen was diagnosed. Pneumonia occurred in 42.9% of patients (n=6), peritonitis- in 21.4% (n=3) of cases. In one case simultaneous occurrence of pneumonia and blood infection was diagnosed, which made 7.1% of tested patients. Coincidence of pneumonia and peritonitis caused by *Acinetobacter* was diagnosed in 14.3% (n=2) of patients.

Conducted genetic research of *Acinetobacter* spp. strains with the use of rep-PCR method allowed to detect 5 clones in the tested group of patients. Identified clones were marked with capital letters of the alphabet starting from A to E. Strain No 1 was closely related to clone A, strain No 12 to clone D and strain No 16 to clone E. Strain No 17 was unrelated to any of the detected clones *(Fig.1)*. Frequency of isolation of multiresistant strains of *Acinetobacter* spp. from particular biological specimen collected from tested group of patients is presented in *Table 2*. Strains of *Acinetobacter* spp. susceptible to colistin and amikacin belonged to clones C, D, E. One strain of clone B was susceptible to colistin and minocycline. In addition one strain of the same clone was characterized by susceptibility to colistin, minocycline and trimethoprim/sulfamethoxazole. Strains of *Acinetobacter* spp. included to clone A, as well as one strain of clone B were susceptible to colistin only. Strains of *Acinetobacter* spp. No 1 and No 16 were susceptible to colistin and amikacin and strain No 17 was susceptible to colistin and tobramycin. Colistin, amikacin and rifampicin were active in vitro against strain No 12 *(Tab. 3)*. The largest number of patients with infections caused by multiresistant strains of *Acinetobacter* spp. occurred in October 2010 (6 patients/ 3 clones of *Acinetobacter* spp. - C, D, E and 1 strain closely related to clone D**)**, in February 2011 (2 patients/ 3 clones of *Acinetobacter* spp. - A, B, E and 1 strain closely related to clone E) and in April 2011 (2 patients/ 2 clones of *Acinetobacter* spp. - A, B and 1 strain unrelated to any of the clones) – results presented in *Table 4*. Epidemiological data concerning patients with multiresistant strains of *Acinetobacter* spp. are gathered in *Table 5*.

DISCUSSION

During the 12-months long period, presence of multiresistant strains of *Acinetobacter* spp. was diagnosed in 14 patients treated in Intensive Care Unit of Emergency Department of University Hospital in Bialystok. All of the patients were in severe general condition (APACHE II score – average – 24.5 points) and required using intensive care procedures such as: intubation, ventilation, invasive monitoring of circulatory system, catheterization of urinary bladder, catheterization of central and peripheral vessels, and renal replacement therapy in some cases. In two cases colonization of lower respiratory tract by multiresistant strains of *Acinetobacter* spp. was diagnosed, and in 12 cases an infection caused by this microorganism was diagnosed. According to data provided by literature, bacteria such as *Acinetobacter* are responsible for various types of infection: pneumonia, urinary tract infection, wound infection, surgical wound infection, blood stream infection and central nervous system infection [8,10,11,13,14]. In conducted research- pneumonia was diagnosed in 42.9% of patients (n=6), peritonitis was diagnosed in 21.4% (n=3), simultaneous occurrence of pneumonia and blood stream infection in 7.1% (n=1) and simultaneous occurrence of pneumonia and peritonitis in 14.3% (n=2) of tested patients. In tested population infections caused by multiresistant strains of *Acinetobacter* spp. were dominant and made 85.7% (n=12), whereas colonization was diagnosed in 14.3% of cases (n=2). Obtained results differ from some previous publications in which *Acinetobacter* caused infection in 53% of cases, and colonized organism in 43.4% of cases [15]. Some authors point out that strains of *Acinetobacter baumannii* isolated from secretion of respiratory tract and samples of urine more often colonize organism than are a cause of infection [13]. According to publications, colonization of lower respiratory tracts by Gram-negative bacteria in patients hospitalized in Intensive Care Unit occurs after 60 – 84 hours of hospitalization [16]. Drakulovic *et al.* [17] demonstrated that colonization of the trachea with hospital acquired pathogens is present in every tenth intubated patient during the first 24 h after admission to Respiratory Intensive Care Unit. In conducted research colonization by multiresistant strain of *Acinetobacter* spp. was diagnosed in two cases- patient No IV and patient No XII – details are shown in *Table 5.* Patient No IV had epidemiological history indicating that colonization by multiresistant *Acinetobacter* spp. could take place before admission to Emergency Department (chronic hemodialysis, long-lasting stays in multiple hospitals, broad spectrum antibiotic therapy before to admission to ED). Whereas patient No XII, although he didn't have any risk factors predisposing him to become carrier of multiresistant microorganisms (except for alcoholism),

Figure 1. **Results of genotyping using rep-PCR method in DiversiLab system of multidrug resistant strains Acinetobacter spp., isolated in investigated group of patients – belonging to different clones.**



