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Determination of Possible Resistance Mechanisms Using Disc Diffusion Test in Gram Negative Bacteria

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Abstract

Infections due to resistant microorganisms at hospitals have recently and more importantly posed an increasing problem. Interpretative reading of antibiograms with the help of disk diffusion method, which is more practical, cheap and efficient in short time, can provide important hints about the mechanism of resistance to antibiotics. In this study, our aim is to determine the possible resistance mechanisms and their frequency, to interpret antibiograms depending on resistance, and to help physician choose the most suitable antibiotics for the patients by using only antibiotic susceptibility tests on gram negative bacteria isolated in our hospital.

This study covers 100 *Klebsiella spp.*, 100 *Escherichia coli*, 30 *Enterobacter spp.* and 100 *Pseudomonas aeruginosa* isolates. It has been tried to determine possible resistance mechanisms in antibiograms with the help of disk diffusion tests. Test results were evaluated according to CLSI.

In our study, *Klebsiella spp*. isolates produced classical and low SHV-1/K1 (17%). 1% of *Klebsiella spp*. isolates produced penicillinase-high, 74% of *Klebsiella spp*. isolates produced extended-spectrum beta lactamase (ESBL), and 5% high amount of Kl enzymes. Production of classical (31%), low penicillinase (16%), high penicillinase (14%) and ESBL (39%) were observed in *E. coli* isolates. Production of classical AmpC inducible (63.4%), penicillinase (6.6%), ESBL (3.3%) derepressed AmpC (23.4%), were observed in *Enterobacter* isolates. Resistance mechanism in an *Enterobacter* isolate could not be interpreted as phenotypic. Production of classical (27%), penicillinase (14%), derepressed AmpC (7%) were found in *P. aeruginosa* isolates. Additionally, derepressed AmpC at the rate of 36% was considered to be found possibly along with the loss of OprD porin, a metallo-/3 lactamase, GES-2 having ESBL characteristics, or overproduction of derepressed AmpC enzyme could be considered. Availability of derepressed AmpC and high level penicillinase (5%), increased efflux (6%), and of multiple resistance mechanisms in *P. aeruginosa* isolates was thought. As a result, biochemical mechanisms of resistance can be figured out through interpretative reading taking care of phenotypic characteristics. Interpretative reading not only provides a better treatment but enable clinical antibiotics to have a longer life and low level resistance to be determined as well. Resistance phenotypes in the subject mechanism can be pointed out in advance through interpretative reading. Although interpretative reading has a considerable importance in diagnostic microbiology, it cannot be substituted for genetic and biochemical methods to determine resistance mechanisms.

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Introduction

Infections caused by resistant microorganisms at hospitals become a bigger problem with each passing day. For this reason, showing resistance mechanisms to microorganisms both in society and in hospitals and also finding out in which microorganisms these mechanisms are frequently found will be a big step in preventing the resistance developed against antibiotics [1]. In microorganisms, it is possible to show resistance to antibiotics only through molecular methods. However, molecular methods are both difficult to work and very expensive. Interpretative reading of antibiograms with the help of Kirby-Bauer disk diffusion method, which is more practical, cheap and efficient in short time, can provide important hints about the mechanism of resistance to antibiotic [1,2]. The purpose in interpreting antibiograms is to prevent erroneous results and to reach a result as correct as possible by taking into consideration the bacteria identification of sensitivity experiment results, statistical information about the frequency of antibiotic resistance in bacteria, resistance mechanisms in bacteria and the antibiotics influenced by these mechanisms [1].

In order to better understand resistance mechanisms, antibiotics which are not used in treatment and which are not reported to physicians are also used in experiments. These antibiotics are indicators and they can be used to find out some of the resistance mechanisms of bacteria easily. Thus, instead of tests which are difficult, expensive and which cannot be done in all laboratories, the easy application of disk diffusion test, which is a simple and cheap method that can find these characteristics of bacteria, will contribute to laboratories. This method helps to find out the possible resistance mechanism to bacteria and the accuracy of the identification of bacteria [3,4]. Gram negative bacilli, which cause a great number of infections in humans, are also found as the most frequent hospital infection agents in hospitals. Revealing these resistance mechanisms will be a guide for early and fast treatment of agents. Different studies conducted on the subject have generally focused on molecular and advanced tests. Methods with more simple techniques that can easily be applied by each laboratory are forgotten [5].

