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PHENOTYPIC AND GENETIC RELATIONSHIP OF *ACINETOBACTER BAUMANNII* ISOLATES

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A b s t r a c t: The interest in *Acinetobacter* continues to rise. One of the main reasons is the emergence of multi-resistant strains, which cause outbreaks of infection involving several patients in a ward, in the intensive care unit and in different areas of the hospital. Many outbreaks of its infection or colonization in surgical, neonatal and burn intensive care units have been reported, but the epidemiology of these infections remains unclear.

Aim: To investigate the relationship among the isolates of *Acinetobacter baumannii*, comparing some of their phenotypic and genetic features.

Material and Methods: A total of 20 *Acinetobacter baumanni* isolates were included in the study. 12 strains of *Acinetobacter baumannii* were obtained within a week in July 2010, from neonates hospitalized at the paediatric intensive care unit and on the neonatal ward. Three strains were isolated from neonates at the paediatric intensive care unit three months ago. All the *Acinetobacter baumannii* strains were isolated from tracheal aspirates obtained from neonates with infection of the lower respiratory tract. Five additional *Acinetobacter baumannii* strains were included in the study as controls. They were isolated from wound swabs taken from adult patients with wound infection, hospitalized at the University Traumatology Clinic. Susceptibility of the bacterial strains to 13 different antimicrobial agents was determined by the disk diffusion method (Kirby-Bauer). Additional testing of the susceptibility was performed by the VITEK 2 system. RAPD-PCR fingerprinting was carried out using the following primer (5' GAAACAGCTATGACCATG -3').

Results: All *A. baumannii* isolates were multi-drug resistant. Antibiotic susceptibility-testing by the disk-diffusion method and automated VITEK 2 system showed 3 and 2 antimicrobial susceptibility patterns, respectively. RAPD-PCR assay of *A. bau-*

mannii strains revealed two different RAPD-fingerprints. All the strains of *A. baumannii* isolated within a week in July 2010 from tracheal aspirates taken from neonates in the paediatric intensive care unit and neonates in the paediatric ward revealed the same RAPD-fingerprint, as well as 3 strains of *A. baumannii* isolated from tracheal aspirates taken from neonates in the paediatric intensive care unit three months ago. 5 strains of *A. baumannii* isolated from wound swabs of patients hospitalized at the Traumatology Clinic revealed a different RAPD-fingerprint.

Conclusion: All the strains of *A. baumannii* isolated from neonates in the paediatric intensive care unit and paediatric ward were multi-drug resistant. Investigating the resistance patterns in multi-resistant isolates of *Acinetobacter* is a useful method which can predict the strain relationship. This method could be completed by at least one molecular method, such as the RAPD-PCR technique, which has shown itself to be a convenient and more reliable in interpreting the strain relationship of the *A. baumannii* isolates. Good infection control procedures, including phenotypic and molecular typing of *A. baumannii* isolates, are essential for preventing outbreaks of multi-drug resistant *A. baumannii* infections in our hospitals.

Key wards: *Acinetobacter baumannii,* phenotypic relationship, genetic relationship, resistance patterns, RAPD-PCR fingerprints.

Introduction

Bacteria of the genus *Acinetobacter* are non-motile, non-fermentative, aerobic, Gram-negative coccobacilli [1]. Because of the simplicity of their growth requirements and their high tolerance of environmental conditions, these bacteria are ubiquitous in the environment. As commensal bacteria they are usually part of the human bacterial flora, but they are also known as opportunistic pathogens. In the last decade, they have been increasingly reported as significant microorganisms involved in various nosocomial infections, including pneumonia, septicaemia, urinary tract infections and wound infections, especially in patients admitted to intensive care units [2]. *A. baumannii* is the species most frequently isolated from patients and the hospital environment [3]. Many outbreaks of its infection or colonization in surgical, neonatal and burn intensive care units have been reported. The epidemiology of these infections remains unclear, because it is ubiquitous and infections may occur on either a sporadic or an epidemic basis [4].

Extensive use of antimicrobial chemotherapy within hospitals is the main reason for the increasing number of *A. baumannii* strains resistant to a wide range of antibiotics, including broad-spectrum beta-lactams, aminoglycosides and fluoroquinolones [5]. Due to the multiple antibiotic resistance and frequency of nosocomial infections caused by *A. baumannii*, these bacteria were found difficult to treat. These therapeutic difficulties are associated with the abi-

lity of these bacteria to survive in the hospital environment, thus favouring transmission between patients, either via human reservoirs or via inanimate materials [6]. A lot of traditional and molecular typing methods have been used for epidemiological investigation of outbreaks caused by *Acinetobacter spp*. [7]. RAPD-PCR is one of the most rapid and simple methods that generate fingerprints, and it can be applied to detect polymorphism in a wide variety of organisms.

