A Six-Month Survey of the Frequency of Extensively Drug-resistant Gram-Negative Bacteria by VITEK 2 System in 2020

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ABSTRACT

Background and Aim: Increasing of resistant bacteria is a major concern globally. The emergence of XDR gram-negative bacteria is a more serious problem due to treatment limitations. This study aimed to evaluate the frequency of extensively drug-resistant (XDR) gram-negative bacterial isolates in different clinical samples from Payvand Clinical and Specialty Laboratory, Tehran, Iran, for 6 months by VITEK 2 system.

Materials and Methods: During March 2020-September 2020, different clinical samples were collected from patients referred to Payvand Clinical and Specialty Laboratory. Bacterial identification and antimicrobial susceptibility test (AST) were performed applying an automated VITEK 2 system. The frequency of identified bacteria, their resistance to common antibiotics and also XDR bacteria were calculated and reported, respectively.

Results: Overall, 4125 urine specimens, 34 sputum samples, and 1 tracheal aspirate tube were submitted to Payvand Laboratory during 6 months. Of them, 486 urine, 32 sputum, and a tracheal aspirate tube samples were culture positive. Gram-negative isolated bacteria were included in this study. Based on AST, 63.3% of the isolated *Klebsiella pneumoniae*, 100% of *Pseudomonas aeruginosa*, and all *Acinetobacter baumannii* isolates were susceptible to amikacin and colistin. Totally, 31 XDR gram-negative bacteria, including: *K. pneumonia (ssp. pneumonia* (n=20), and *ozaenae* (n=2), *Escherichia coli* (n=3), *P. aeruginosa* (n=5), *and A. baumannii* (n=1) were identified from 18 urine samples, 12 sputum specimens, and a tracheal aspirate tube.

Conclusion: The rate of XDR bacteria was high in the investigated laboratory in this study. Therefore, accurate screening and antimicrobial stewardship is recommended in different medical centers of Iran.

Keywords: Automation, Drug resistance, Gram negative bacteria, antibiotics

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1. Introduction

Many pathogenic bacteria, such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Enterococcus* spp-., and many members of *Enterobacteriaceae*, namely *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus* spp. are increasingly developing resistance **(1, 2)**. Because of the importance of drug resistance, the World Health Organization named the year 2011 "Combat Antimicrobial Resistance" to warn about the increase in resistant bacteria worldwide. Such global challenge is elevating rapidly and is life-threatening **(3-**

5). Therefore, different terms are used to categorize these bacteria, such as multiple drug-resistant (MDR), extensively drug-resistant (XDR), and pan-drug resistant (PDR) bacteria **(6)**.

According to the Centers for Disease Control and Prevention and European Center for Disease Prevention and Control, MDR bacteria are resistant to the member of three or more antibiotic families. In addition, XDR is defined as the bacteria resistant to the

members of all antibiotic families except one or two families, which are usually old antibiotics. PDR is usually referred to as *Mycobacterium tuberculosis*, which is resistant to all existing antibiotic families **(1, 7)**. Various studies showed that the early detection of gram-negative infections is crucial because of their life-threatening role and the importance of starting rapid suitable antimicrobial treatment **(8, 9)**.

VITEK 2 is a rapid, fully automated system for bacterial identification and antimicrobial susceptibility test (AST). This system uses a fluorogenic method for bacterial identification and a turbidimetric technique for susceptibility testing using a 64-well card **(10)**. The present study aimed to evaluate the frequency of XDR gram-negative bacteria isolated from different clinical samples in Payvand Clinical and Specialty Laboratory, Tehran, Iran in 2020.

2. Materials and Methods

Sample Collection

All samples, which were referred to Payvand Clinical and Specialty Laboratory for culture during March 2020-September 2020 (6 months), were included in the present study. The demographic data were submitted during sample collection. The information of all patients was kept private during data analysis and manuscript preparation. All the isolated microorganisms, including gram-positive and gram-negative bacteria, as well as isolated yeasts, were stored in a -70°C freezer in trypticase soy broth (TSB) with 15% glycerol. But based on the aims of this study only gram negative bacteria were included for further study and the remained were role out.

Bacterial Identification and AST

Bacterial identification and AST were carried out using an automated VITEK 2 system (BioMerieux, France). In this system, pure cultures are needed for bacterial inoculation preparation. Consequently, different media were used for isolation based on the clinical samples. Sheep blood agar and MacConkey agar (QUELAB, UK) were utilized for urine samples, and blood agar, MacConkey agar, chocolate agar, and Sabouraud dextrose agar (QUELAB, UK) were applied for sputum specimens. In addition, all the four mentioned media and TSB were used for tracheal tube culture **(11)**.