** strain 1 - closely related to clone A; ** strain 12 - closely related to clone D; *** strain 16 - closely related to clone E; **** strain 17 - unrelated to any of the clones*

growth of a multiresistant strain of *Acinetobacter* spp. was obtained from his lower respiratory tract in the first twenty-four hours of hospitalization. This observation can indicate diagnostic error, but it can also suggest that colonization by multiresistant strain of *Acinetobacter* spp. of the patient requiring intubation and ventilation, can take place in a period of time shorter than twenty-four hours from admission to Emergency Department. An observation was made during the research which indicated presence of multiresistant strains of *Acinetobacter* spp. in urban post hospital environment and suggested existence of local risk factors of carrier state of this pathogen in northeast Poland, other than described in the guidelines by American Thoracic Society and Infectious Diseases Society of America [18]. It has to be noted that pandrug – resistant *Acinetobacter baumannii* strain with an unusual phenotype could persist in humans for long periods and be widely disseminated throughout the hospital, what was presented by Kuo *et al.* [19].

Villalón *et al.* [20] showed the presence of clonal diversity of nosocomial epidemic *Acinetobacter baumannii* strains. In our work, using genotyping with rep-PCR method in DiversiLab system 5 clones of multiresistant strains of *Acinetobacter* spp. were found in tested group of patients, they were marked with letters of the alphabet: A, B, C, D, E. It was observed the presence of three closely related strains (strain No 1 to clone A, strain No 12 to clone D, strain Nr 16

to clone E) and one strain that was not related to any clone found in the studied group of patients.

It was recognized that strains of *Acinetobacter* spp. which differ from each other with phenotype and have different antibiotic-susceptibility profile, belong to the same clone- strains No 4, 5, 6 belonging to clone B *(Tab. 3)*. It was noticed that strains of *Acinetobacter* spp. which have the same phenotypic susceptibility to antibiotics belong to different

***Table 2.* Frequency isolation of multiresistant strains *Acinetobacter* spp. from particular biological specimens collected from tested group of patients.**

Type of biological material	Number of isolated strains of Acinetobacter spp.		Clone/Strain
	n	%	
Broncho - alveolar lavage	11	64.7	Strain No1 - closely related to clone A Clone B Clone C Clone D Strain No12- closely related to clone D Clone E Strain No17- unrelated to any of the clones
Peritoneal fluid	5	29.4	Clone A Clone C Clone E
Blood	1	5.9	Strain No 16 - closely related to clone E

***Table 3.* Antibiotic susceptibility of strains *Acinetobacter* spp. isolated in tested group of patients.**

Strains of *Acinetobacter* spp. (No)	Patient (No)	Antibiotics active in vitro against particular strain	Clone/Strain
1	I	Colistin Amikacin	Strain No 1 – closely related to clone A
2	II	Colistin	Clone A
3	III	Colistin	
4	II	Colistin Minocycline	Clone B
5	IV	Colistin Minocycline Trimethoprim/sulfamethoxazole	
6	III	Colistin	
7	V	Colistin Amikacin	Clone C
8	VI	Colistin Amikacin	
9	VIII	Colistin Amikacin	Clone D
10	VII	Colistin Amikacin	
11	IX	Colistin Amikacin	
12	X	Colistin Amikacin Rifampicin	Strain No 12 – closely related to clone D
13	XI	Colistin Amikacin	Clone E
14	XII	Colistin Amikacin	
15	XIII	Colistin Amikacin	
16	XI	Colistin Amikacin	Strain No 16 – closely related to clone E
17	XIV	Colistin Tobramycin	Strain No 17- unrelated to any of the clones

Table 4. **Occurrence of particular clones *Acinetobacter* spp. in patients hospitalized in Intensive Care Unit of Emergency Department in a period from 1.08.2010 to 1.08.2011.**

Year / month	Patient / Clone/ Strain
2010	
August	Patient No IX/ Clone D
September	Patient No VI/ Clone C
October	Patient No V/ Clone C Patient No VII/ Clone D Patient No VIII/ Clone D Patient No X / strain No 12 – closely related to clone D Patient No XII/ Clone E Patient No XIII/ Clone E
November	No strains of Acinetobacter spp.
December	Patient No I/ strain No 1 – closely related to clone A
2011	
January	Patient No IV/ Clone B
February	Patient No II / Clone A and Clone B Patient No XI / Clone E and strain No 16 – closely related to clone E
March	No strains of Acinetobacter spp.
April	Patient No III / Clone A and Clone B Patient No XIV / Strain No 17 – unrelated to any of the clones
May	No strains of Acinetobacter spp.
June	No strains of Acinetobacter spp.
July	No strains of Acinetobacter spp.
August	1st August 2011 – End of the study period

clones- strains No 7 and 8 belonging to clone C, strains No 9, 10, 11 belonging to clone D, strains No 13, 14, 15 belonging to clone E, strains No 2, 3 belonging to clone A and strain No 6 belonging to clone B *(Tab. 3).* Before introduction of genotyping with rep-PCR method for diagnostics of multiresistant strains in patients hospitalized in ICU of Emergency Department, epidemiological proceedings in our department was limited to comparison of phenotypes of pathogenic microorganisms, which apparently has very limited clinical value according to conducted research and obtained results.