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Our aim in this study was to determine the possible resistance mechanisms and their frequency, to interpret antibiograms depending on resistance, and to help physician choose the most suitable antibiotics for the patients using only antibiotic susceptibility tests on gram negative bacteria which are frequently isolated in our hospital.

Method

Isolation and identification of bacteria

A total of 100 *Klebsiella spp*., 100 *Escherichia coli*, 30 *Enterobacter spp*. and 100 *Pseudomonas aeruginosa* isolates, which were isolated from samples that came to Ondokuz Mayis University, Medical Faculty central laboratory from various clinics, were included in our study. After the bacteria were isolated from the samples, they were identified by using conventional methods in accordance with the recommendations of the Api 32E (bioMeriux, France) and Api 20 NE (bioMeriux, France) kit producing company.

Sensitivity tests

Pure bacteria cultures isolated from the medium were studied daily with Kirby Bauer disk diffusion method. For beta-lactam induction test to the same strains, imipenem (10 mg), ceftazidime (30 mg), aztreonam (30 mg) discs (BD, ABD) were used. For double disc synergy test (DDST), amoxicillin/clavulanic acid (20/10 mg), cefotaxime (30 mg), ceftazidime (30 mg), aztreonam (30 mg), cefoxitin (30 mg), discs (BD, ABD) were used. For metallo-beta-lactamase identification with double disc synergy test, imipenem and EDTA disc or ceftazidime (30 mg) and 2-mercaptopionic acid were used. The results of these tests were interpreted as stated by Livermore [4]. The antibiotics applied on bacteria were chosen according to CLSI criteria and the results were interpreted according to these criteria [3].

Results

In our study, in 17 (17%) of *Klebsiella spp*. isolates, all beta-lactams including all beta-lactam/beta-lactam combinations except penicillin were found to be sensitive. This resistance pattern was interpreted as classical or low SHV-1 phenotype. While one *Klebsiella spp*. isolate was found to be resistant to ampicilline, ticarcillin, piperacillin, cephalothin and beta-lactam/beta-lactamase inhibitor combinations, it was found to be sensitive to other beta-lactam antibiotics. Resistance mechanism causing this phenotype was interpreted as high penicillinase phenotype. In 71 (71%) of *Klebsiella spp*. isolates, DDST and extended-spectrum beta lactamase (ESBL) were found and four different ESBL types were found according to sensitivity results. Resistance to all beta-lactam except cefoxitin and carbapenem and beta-lactam/beta-lactamase inhibitors in 26 species was interpreted as high production ESBL enzyme phenotype. Ceftazidime resistance which was found more when compared with other cephalosporins in one of *K. pneumoniae* isolates was interpreted as ESBL positive ceftazidimase resistance phenotype. Since a total of 14 (14%) *Klebsiella spp.* isolates- 7 DDST positive and 7 were DDST negative- were resistant to all beta-lactams except carbapenem, they were interpreted as AmpC or impermeability resistance phenotype. Since resistance was found in 5 (5.9%) of the *K. pneumoniae* isolates to all beta-lactams except imipenem and cefepime, the resistance mechanism in these isolates was interpreted as acquired Armp-C enzyme. In five (33.3%) of the K. oxytoca isolates, while resistance was found to all penicillin including beta-lactam/beta-lactamase inhibitor combinations, 1st generation cephalosporins, cefuroxime and aztreonam, sensitivity was found to other cephalosporins and carbapenems. Phenotypically, this resistance pattern was interpreted as high amount of K1 enzyme production. Resistance mechanism of an isolate in *Klebsiella spp*. was not interpreted as phenotypic. The distribution of five different resistance mechanisms found for *Klebsiella spp*. . According to type is given in Table 1.