All inoculated plates were incubated at 37ºC for 24 h. Afterward, gram staining was performed, and using two or three pure colonies, a bacterial suspension was prepared by special PBS of the VITEK 2 system from each bacterial sample with turbidity equal to standard 0.5 McFarland (1.5×10^8 CFU/mL). The OD of bacterial and fungal suspensions by VITEK 2 spectrophotometer must be in the ranges of 0.5-0.63 and 1.8-2.2, respectively. Further dilution was executed for AST based on the manufacturer's protocol. Moreover,

especial GP, GN, N240, GN76, AST-STO3, and AST-GP75 cards were used for microbial isolation and AST. According to the protocol, all the bacterial suspensions must be used 20 min after preparation. For quality control, standard ATCC bacteria, namely *E. coli* 25922, *K. pneumoniae* ATCC700603, and *P. aeruginosa* ATCC27853 were injected into the VITEK 2 system simultaneously with the clinical samples.

Confirmation of ESBls Production by Disk Synergy Test

Flagged samples as extended-spectrum beta-lactamase (ESBLs) producers by VITEK 2 system were confirmed by manual double-disk synergy test (DDST). In order to complete the DDST, a bacterial suspension with 0.5 McFarland turbidity (1.5×10^8 CFU/mL) was prepared and inoculated to Mueller-Hinton agar. A ceftazidime disk alone and a ceftazidime-clavulanic acid disk were placed by sterile forceps at a distance of 20 mm from the center. All plates were incubated for 24 h at 37ºC. A difference of ≥ 5 mm between the diameter of the zone of inhibition around ceftazidime-clavulanic acid disk versus ceftazidime disk alone was reported as ESBL producer **(12-14)**.

Statistical Analysis

The frequency of isolated XDR bacteria, as well as resistant and susceptible bacteria, was entered in an excel file and was reported after percentage calculation.

3. Results

In the current study, 4125 urine, 34 sputum, and 1 tracheal tube samples were submitted to Payvand Clinical and Specialty Laboratory. We found that 486 urine, 32 sputum, and 1 tracheal tube specimens were positive in culture. The isolated gram-positive and gram-negative bacteria, as well as Candida species are shown in Table 1. We included the gram-negative bacteria for further analysis in this study.

Two different microorganisms were isolated from eight sputum, four urine, and one tracheal tube samples. The isolated bacteria were *K. pneuminiae ssp. pneumonia* and *E. coli* from three of four urine samples, in addition to *E. coli* and *P. aeruginosa* from the remaining urine specimens. Furthermore, two isolated bacteria from eight sputum samples included *P. aeruginosa* and *C. glabrata* from sample one, *Stenotrophomonas maltophilia* and *K. pneumonia* from sample two, *C. krusei* and *K. pneumoniae* from sample three, *P. aeruginosa* and *K. pneumoniae* (ESBL+) from sample four, *P. aeruginosa* and *K. pneumoniae* from sample five, *E. coli* and *C. glabrata* from sample six, *K. pneumoniae* and *C. albicans* from sample seven, and *P. aeruginosa* and *K. pneumonia* from sample eight. *K. pneumoniae* and *P. aeruginosa* were isolated from the only tracheal tube. The isolated bacteria are listed in Table 1. The results of AST for all gram-negative isolates are demonstrated in Table 2.

Based on the results of VITEK 2 and manual DDST, 123/265 (46.1%) *E. coli* and 10/78 (12.82%) *K. pneumoniae* isolates were ESBL-positive, respectively. Moreover, we observed that 31 (7.43%) of gram-negative isolates were XDR, namely (n=18) *K. pneumoniae* subspecies, (n=3) *K. pneumoniae* ssp. ozaenae, (n=3) *E. coli*, 6 *P. aeruginosa,* and (n=1) *A. baumannii*. The results of AST are shown in Table 2.

Out of 18 isolated *K. pneumoniae* ssp. *pneumonia*, 11 and 7 were from urine and sputum samples, respectively. All 3 *K. pneumoniae* ssp. ozaenae were isolated from urine. In addition, 2 and 1 *E. coli* isolates were from urine and sputum specimens, respectively. It was found that 2 and 6 *P. aeruginosa* isolates were from urine and sputum samples, respectively. The only *A. baumannii* isolate was isolated from urine.

