Knowledge of antibiotic resistance mechanisms is absolutely necessary to successfully fight against multi-resistant bacteria, the solutions to this issue being a direct consequence of understanding the mechanisms underlying its occurrence. Considering that in Romania more than 92.3% of patients have been receiving antimicrobial prophylaxis in surgery for more than one day, and empirical therapy is very common, the increasing antibiotic resistance is an important problem. The experiments were performed at the Military Medical Research Center on the 26 multiple drug resistance (MDR) bacterial strains from health care-associated infections (HAI): Escherichia coli (3), Klebsiella spp. (4), Pseudomonas aeruginosa (10), Proteus mirabilis (1), Staphylococcus aureus (7) and Enterobacter cloacae (1). They were isolated and initially identified by the Medical Analysis Laboratory of the Dr. Al. Gafencu Emergency Military Hospital, Constanta and the Microbiology Laboratory from the Military Medical National Institute for Research and Development Cantacuzino of Bucharest. Bacterial strains were reseeded on specific culture media, and identification was based on culture, morpho-tinctorial characters and biochemical properties. Antibiotic susceptibility testing was performed by the Kirby-Bauer diffusion method, following the CLSI 2016 guidelines. The results obtained lead to the idea of reconsidering the strategy for the use of antimicrobial substances by the following actions: performing in vitro sensitivity tests, close collaboration between the clinician and microbiologist, finding additional methods for assessing the effective concentrations of the antibiotic at the level of the infections. The observed percentage of antibiotic resistance in our study was 85.72%, that being much higher than the mentioned percentage by the European Antibiotic Surveillance Report (EARS-Net) for Romania in 2013 (25-50%).

Keywords: antimicrobial resistance, multiple drug resistance (MDR), healthcare-associated infections (HAI)

Large amounts of antibiotics used in current human therapy, in animal and domestic bird therapy and even in aquaculture have led to the selection of multi-resistant pathogenic bacteria. Because of this, there are currently almost no antibiotics that can be used against these bacteria. Solutions to the problem of antimicrobial resistance are a direct consequence of understanding the mechanisms underlying its occurrence [1-5]. Therefore, knowing molecular and genetic mechanisms of antibiotic resistance is absolutely necessary to successfully fight against multi-drug resistance (MDR) [6]. Bacterial DNA, whether of its nature (chromosomal, plasmid or phagocytic), may include genes encoding antibiotic resistance mechanisms such as beta-lactam inactivating enzymes, efflux pumps, modification of the antibiotic target etc. [1,5]. Given that in Romania over 92.3% of patients received antimicrobial prophylaxis in surgery more than one day [7], for pathologies such as skin tumors, diabetic feet, trauma [8-10] and self-therapy antibiotic by ear is very frequent (causing clostridium difficile infection in most cases) [11], the increases of antibiotic resistance is a major concern [12,13].

Experimental part

Material and methods

Experiments were performed on several bacterial strains (26 strains) from antibiotic-resistant infections associated with antibiotics, namely Escherichia coli (3), Klebsiella (4), Pseudomonas aeruginosa (10), Proteus mirabilis (1), Staphylococcus aureus (7) and Enterobacter cloacae (1). These were initially isolated and identified in the Medical Analysis Laboratory of the Emergency Military Hospital Dr. Al. Gafencu University of Constanta and in the Microbiology Laboratory of the National Institute for Military-Medical Research and Development Cantacuzino of Bucharest. In data processing it was intended to observe ethical rules in research [14].

The 26 strains were cultivated in the Epidemiology and Emergency Laboratory of the Center for Scientific Medico-Military Research of Bucharest. Bacterial strains were seeded on specific culture media, and identification was based on culture, morpho-tinctorial characters and biochemical properties [12]. Antibiotic sensitivity testing was performed by the Kirby-Bauer diffusion method, following the CLSI 2016 guidelines. Antibiotic quality control was performed with Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923 [13].