In standard PCR procedure a primer sequence is used specifically to amplify a known sequence of an organism's genome. In RAPD-PCR, random primer sequences may be used in organisms where a specific genome sequence is not known. Random parts of the organism genome are produced, which are expected to be identical among related species, and so similar banding patterns should be produced in gel electrophoresis. This technology is proving to be quite useful in typing strains of bacteria involved in nosocomial (hospital acquired) outbreaks of infectious diseases [7]. Typing is particularly useful for new strains arising due to the use of broad-spectrum antibiotics to which certain organisms develop a resistance. Patients in hospitals settings are generally more susceptible than the general population to these types of epidemics due to pre- or post-surgical procedures, skin trauma (burns, wounds), catheterization, and prolonged hospitalization.

The aim of our study was to investigate the relationship among the isolates of *Acinetobacter baumannii*, comparing some of their phenotypic and genetic features.

Materials and methods

A total of 20 *Acinetobacter baumannii* isolates were included in the study. 12 strains of *Acinetobacter baumannii* were obtained within a week in July 2010. 6 of them were isolated from neonates hospitalized in the paediatric intensive care unit and 6 strains were from neonates in the neonatal ward, situated in the same building. 3 strains were isolated from neonates in the paediatric intensive care unit three months ago (in April 2010). All *Acinetobacter baumannii* strains were isolated from tracheal aspirates obtained from neonates with infection of the lower respiratory tract. 5 additional *Acinetobacter baumannii* strains were included in the study as controls. They were isolated from wound swabs taken from adult patients with wound infection, hospitalized at the University Traumatology Clinic.

All the specimens were cultured on blood agar, under aerobic conditions and at a temperature of 37°C for 24 hours. Isolates were identified as members of the genus *Acinetobacter* by Gram staining and biochemical analyses. All the isolates were Gram-negative, non-motile coccobacilli, oxidase nega-

tive, glucose non-fermentative. They were also confirmed as *A. baumannii* by the automated system VITEK 2 (bioMerieux) used at the Institute of Microbiology and Parasitology, Medical Faculty, Skopje, to perform microbial identification as well as the antimicrobial susceptibility testing of the isolates.

Susceptibility of the bacterial strains to 13 different antimicrobial agents was determined by the disk diffusion method (Kirby-Bauer). The following antimicrobial agents were used at the concentrations indicated : cefixime (5 μg), ceftriaxone (30 μg), amoxycillin-clavulanic acid (20/10 μg), piperacillin (10 μg), gentamicin (10 μg), ofloxacin (5 μg), ciprofloxacin (5 μg), trimethoprim/-sulfamethoxazole (25 μg), cefuroxime (30 μg), amikacin (30 μg), imipenem (10 μg), cefepime (30 μg) and piperacillin-tazobactam (100/10 μg). The VITEK 2 system enabled us to prolong the list of antimicrobials wth 5 other antibiotics: meropenem, tobramycin, minocycline, colistin and rifampicin. Gentamicin and trimethoprim/sulfamethoxazole were also included in this automated system for antimicrobial susceptibility testing.

DNA extraction: Bacterial strains were cultured aerobically in Lauria broth (LB) at 37°C. Two millilitres of overnight culture were centrifuged at 4000 rpm for 20 minutes. The pellet was resuspended in 500 μl of lysis buffer (10 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, pH = 8) containing 1% Sodium lauryl sulfate (SDS) and 0.4 μg/ml of proteinase K. The mixture was incubated for one hour at 56°C and then for one hour at 100°C. An equal volume of phenol/chloroform/isoamylalcohol was added to the mixture and centrifuged at 10000 rpm for 10 minutes. The supernatant was added to an equal volume of chloroform. After being centrifuged at 10000 rpm, the top layer was collected and DNA was precipitated with two volumes of cold isopropanol at -20°C for 10 minutes. The pellet was obtained by centrifugation for 20 minutes and washed with 1.5 ml of 70% cold ethanol. Finally, the pellet was resuspended in 100 μl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8). 10 μl of the pellet was used for the RAPD-PCR mixture.