While sensitivity was found in 31 (31%) E. coli isolates to aminopenicillins, carboxypenicillins, ureidopenicillins, first, second and third generation cephalosporins, monobactam and carbapenems, resistance was interpreted as classical or low SHV-1/K1. 16 (16%) strains which were found to be resistant to Ampicilline and ticarcillin, and moderately sensitive to piperacillin and cephalothin and sensitive to all other beta-lactam antibiotics was interpreted as low penicillinase resistance phenotype. Since 14 (14%) strains were found to be resistant to ureidopenicillins and first generationcephalosporins in addition to aminopenicillins, carboxypenicillins; and moderately sensitive or resistant to beta-lactam/beta-lactamase inhibitor combinations, it was interpreted as high penicillinase phenotype. 39 (39%) of the isolates were found to be ESBL resistant to all beta-lactamase except for cefoxitin and carbapenem (Table 2).

Resistance mechanism	K. pneumoniae n (%)	K. oxytoca n (%)	Total n (%)
Classical or low SHV-1/K1	15 (17.6)	2 (13.4)	17
High penicillinase	1 (1.2)	-	1
K1 enzyme	-	5 (33.3)	5
Acquired Amp C	5 (5.9)	-	5
Mechanism not found	1 (1.2)	-	1
ESBL type			
ESBL	22 (34.9)		22
ESBL over production	26 (41.2)		26
ESBL ceftazidimase	1 (1.5)		1
DDST (+)/Amp C or impermeability	7 (11.2)		7
DDST (-)/Amp C or impermeability	7 (11.2)		7

Table 1: Distribution of resistance mechanisms found for *Klebsiella spp*.
ESBL: Extended spectrum beta-lactamase. DDST: Double disc synergy test. n: Number of bacteria.

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Resistance mechanism	*E. coli* n (%)
Classical or low SHV-1/K1	31 (31)
High penicillinase	14 (14)
Low penicillinase	16 (16)
ESBL	39 (39)
Total n	100

Table 2: Distribution of resistance mechanisms found for *E. coli*.
ESBL: Extended spectrum beta-lactamase. n: number of bacteria.

A total of 19 (63.4%) of *Enterobacter* isolates which were found to be resistant to ampicilline, amoxicillin/clavulanic acid, cephalothin and cefoxitin and sensitive to beta-lactam antibiotics except these were interpreted as classical inducible AmpC resistance phenotype. 2 (6.66%) of the isolates which were found to be resistant to ticarcillin and piperacillin in addition to ampicilline, amoxicillin/clavulanic acid, cephalothin and cefoxitin were interpreted as penicillinase resistance phenotype. One isolate (3.3%) which was sensitive to imipenem, resistant to all other cephalosporins, beta-lactam/beta-lactamase inhibitor combinations and DDST positive was interpreted as ESBL positive resistance phenotype. While four isolates were found to be resistant to all beta-lactam antibiotics and inhibitor combinations except carbapenems and cefepime, one was found to be resistant to all beta-lactam antibiotics including cefepime except for carbapenem. Two isolates were moderately resistant to aztreonam, unlike the other four isolates. All these seven strains were interpreted as derepressed AmpC (23.4%) resistance phenotype. Resistance mechanism in one *Enterobacter spp.* isolate which was resistant to Carbapenem, monobactam and cephalosporins could not be interpreted as phenotypic (Table 3).

Resistance mechanism	n (%)
Classical inducible Amp C	19 (63.4)
Derepressed Amp C	7 (23.4)
Penicillinase	2 (6.6)
ESBL	1 (3.3)
No mechanism found	1 (3.3)
Total n	30 (100)

Table 3: Distribution of resistance mechanisms found for *Enterobacter spp*.
ESBL: Extended spectrum beta-lactamase. n: Number of bacteria.