Results and discussions

The antibiotic resistance profile for the bacterial strains studied was analyzed and interpreted. Classification of antibiotics by groups was performed according to the Angelescu Guide 2012 [15]. After reading the inhibition diameters of the anti-infectious chemotherapies and the expression of sensitivity (S) / moderate sensitivity or intermediate (I) / resistance (R) results, the phenotypes of resistance were made [16-19].
Antibiotic resistance phenotypes in Pseudomonas aeruginosa

Antibiotic susceptibility testing of isolates of Ps. aeruginosa revealed that all 10 strains studied showed multiple antibiotic resistance (MDR). Analysis of resistance profiles showed different resistance levels, namely: Ps. aeruginosa strains are resistant to oxacillin, cefuroxime, cefotaxime, trimethoprim-sulfamethoxazole in 100%. High resistance was seen in ceftriaxone (60%), gentamycin (60%), ceftazidime (60%), colistin (50%) and doxycycline (50%). In contrast, Ps. aeruginosa was sensitive to: cefepime (90%), ciprofloxacin (90%), norfloxacin (90%), levofloxacin (90%), tobramycin (90%) and amikacin (80%). Testing to other antimicrobials, clinically relevant for the group of Gram-negative, whose weight in infectious pathology has recently increased greatly meaning to the fermentation bacilli (Enterobacteriaceae) revealed a high resistance to cefoxitin (100%), cefuroxime (100%), doxycycline (100%), erythromycin (100%), nitrofurantoin (100%), tetracycline (100%) and trimethoprim-sulfamethoxazole (100%). In contrast, the tested strains were 100% sensitive to rifampicin.

Analysis of resistance profiles for isolated Ps. aeruginosa strains showed:

Beta-lactam resistance phenotypes characterized by resistance to aminopenicillins, cephalosporins of generation I and II (cefuroxime) and an intermediate sensitivity to some third generation cephalosporins (cefotaxime, ceftriaxone). Cephalosporins of the third generation (ceftazidime) remained active only in 50% of the studied strains. Only cephalosporins of the fourth generation (cefpim) remained active. A-lactamase inhibitors restored the activity of penicillins (piperacillin-tazobactam) to only 50% of the studied strains. The tested strains exhibited an inherent resistance to penicillins, cephalosporins and aztreonam, but were, in turn, sensitive to imipenem.

Among the aminoglycoside acquired phenotypes, 5 strains of Ps. aeruginosa showed associated resistance to gentamicin (G).

Quinolone resistance phenotypes. The resistant phenotype is characterized by the decrease of the inhibition diameters to fluoroquinolones. One strain showed resistance to this class of antibiotics. A number of 4 strains producing penicillinases and extended spectrum beta-lactamases showed associated sulfamidine resistance (SXT). A number of 8 strains were resistant to beta-lactam and showed no associated resistance in 2 classes of antibiotics: aminoglycosides or fluoroquinolones.

Our study highlighted the high levels of antibiotic resistance of isolated Ps. aeruginosa strains, and the knowledge of circulating resistance phenotypes is of interest to the practitioner and is of real use in preventing and controlling infections associated with medical care.

Resistance phenotypes encountered in test strains of the Enterobacteriaceae family

Resistance phenotypes of Klebsiella spp. strains

All 4 strains of Klebsiella spp. have associated resistance to beta-lactam antibiotics, aminoglycosides, quinolones, tetracycline, trimethoprim-sulfamethoxazole and nitrofurantoin, as follows:

Resistance to cefoxitin (75%), ciprofloxacin (100%), cefuroxime (100%), ceftazidime (75%), ceftriazone (100%), ceftazidime (75%), levofloxacin (100%), norfloxacin (100%), nitrofurantoin (100%), rifampicin (75%), piperacillin-tazobactam (100%), trimethoprim-sulfamethoxazole (100%). Sensitivity to colistin (75%) and amikacin (75%) of the studied sublot.
A number of other antimicrobial substances not usually administered in infections caused by Klebsiella spp. have been tested and analysis of the results indicated that they are resistant to: clarithromycin (100%), clindamycin (100%), erythromycin (100%), linezolid (100%), quinupristin-dalfopristin (100%), oxacillin (100%), teicoplanin (100%) and vancomycin (100%).

All tested strains present resistance to betalactam, aminoglycoside, quinolone, tetracycline, trimethoprim-sulfamethoxazole, and nitrofurantoin, thus resulting in the corresponding resistance phenotypes.

Resistance phenotypes of Escherichia coli strains

In our study, Escherichia coli strains exhibited a broad-spectrum beta-lactamase-producing phenotype (BLEL) phenotype, BLSE production being responsible for the absence of the inhibition diameter around the third-generation cephalosporin disks: ceftazidime and cefuroxime. In order to establish the aminoglycoside resistance phenotypes we used 3 types of antibiotic microcompresses: gentamicin, amikacin and tobramycin, and the results showed that the Escherichia coli strains exhibit AT (33%) and GT phenotypes (66%).