RAPD-PCR: The RAPD-PCR fingerprinting was carried out using one primer (5' GAAACAGCTATGACCATG -3'). DNA templates were amplified in a total reaction volume of 50 μl containing: 50 pmol of the primer, 200 μM of each deoxynucleotide, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH = 8.3) and 2.5 U Taq DNA polymerase. Amplification was carried out with denaturation at 94^0C for five minutes, followed by 40 cycles according to the following programme: 94°C for 30 seconds, 40°C for one minute and 72°C for one minute, plus a final extension of 10 minutes at 72°C to complete partial polymerizations. 12 μl of PCR products stained with ethidium bromide were subjected to electrophoresis in 1.5% agarose gels. After electrophoresis, the results were displayed under UV light. The molecular size of each fragment generated by electrophoresis was determined by comparison with molecular weight standards running

simultaneously. The fragments of each strain were compared by visual inspection. If all the visable bands of 2 isolates were the same distance apart, then the fingerprints were considered the same. Neither the variations in the intensity of the bands nor the shapes of the bands were taken into account, according to some studies [8].

Results

Analysis of antimicrobial susceptibility patterns obtained by the disk diffusion method showed that all the *A. baumannii* isolates were multi-drug resistant. All the isolates were resistant to at least 11 tested antimicrobial agents (cefixime, ceftriaxon, amoxycillin-clavulanic acid, piperacillin, gentamicin, ofloxacin, ciprofloxacin, trimethoprim/sulfamethoxazole, cefuroxime, amikacin, imipenem, cefepime and piperacillin-tazobactam), as shown in Table 1.

Table 1

Antimicrobial susceptibility of A. baumannii isolates determined by disk-diffusion method

Antibiotic	**Antimicrobial susceptibility of *A. baumannii* isolates**		
	R	**I**	**S**
	Number (%)	**Number (%)**	**Number (%)**
1. Cefixime	20 (100)	0	0
2. Ceftriaxon	20 (100)	0	0
3. Amoxycillin-clavulanic acid	20 (100)	0	0
4. Piperacillin	20 (100)	0	0
5. Gentamycin	7 (35)	13 (65)	0
6. Ofloxacin	20 (100)	0	0
7. Ciprofloxacin	20 (100)	0	0
8. Trimethoprim/sulfamethoxasole	0	2 (10)	18 (90)
9. Cefuroxime	20 (100)	0	0
10. Amikacin	20 (100)	0	0
11. Imipenem	20 (100)	0	0
12. Cefepime	20 (100)	0	0
13. Piperacillin-Tazobactam	20 (100)	0	0

R – resistant, I – intermediate susceptible, S – susceptible

7 (35%) and 13 (65%) out of 20 *A. baumannii* strains were resistant and intermediately susceptible to gentamycin, respectively. Only 2 (10%) out of 20 strains were intermediately susceptible to trimethoprim/sulfamethoxazole and 18 (90%) of them were susceptible to this antimicrobial agent (Table 1).

These analyses showed 3 antimicrobial susceptibility patterns, as shown in Table 2.

Table 2

Acinetobacter baumannii antimicrobial patterns

Type of antimicrobial pattern (Number of strains)	CFM	CRO	AMC	PIP	G	OFL	CIP	TXS	CFX	AK	IMI	FEP	TZP
I (2)	R	R	R	R	R	R	R	**I**	R	R	R	R	R
II (13)	R	R	R	R	**I**	R	R	**S**	R	R	R	R	R
III (5)	R	R	R	R	R	R	R	**S**	R	R	R	R	R

CFM – Cefixime; CRO – Ceftriaxone; AMC – Amoxycillin-clavulanic acid; PIP – Piperacillin; G – Gentamycin; OFL – Ofloxacin; CIP – Ciprofloxacin; TXS – Trimethoprim/Sulfamethoxazole; CFX – Cefuroxime; AK – Amikacin; IMI – Imipenem; FEP – Cefepime; TZP – Piperacillin-Tazobactam

The first antimicrobial pattern consisted of 2 (10%) out of 20 *Acinetobacter* isolates which were intermediately susceptible to trimethoprim/sulphamethoxazole and resistant to the rest of the 12 antibiotics tested by the disk diffusion method. 13 (65%) of 20 the strains were intermediately susceptible to gentamicin, susceptible to trimethoprim/sulfamethoxazole and resistant to the rest of the 11 antibiotics and they belonged to the second antimicrobial pattern. 5 (25%) out of 20 strains which were susceptible only to trimethoprim/sulfomethoxazole and resistant to the rest of 12 antibiotics were included in the third antimicrobial pattern.