In case of patients with diagnosed infections of two systems, genotyping with rep-PCR method in DiversiLab system enabled to learn that different clone of multiresistant strain of *Acinetobacter* spp. is responsible for infection of each system. In case of patients No II and No III: clone A caused peritonitis, and clone B caused pneumonia. In case of patient No XI: clone E was responsible for pneumonia, and strain No 16 (closely related to clone E) was responsible for blood stream infection. In case of patients No III and No XI: it was impossible to differentiate strains on the basis of phenotypic susceptibility to antibiotics, which was enabled only by use of genotyping with rep-PCR method.

Microbiological monitoring conducted during the period of 12 months enabled to diagnose presence of 17 multiresistant strains of *Acinetobacter* spp. in 14 patients. This observation could suggest presence of strains of *Acinetobacter* spp. in hospital environment or carrier state among the staff. Microbiological examination of hospital environment and staff of Emergency Department did not show presence of multiresistant strains of *Acinetobacter* spp. Epidemiological investigation combined with genotyping with rep-PCR method in DiversiLab system allowed to uncover cross infections in two cases- transmission of clone D between patients No VII and No VIII, transmission of clone E between patients No XII and No XIII *(Tab. 4, Tab. 5).* This observation allowed to verify and improve internal department procedures aiming to reduce number of hospital acquired infections. Continuous microbiological monitoring was conducted in ED, there weren't any cases of *Acinetobacter* spp. detection in a period from May to August 2011.

Observation of 5 clones of multiresistant strains of *Acinetobacter* spp., 3 closely related strains and 1 unrelated strain in tested population of patients and obtaining negative results of environmental examinations indicates that in most cases infections are caused by pathogens acquired prior to admission to Emergency Department. Their mechanisms of transmission require further research. Zeana *et al*. [21] report of the absence of multidrug-resistant strains of *Acinetobacter* in the community. On the other hand Abbo *et al*. [22] in their study on multidrug – resistant *Acinetobacter baumanni* infections report that 10% of infections were imported into the hospital by patients with recent exposure to the health – care system. Furthermore, Chen *et al*. [23] inform on two cases of severe community acquired pneumonia caused by multidrug – resistant *Acinetobacter baumanni* which suggests

Table 5. **Risk factors of infection caused by multiresistant strain Acinetobacter spp. in tested group of patients.**

Patient (No)	Clone/ Strain	Risk factors of infection existing prior to admission to Emergency Department	Period of time from admission to Emergency Department to collecting sample in which *Acinetobacter* spp. was isolated	Classification of infections
I	Strain No 1	- hospitalization prior to admission to ED - immunosupression - prior hospitalization in ICU - broad spectrum antibiotic therapy - using invasive procedures	< 24 h	- HCAP
II	A, B	- long-term hospitalization - broad spectrum antibiotic therapy - operation prior to admission to ED	72 h	- Postoperative peritonitis - HCAP
III	A, B	- long-term hospitalization - broad spectrum antibiotic therapy - operation prior to admission to ED	<24 h	- Postoperative peritonitis - HCAP
IV	B	- chronic hemodialysis - long-lasting stays in multiple hospitals - broad spectrum antibiotic therapy	<24 h	- Colonization of lower respiratory tract
V	C	- chronic alcoholism - craniocerebral injury - operation prior to admission to ED	72 h	- Early HAP
VI	C	- past acute pancreatitis - surgical procedure in last 30 days - prior hospitalization in ICU - long-lasting hospital treatment	< 24 h	- Postoperative peritonitis
VII	D	- long-lasting outpatient treatment of deep venous thrombosis	96 h	- Early HAP
VIII	D	- chronic alcoholism	96 h	- Early HAP
IX	D	- hospitalization prior to admission to ED - operation prior to admission to ED	120 h	- Late HAP
X	Strain No 12	- chronic alcoholism - hospitalization prior to admission to ED	< 24 h	- HCAP
XI	E, strain No 16	- multiple hospitalizations prior to admission to ED - surgical procedure in last 6 months	< 24 h	- HCAP - BSI
XII	E	- chronic alcoholism	< 24 h	- Colonization of lower respiratory tract
XIII	E	- operation prior to admission to ED - antibiotic therapy - prior hospitalization	< 24 h	- Postoperative peritonitis
XIV	Strain No 17	- prior hospitalization - prior surgical procedure - antibiotic therapy	< 48 h	- HCAP

ED – Emergency Department, ICU – Intensive Care Unit, HCAP – Healthcare Associated Pneumonia, HAP – Hospital-acquired Pneumonia, BSI – Bloodstream infection

possibility of occurrence of these strains in community environment, which converges with our hypothesis.

CONCLUSIONS

Results of conducted research suggest that genotyping with rep-PCR method in DiversiLab system is a useful tool in diagnostics of clones of multiresistant pathogens isolated from patients requiring intensive care, hospitalized in Emergency Department. Genotyping with rep-PCR method combined with epidemiological investigation enables to establish ways of spreading of multiresistant strains of *Acinetobacter* spp. in Emergency Department.

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