Twenty-seven (27%) *P. aeruginosa* isolates were sensitive to ureidopenicillins, carboxypenicillins and cephalosporins and they were interpreted as classical phenotype. A total of 14 (14%) of these isolates were also resistant to ticarcillin, piperacillin and the beta-lactamase inhibitor combinations of these antibiotics and sensitive to other beta lactams and they were interpreted as penicillinase phenotype. Seven (7%) isolates which were found to be resistant to all beta-lactams except cefepime in various degrees were interpreted as derepressed AmpC phenotype. A total of 36 (36%) strains resistant to all beta-lactams and sensitive to quinolons which were resistant to all other cefepime sensitive beta-lactams were interpreted as a combination of derepressed AmpC and OprD porin loss. Five (5%) *P. aeruginosa* isolates which were found to be resistant to ticarcillin, piperacillin, imipenem and meropenem and sensitive to other cephalosporins, monobactam were interpreted as loss of OprD porin and high level penicillinase phenotype. 6 (6%) isolates resistant to ticarcillin, piperacillin and beta-lactamase inhibitor combinations of these antibiotics and sensitive to imipenem which were resistant to cephalosporins, monobactam and meropenem were interpreted as efflux phenotype. Since 5 (5%) isolates were resistant to all beta-lactam and quinolons, they were thought to have multiple resistance mechanisms (Table 4).

Resistance mechanism	n (%)
Classical	27 (27)
Penicillinase	14 (14)
Amp C completely depressed	7 (7)
Amp C completely depressed + OprD loss	36 (36)
OprD loss + high level penicillinase	5 (5)
Efflux	6 (6)
Multiple resistance	5 (5)
Total	100 (100)

Table 4: Distribution of resistance mechanisms found for *P. aeruginosa*.
OprD: Outer membrane porin protein D, n: Number of bacteria.

Discussion

Detection and reporting of possible resistance mechanisms in bacteria guide the clinician to correct treatment. There are a great number of methods that can show the resistance mechanisms in bacteria. It is of importance for the method to be used in the detection of resistance to be applicable in every laboratory, to be cheap and easy to work. Our study found out the possible resistance mechanisms that can be found in gram negative bacteria that are most frequently seen in hospitals with the help of disk diffusion test, which is a simple test requiring low cost. In our study, the resistance type found the most in *Klebsiella spp.*, 17 (17%) was chromosomal class A beta-lactamase enzyme phenotype. This phenotype is seen in the presence of SHV-/K1 enzyme. It represents resistance to ampicilline structurally [6]. At the same time, the only difference of high penicillinase enzyme presence we found in one (1%) of the *Klebsiella spp.* isolates from classical SHV-1/KI phenotype is causing resistance to beta-lactam/beta-lactamase inhibitor combinations and first generation cephalosporin in addition. When SHV-1 and TEM-1 β-lactamase enzymes are produced in normal levels in *Klebsiella spp*, they remain sensitive to beta-lactam/beta-lactamase inhibitors. However, if the enzyme is produced excessively, it is reported to develop resistance to these antibiotics and cephalothin [7,8]. The other phenotype we found in *Klebsella spp.* isolates was ESBL. This resistance phenotype is more frequent in *Klebsiella spp.* when compared with other gram negatives and this frequency increases gradually [9]. In a multi central study, ESBL positivity in *Klebsiella spp.* isolates has been reported to be over 80% [10,11]. The resistance developed against beta-lactams in these strains was developed by resistance against beta-lactamase inhibitors with a rate of over 30% [10]. Acquired AmpC was found in 5.9% of our *Klebsiella spp.* isolates. In a study the conducted, Coudron et al. [12] found that 1.2% of the clinic isolates of *E. coli*, *K. pneumoniae* and *P. mirabilis* which were found to have resistance to all beta-lactams except cefepime and carbapenem released AmpC beta-lactamase [13]. The resistance phenotype of the *K. pneumoniae* isolates which were found to have DDST and positive/AmpC resistance with a rate of