A total number of 15 *A. baumannii* strains from the first and the second antimicrobial patterns were isolated from tracheal aspirates taken from neonates hospitalized in the paediatric intensive care unit and neonates in the paediatric ward. 5 *A. baumannii* isolates, which belonged to the third antimicrobial pattern, were isolated from wound swabs taken from adult patients hospitalized at the Traumatology Clinic.

The VITEK 2 automated system showed that all the strains were susceptible to: tobramycin, minocyclin, colistin, rifampicin, trimethoprim/sulfamethoxazole and intermediately susceptible to meropenem. 7 (35%) and 13 (65%) of the 20 *A. baumannii* strains were resistant and intermediately susceptible to gentamicin, respectively (Table 3).

Table 3

Antimicrobial susceptibility obtained by VITEK 2 system

Antibiotic	**Antimicrobial susceptibility of *A. baumannii* isolates determined by VITEK 2 system**		
	R	**I**	**S**
	Number (%)	**Number (%)**	**Number (%)**
1. Gentamicin	7 (35)	13 (65)	0
2. Meropenem	0	20 (100)	0
3. Tobramycin	0	0	20 (100)
4. Minocyclin	0	0	20 (100)
5. Colistin	0	0	20 (100)
6. Rifampicin	0	0	20 (100)
7. Trimethoprim/sulfamethoxasole	0	0	20 (100)

R – resistant, I – intermediate susceptible, S – susceptible

These analyses showed 2 antimicrobial susceptibility patterns of *A. baumannii* isolates tested by the VITEK 2 system (Table 4).

Table 4

Antimicrobial pattern obtained by VITEK 2 system

Antimicrobial pattern (Number of strains)	G	Mer	Tob	Min	Col	Rif	TXS
I (13)	I	I	S	S	S	S	S
II (7)	R	I	S	S	S	S	S

G – Gentamycin; Mer – Meropenem; Tob – Tobramycin; Min – Minocyclin; Col – Colistin; Rif – Rifampicin; TXS – Trimethoprim/Sulfamethoxazole

5 (71.3%) out of 7 *A. baumannii* strains with the second antimicrobial pattern were isolated from adult patients hospitalized at the Traumatology Clinic and 2 (29.9%) of them, together with the 13 *A. baumannii strains* with the first antimicrobial pattern, were isolated from neonates hospitalized in the paediatric intensive care unit and the paediatric ward.

RAPD-PCR assay of *A. baumannii* strains revealed two different RAPD-fingerprints as shown in Fig. 1. All the strains of *A. baumannii* isolated within a week in July 2010 from tracheal aspirates taken from neonates in the paediatric intensive care unit and neonates in the paediatric ward revealed the same RAPD-fingerprint designated as fingerprint A. 3 strains of *A. baumannii* which were isolated from tracheal aspirates taken from neonates in the paediatric intensive care unit three months ago revealed the same RAPD-fingerprint A. 5 strains of *A. baumannii* isolated from wound swabs of patients hospitalized

at the Traumatology Clinic revealed a different RAPD-fingerprint, designated as fingerprint B.

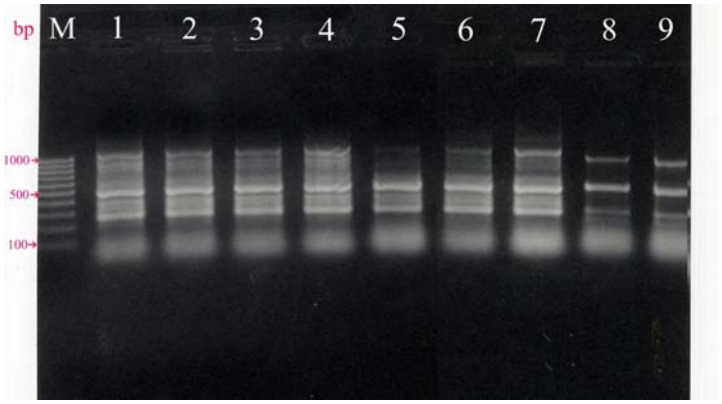

Figure 1 – Acinetobacter baumannii RAPD-PCR fingerprints
RAPD-PCR patterns. Lanes: 1–7 represent RAPD – PCR fingerprint A; lanes 8 and 9 represent RAPD – PCR fingerprint B; MW: DNA size marker 100 bp