Table 1

<table>
<thead>
<tr>
<th>Phenotype</th>
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<tr>
<td>AT</td>
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Legend: G = gentamicin, A = amikacin, T = tobramycin, R = resistant, S = sensitive.

Quinolone resistance phenotypes were highlighted by levofloxacin, norfloxacin and ciprofloxacin strains. In our study, resistance to fluoroquinolones is crossed between all substances in this class (norfloxacin, ciprofloxacin, levofloxacin), all strains exhibiting 100% resistance.

Phenotypes of resistance encountered in the strain of Proteus mirabilis

Proteus mirabilis strains show resistance to: cefuroxime, cefepime, ceftazidime, colistin, doxycycline, gentamicin, levofloxacin, nitrofurantoin, tetracycline and trimethoprim-sulfamethoxazole. The Pr. mirabilis strain is intermediate sensitive to: amikacin, ciprofloxacin, norfloxacin and piperacillin-tazobactam and susceptibility to cefoxitin, ceftriazone, rifampicin and tobramycin.

A series of other antimicrobial substances which are not routinely administered in infections caused by Proteus spp. have been tested, and analysis of the results indicated that they have resistance to: clarithromycin, clindamycin, erythromycin, linezolid, quinupristin-dalfopristin, oxacillin, teicoplanin and vancomycin.

Antibiotic resistance of Enterobacter cloacae strain

A high level of resistance of the Enterobacter cloacae strain to the tested antibiotics was observed to: cefoxitin, cefuroxime, cefepime, ceftriazone and ceftazidime.

The level of resistance of Enterobacter cloacae isolated to aminoglycosides was also analyzed, but it was susceptible to amikacin, gentamicin and tobramycin.

The level of sensitivity to fluoroquinolones was evaluated: intermediate to ciprofloxacin (CIP), respectively sensitivity to norfloxacin and levofloxacin.

In vitro sensitivity to doxycycline and tetracycline has been preserved. As, susceptibility to rifampicin and trimethoprim-sulfamethoxazole, respectively, colistin, piperacillin-tazobactam and nitrofurantoin have been observed. A series of other antimicrobials that should not be used commonly in infections caused by Enterobacter spp. were tested, and analysis of the results indicated that they showed resistance to: clarithromycin, clindamycin, erythromycin, linezolid, quinupristin-dalfopristin, oxacillin, teicoplanin and vancomycin.

Phenotypic evidence of antibiotic resistance in Staphylococcus aureus strains

Staphylococcus resistance to beta-lactams. In the seven staphylococcal strains studied, plasmid-mediated broad-spectrum beta-lactamases, which encode resistance to cephalosporins, were identified: resistance to cefuroxime (100%), and resistance and intermediate to ceftazidime (100%). In contrast, S. aureus strains retained their sensitivity to: cefoxitin (100), ceftriaxone (100%), cefepime (100%) and oxacillin (100%).

Oxacillin is a staphylococcal beta-lactamase resistant penicillin, being preferred for the stability and reproducibility of the results. It has been recommended since 2004 for the treatment of staphylococcal infections by the European Antibiotic Resistance Surveillance System. Since 2005, the Institute for Clinical Laboratory Standards recommended the cefoxitin disk diffusion test.

Staphylococcus resistance to aminoglycosides. A total of 6 strains (85.72% of the cases) had resistance to gentamicin, respectively, tobramycin; only 2 strains were resistant, and other 2 showed the amikacin intermediate sensitivity phenomenon (28.57%).

Aminoglycosides are a group of antibiotics commonly used in human therapy for staphylococcal infections. Resistance to these antibiotics is determined by a gene, responsible for the synthesis of the aminoglycoside acetyltransferase, at either the plasmid or chromosome. An enzyme which induces resistance. Although several antibiotics are included in this group, only resistance to kanamycin, tobramycin and gentamicin is tested in practice. Resistance to aminoglycosides being enzymatic extends to amikacin and netilmicin, which further alter...
their bactericidal activity than bacteriostatic activity, resulting in the loss of synergism with beta-lactam antibiotics. Resistance to kanamycin (K phenotype), kanamycin and tobramycin (KT phenotype) are predictive of resistance to amikacin, netilmicin, however, remains active. Resistance to kanamycin, tobramycin and gentamicin (KTG phenotype) is predictive of resistance to amikacin and netilmicin.