8.2% was found in a *K. pneumoniae* isolate in the intensive care unit of a hospital in Greece [13]. In our study, 7 (8.2%) of *K. pneumoniae* isolates were found to have sensitivity to imipenem and resistance to all beta-lactams including cefoxitin and DDST was found to be negative in these isolates. Resistance to cephamycins and carbapenems is not observed in ESBL producing *Klebsiella*. In isolates which have loss of porin with ESBL, resistance to cephamycins is observed and this contributes to increase in resistance to other cephalosporins [13,15]. In a study conducted by Domenech-Sanchez [13], porin presence was found in 50 of 65 ESBL producing *K. pneumoniae* isolates. The fact that these isolates also had cefepime resistance was interpreted as AmpC beta-lactamase phenotype which brought to mind loss of porin [8,16]. For 14 isolates in our study which were DDST negative or positive and which also had cefoxitin resistance, the exact reason for resistance can be enlightened with molecular studies. It is thought that more than one mechanism plays a role in this resistance [17].

Although beta-lactamase which are generally coded as chromosomal in *Enterobacteriaceae* family are examined in class C, they are found in class A group in *K. oxytoca* species. These enzymes are structurally produced in low levels and they cause ampicilline, amoxicillin, carbenicillin and ticarcillin resistance [18]. Five (53.3%) *K. oxytoca* isolates in our study showed this phenotype and it was interpreted as the presence of K1 enzyme in these strains. As in Livermore et al.'s study, these strains were sensitive to all cephalosporins, carbapenems except aztreonam and cefuroxime [4]. In a study conducted by Fournier et al. [19], excessive K1 enzyme presence was found in 63% of *K. oxytoca* strains, while there are also studies which reported this rate as 8-10%. This rate differs between 10-20% in Europe [20-22]. In our study, *E.coli* strains, which were the most important pathogen of nosocomial infections, had classical or low SHV- resistance phenotype with a rate of 31%. This resistance on plasmid represents high resistance to ampicilline and it is reported that it can be found as positive on 50% of strains [23]. Amoxicilline resistance in E. coli differs from country to country and even from hospital to hospital. This resistance is over 10-68% in Europe and America [24]. In our study, the rate of low and high level penicillinase which caused this resistance was found as 30%. The rate of 39% which we found about E. coli isolates producing ESBL was higher than the rates in America and Europe [25,26]. This rate was also found to be high in studies conducted at different times in our country [27,28]. The rate was lower in studies conducted previously in our hospital [29,30]. This difference is probably a result of change in intense antibiotic use. 19 (63.3%) of the *Enterobacter* isolates in our study were found to have inducible AmpC. Studies conducted have shown that *Enterobacter* species have inducible beta-lactamase enzyme and the frequency of this was found as 80% in these bacteria types [31-33]. Although ESBL has been reported in *Enterobacter* species in studies, this rate is too small in bacteria of other enterobacter families [34,35]. In our study, we found ESBL in only one isolate. While derepressed AmpC phenotype that we found with a rate of 23.4% in *Enterobacter spp.* in our study was found with a rate of 70% in 12 hospitals of Athens and with a rate of only 5% in a hospital in Connecticut. It has been reported that the different resistance rates found can be due to bacteria intensity in the population, level of antibiotics, antibiotics used and hospital microflora [20]. It was not possible to interpret the resistance phenotype in an isolate which was resistant to all beta-lactam antibiotics including carbapenems with the method we used and it was thought that molecular methods are necessary to show the resistance mechanism of such strains. 27% of the *P. aeroginosa* strains in our study were found to have resistance phenotype depending on classical enzyme release. This is a resistance phenotype described by Livermore [36]. In 124% of these strains, penicillinase was interpreted as the presence of resistance phenotype. This extremely active enzyme is class A penicillinase. It is found very rare in *P. aeruginosa* isolates [20,37,38]. It has been reported as 52% in a study conducted in our country [39]. This brought to mind that with penicillinase production, inducible beta-lactamase presence can occur.