Discussion

Hospital-acquired infections caused by multiple resistant Gram–negative bacilli have been a problem over the last 20 years. An increasing incidence of resistant members of the *Enterobacteriaceae* family involved in nosocomial infections was observed following the therapeutic introduction of newer broad-spectrum antibiotics in hospitals. A remarkable increase in the importance of strictly aerobic Gram-negative bacilli was observed including *Pseudomonas aeruginosa, Stenotrophomonas maltophila* and *Acinetobacter spp* [1].

The *Acinetobacter* isolates used in our study belonged to the species *A. baumannii* and were multi-drug resistant. All of them revealed 100% resistance to at least 11 antibiotics. This presented a huge ability for *Acinetobacter* to acquire resistance genes and cause serious infections in humans.

Despite the increasing frequency of multiple resistant *Acinetobacter* isolates, many clinicians still do not take sufficient care about the importance of these bacteria in hospitals. *Acinetobacter baumannii* is dangerous because it has no fastidious growth requirements and it is able to grow at various temperatures and in pH conditions.Thus it can persist in moist and dry conditions in the hospital environment, contributing to its transmission. the combination of this fact with its intrinsic resistance to many antimicrobial agents, enables these bacteria to spread very easily in hospitals [9]. Similar to our observations, two

separate studies report that all *A. baumannii* isolates were resistant to cefixime, amoxicillin-clavulanic acid, amikacin, imipenem, piperacillin, tazobactam and sensitive to rifampicin, minocyclin and colistin [10, 11], emphasizing the low clinical value of the last antibiotic because of its toxicity.

Testing antimicrobial susceptibility by disk diffusion method could show minor variations in the results, as in two *A. baumannii* isolates which were detected as intermediately susceptible to trimethoprim/sulfamethoxazole. The same isolates revealed good susceptibility to trimethoprim/sulfamethoxazole by the VITEK 2 system. These minor variations could be frequently observed among isolates tested by the disk diffusion method only. The VITEK 2 system is a more precise method for antimicrobial susceptibility testing and enables us to make clear our doubtful results. The resistance of 7 *A. baumannii* strains to gentamicin indicates that they contain plasmids with genes encoding adenyltranspherases or acetyltranspherases. Antimicrobial susceptibility patterns may be suitable as a screening method in epidemiological investigations, but requires confirmation by complementary techniques. RAPD-PCR fingerprinting has a particular significance in the epidemiological tracing because of the nature of RAPD profiling, which depends on DNA analyses. RAPD typing is more conservative and stable than typing based on plasmid profiles. Plasmids cannot be used as DNA templates by arbitrary primers, so that plasmid-free and plasmid-containing strains usually display the same fingerprint.

Based on RAPD-PCR analyses, all 12 *A. baumannii* strains isolated within a week in July 2010 as well as 3 *A. baumannii* strains isolated 3 months ago revealed the same RAPD fingerprint. This finding suggests a high epidemiological relatedness among all these 15 strains. There is a high probability that one and a unique strain of *A. baumannii* is the cause of low respiratory tract infections in neonates in the paediatric intensive care unit and neonates in the paediatric ward, as a result of their transmission between these two places. Detection of the same RAPD fingerprint of the *A. baumannii* strains isolated 3 months ago emphasized the high probability that the same strain has circulated for this period of time and suggests that this strain could be an endemic strain of *A. baumannii* in this setting.

Molecular typing by RAPD-PCR of 5 *A. baumannii* strains isolated from patients hospitalized in the Traumatology Clinic revealed a different RAPD fingerprint. This finding proved that these 5 strains belong to a completely different strain of the species *A. baumannii* and that RAPD-PCR is a precise molecular method which can make a differentiation between the strains from the same species.

In the present study, the issued antibiotic profiles were associated with RAPD-PCR profiles of *A. baumannii*. This provided evidence for using more advanced molecular typing methods for tracing and predicting *A. baumannii*

resistance to antibiotics. In fact, traditional methods for recognizing and typing *Acinetobacter* have often lacked sufficient reproducibility, typeability and discriminatory powers [12]. Antimicrobial susceptibility patterns may be suitable as screening methods in epidemiological studies, but they need to be confirmed by more precise and complementary techniques.