Staphylococci resistance to macrolides, tetracyclines and other antibiotics. To determine other antibiotic resistance phenotypes of isolated Staphylococcus aureus strains, we determined the susceptibility of these strains to macrolide-lincosamide-streptogramin B (MLSB). The tested strains showed the form of intermediate resistance (I) to erythromycin in 100% of cases, and the resistant + intermediate formulations to clindamycin - 71.4%. In contrast, clarithromycin sensitivity was observed in 71.4% of the tested strains. Erythromycin-resistant and clindamycin susceptible strains have MLSB phenotype. The strains tested with the MLSB phenotype were reported to have been clindamycin-resistant. Staphylococci resistance to tetracyclines is encoded by the plasmid and chromosomal tet gene, common to all antibiotics in this group. All susceptible strains retained sensitivity to tetracyclines (doxycycline and tetracycline). In the case of rifampicin, considered the drug of choice for staphylococci, it has not been reported as a subgroup of drug resistant strains studied. The same susceptibility maintained in 100% of cases was also observed with quinupristin-dalfopristin and trimethoprim-sulfamethoxazole. All strains were resistant to vancomycin. Also, no strain was sensitive to ticoplanin and nitrofurantoin. With linezolid, the percentage of resistance of S. aureus is high (57.14%). The strains tested had high resistance to cefazidime (100%) and cefuroxime (85.72%) and the same percentage (85.72%) showed resistance to gentamicin. We tested the sensitivity to erythromycin, all strains proving to be medically sensitive.

No resistance level has been demonstrated in cefepime and quinolone (ciprofloxacin, norfloxacin and levofloxacin).

The observed percentage of antibiotic resistance in our study was 85.72%, which is much higher than the mentioned percentage by the European Antibiotic Surveillance Report (EARS-Net) for Romania in 2013 (25-50%) [20].

On the other hand, bacteria could be damaged by an excess concentration of oxidative agents - e.g. reactive oxygen species (ROS) -, a process named cellular oxidative stress, which can have a devastating effect on the structure and activity of proteins, and may even lead to cell death [21-23].

Conclusions
The subject of microbial resistances is more important because the literature data about this subject is insufficient for our area. Pseudomonas aeruginosa, Escherichia coli, Proteus spp., Klebsiella spp. and Staphylococcus aureus are nosocomial pathogens and are involved in a wide range of infections, most often difficult to control. Knowing the resistance phenotypes of these bacteria can often be useful in saving patients’ lives by promptly choosing a specific antibacterial agent. A high number of strains from the studied lot showed multiple resistance to most antibiotics, commonly used in anti-infectious therapy: penicillins, cephalosporins, carbapenems, aminoglycosides, tetracyclines, fluoroquinolones, trimethoprim-sulfamethoxazole etc.

Considering all these aspects, it would be useful to reconsider the strategy for the use of antimicrobial substances by the following actions:
- performing in vitro sensitivity tests, because the antibiogram guides the therapeutic decision and highlights the preservation of antibacterial activity considered the miracle of the 20th century;
- close collaboration between clinician and microbiologist, because the microbiologist, through the results that he communicates, assumes responsibility together with the clinician, in the therapeutic act;
- finding additional methods for testing, because the diffusimetric method has the disadvantage that it does not allow the estimation of effective antibiotic concentrations at the level of the infections.

The tested Staphylococcus aureus strains showed increased associated resistance to the second and third generation of cephalosporins, macrolides (erythromycin, clarithromycin), lincosamide (clindamycin) and aminoglycosides (amikacin, gentamicin, tobramycin).

Although clindamycin has high anti-staphilococcal activity, the high percentage of resistance seen at the tested strains, limits the utility of clindamycin in the treatment of infections caused by Staphylococcus aureus. In our study, a number of 7 vancomycin-resistant bacterial strains, with 100% resistance and 4 linezolid resistant bacterial strains, with 57.14% resistance were observed, according to the scientific literature that mentioned the occurrence of such mutagens worldwide. Aminoglycosides should be used in combination therapy because their use as a single antimicrobial agent predisposes to resistance. Even if the clinical efficacy of trimethoprim-sulfamethoxazole is not fully documented, may be a therapeutic option for multi-resistant Staphylococcus aureus, considering the high susceptibility to in vitro testing.

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References

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