Strains with derepressed AmpC resistance phenotype found in *P. aeruginosa* isolates (7%) have been reported to occur as a result of broad spectrum beta-lactam [20,40]. In a study they conducted, Lopez-Yeste et al. showed that the frequency of such stable derepressed mutants was very high in *P. aeruginosa* species with inducible beta-lactamase. These strains are reported to be resistant to aminoglycosides and fluoroquinolones and they are reported to decrease the existing options used in treatment [41]. In our study, it was mentioned that cephalosporinase and metallo beta-lactamase could also be effective in the resistance type found in 36 *P. aeruginosa* isolates which had a combination of loss of porin and derepressed AmpC resistance mechanisms [42-44]. In our study, there are five isolates interpreted as a combination of loss of OprD and penicillinase enzyme. In a study they conducted, Pai et al. [45] reported cephalosporinase and metallo beta-lactamase, loss of porin D, derepressed AmpC resistance, penicillinase in the presence of more than one mechanism in resistance to beta-lactam antibiotics [43]. However, molecular methods should be used for definitive results in explaining these mechanisms [46]. In our study, the resistance mechanism reflected on phenotype in all of the six isolates was interpreted as increased efflux. Efflux is an intrinsic but silently working resistance mechanism in *P. aeruginosa* isolates. This resistance mechanism causes single or multiple resistance to many antibiotics including carbapenems. Carbenicillin resistant strains caused by active efflux are increasing gradually [47]. What is worse, this resistance mechanism can be activated during treatment. Developing resistance during treatment causes very important clinical consequences for *P. aeruginosa* and results in treatment failures [48,49]. This type of resistance developing risk has been reported more with carbapenem use [50] and it can also occur in the form of carbapenem resistance [51]. In our study, five isolates were found with resistance to all beta-lactam antibiotics including carbapenems and in addition to quinolons. We believe that the resistance on the phenotype in these isolates occurred through different resistance mechanisms independent of one another. These mechanisms can occur through with one of or a combination of derepressed AmpC or excessive production of derepressed AmpC enzyme, efflux and loss of OprD. Molecular studies have to be conducted to find out the resistance mechanism in these isolates [52-54].

In *Klebsiella* species, *E. coli, Enterobacter* species and *P. aeruginosa* isolates included our study, difference was found between microorganisms in terms of the variety and frequency of bacterial resistance patterns through interpretative reading. While ESBL production is the most frequent resistance mechanism in *Klebsiella* species and *E. coli*, the most frequent resistance mechanism in *P. aeruginosa* is the combination of more than one resistance mechanism. It has been thought that through interpretation and reading of antibiograms with Kirby-Bauer disk diffusion test, which is an easily applicable, well-standardized, fast and economical method, it will be possible to avoid erroneous results and reach a result as accurate as possible by taking into consideration the frequency of antibiotic resistance, resistance mechanisms in bacteria and the antibiotics

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influenced by these mechanisms with the help of these readings. However, interpretative reading also has some limitations. The most important one is that there are many resistance mechanisms influencing the same group of bacteria and the frequency of these is increasing gradually. Interpretative reading may not show new resistance mechanisms completely. Other limitations of this method are isolates synthesizing small or great numbers of enzymes giving changeable results especially against inhibitors and the presence of more than one resistance mechanism in some strains.

As a conclusion, through interpretative reading, possible biochemical mechanisms of resistance can be understood by starting with phenotypic characteristics. Clinicians' attention can be drawn to the combination of antibiotic-microorganism combinations that pose a risk in terms of treatment. Interpretative reading does not only provide a better treatment, but also contributes to finding out low resistance and antibiotics used in clinic having a longer life. It enables predetermination of the resistance phenotype from the mechanism found. Although interpretative reading has gained a considerable importance in diagnostic microbiology recently, it can't replace genetic and biochemical methods in showing resistance mechanisms.

Competing Interests

The author declare that there is no competing interests regarding the publication of this article.

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