Conclusion

All strains of *A. baumannii* isolated from neonates in the paediatric intensive care unit and paediatric ward were multi-drug resistant, suggesting the wide spread of *A. baumannii* resistance to many antimicrobials. The RAPD-PCR technique was more reliable in interpreting the phenotypic and genetic relationship of the *A. baumannii* isolates. Good infection control procedures, including phenotypic and genetic typing of *A. baumannii* isolates, are essential for preventing outbreaks of multi-drug resistant *A. baumannii* infections in our hospitals.

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Резиме

ФЕНОТИПСКА И ГЕНЕТСКА ПОВРЗАНОСТ НА ИЗОЛАТИТЕ НА *ACINETOBACTER BAUMANNII*

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Апстракт: Во последните дваесетина години интересот за *Acinetobacter* континуирано се зголемува. Една од главните причини е појавата на мултирезистентни соеви, кои можат да предизвикаат инфекции што се шират како епидемии и кои вклучуваат повеќе пациенти од еден оддел, пациенти на интензивна нега и на различни места во болниците.

Цел: Да се испита поврзаноста помеѓу соевите на *Acinetobacter*, компарирајќи некои од нивните фенотипски и генетски карактеристики.

Материјал и методи: Во студијата беа вклучени вкупно 20 изолати на *Acinetobacter baumannii.* Беа добиени 12 изолати во текот на една недела од јули 2010 година, од новороденчиња хоспитализирани на одделот за интензивна нега и неонаталниот оддел при Универзитетската клиника за педијатрија, а 3 изолати беа добиени од истиот оддел за интензивна нега, три месеци претходно. Сите соеви на *Acinetobacter baumannii* беа изолирани од трахејални аспирати, земени од новороденчиња со инфекции на долниот респираторен тракт. Дополнителни 5 изолати на *Acinetobacter baumannii* беа вклучени како контролни соеви. Тие беа изолирани од брисеви од рани земени од возрасни пациенти со инфицирани рани на Универзитетската клиника за

трауматологија. Кај сите изолати беше испитана нивната осетливост кон 13 различни антимикробни средства со помош на диск-дифузониот метод (Kirby-Bauer). Осетливоста на истите изолати беше испитана и со VITEK 2 системот. Од сите изолати беше екстрахирана геномска ДНК која потоа беше користена како калап во молекуларниот метод RAPD-PCR со употреба на прајмерот (5' GAAACAGCTATGACCATG -3').

Резултати: Сите соеви на *A. baumannii* беа мултирезистентни. При тестирање на осетливоста на испитуваните изолати кон антибиотици со диск-дифузониот метод беа добиени 3, а со VITEK 2 системот беа добиени 2 резистограма. Испитувањето на соевите на *A. baumannii* со RAPD-PCR покажа два различни RAPD-фингерпринта. Сите соеви кои беа изолирани во текот на само една недела од јули, од трахејални аспирати на новороденчиња од интензивна нега и новороденчиња на неонаталниот оддел при Универзитетската клиника за педијатрија, покажаа ист RAPD-фингерпринт, како и трите соеви од трахејални аспирати на новороденчиња од интензивна нега добиени три месеци претходно. Петте изолати на *A. baumannii* добиени од Универзитетската клиника за трауматологија покажаа различен RAPD-фингерпринт.

Заклучок: Сите соеви на *A. baumannii* изолирани од новороденчињата на интензивна нега и неонаталниот оддел при Универзитетската клиника за педијатрија беа мултирезистентни. Ова укажува на ширење на резистенцијата истовремено кон повеќе антимикробни средства кај соевите на *A. baumannii* на Универзитетската клиника за педијатрија. Испитувањето на резистограми кај мултирезистентните изолати на *Acinetobacter* е корисен метод кој може да ја предвиди поврзаноста на соевите. Овој метод би можел да се надополни со барем една молекуларна техника, како што е RAPD-PCR, која се покажа како посигурна во интерпретирањето на поврзаноста на соевите на *A. baumannii*. Мерките за спроведување на добра контрола на инфекции, вклучувајќи ги фенотипизирањето и генетското типизирање на соевите на *A. baumannii*, се неопходни за спречување на епидемиските инфекции, предизвикани од мултирезистентни соеви на *A. baumannii* во болниците.

Клучни зборови: *Acinetobacter baumannii,* фенотипска поврзаност, генетска поврзаност, резистограми, RAPD-PCR фингерпринти.

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