Table 1. Frequency of isolated bacteria in this study

Isolated organisms/number											
Frequency of gram positive bacteria											
CoNS	*MRSA*	*MSSA*	*MR-CoNS*	*F. faecium*	*E. faecalis*	*Enterococcus spp.*	*VRE*	*GBS*	*S. dysgalactea*		**Total**
19	**9**	11	20	2	44	1	5	82	1		**194**
Frequency of gram negative Enterobacteriaceae											
E. coli	*K. pneumonia ssp (pneumonia and ozonae)*	*E. cloacea*	*P. mirabilis*	*P. vulgaris*	*E. aeruginosa*	*E. froundi*	*E. abseniae*	*C. koseri*	*S. marcesence*		**Total**
265	**78**	10	8	1	2	1	1	1	2		**369**
Gram negative non fermented bacteria											
P. aeruginosa	*Pseudomonas spp.*	*S. paucimobilis*	*A. lowffi*	*A. baummnnii*	*A. hemolyticus*	*S. maltophila*					**Total**
46	1	2	1	1	1	3					**55**
Candida spp.											
C. albicans	*C. glabrata*		*C. parapsilosis*			*Candida spp.*					**Total**
21	9		4			7					**41**

CoNs: Coagulase negative staphylococcus spp, MRSA: methicillin resistance Staphylococcus aureus, MRSS: methicillin susceptible Staphylococcus aureus, MR-CoNS: methicillin resistance coagulase negative staphylococcus spp, VRE: vancomycin resistant enterococcus, GBS: Group B streptococcus

Table.2. The results of antimicrobial susceptibility test among XDR isolated bacteria

Antibiotics	K. pneumonia ssp pneumonia			K. pneumonia ssp ozonae			P. aeruginosa			E. coli			Acinetobacter spp		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Ampicillin	100%	-	-	100%	-	-	100%	-	-	100%	-	-	-	-	-
Aztreonam	-	-	-	-	-	-	-	-	-	-	-	-	100%	-	-
Piperacillin	-	-	-	-	-	-	-	-	-	-	-	-	100%	-	-
Piperacilin/ Tazobactam	100%	-	-	100%	-	-	100%	-	-	100%	-	-	100%	-	-
cefazolin	100%	-	-	100%	-	-	100%	-	-	100%	-	-			
Cefoxitin	100%	-	-	100%	-	-				100%	-	-			
Ceftazidime	100%	-	-	100%	-	-	100%	-	-	100%	-	-	100%	-	-
Ceftriaxone	100%	-	-	100%	-	-	-	-	-	100%	-	-	-	-	-
Cefepime	100%	-	-	100%	-	-	100%	-	-	100%	-	-	100%	-	-
Ertapenem	100%	-	-	100%	-	-	-	-	-	100%	-	-			
imipenem	100%	-	-	100%	-	-	100%	-	-	100%	-	-	100%	-	-
Meropenem	-	-	-	-	-	-	80%	30% UD	-	-	-	-	100%	-	-
Ciprofloxacin	100%	-	-	100%	-	-	100%	-	-	100%	-	-	100%	-	-
levofloxacin	100%	-	-	100%	-	-	100%	-	-	100%	-	-	100%	-	-
sulfametoxazol / trimetoprime	100%	-	-	100%	-	-	-	-	-	100%	-	-	100%	-	-

Antibiotics	K. pneumonia ssp pneumonia			K. pneumonia ssp ozonae			P. aeruginosa			E. coli			Acinetobacter spp		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Tobromycin	-	-	-	-	-	-	100%	-	-	-			100%	-	-
Gentamicin	58.82%	29.41%	11.76%	100%	-	-	100%	-	-	66.6%	-	33.4%	-	-	-
Amikacin	52.9%	29.1%	18%	-	-	100%	100%	-	-	66.6%	-	33.4%	-	-	-
Nitroforantoin	63%	27%	10%	66.6%	33.4% UD	-	-	-	-	33.3%	33.4% UD	33.3%	-	-	-
Ticarcillin/ clavulanate	-	-	-	-	-	100%	-	-	-	-	-	-	100%	-	-
Colistin	6%	70.5% UD	23.5%	33.4%	66.6% UD				100%	66.6% UD	-	33.4%	-	-	100%

S: susceptibility, I: intermediate, R: resistant, UD: undetected

4. Discussion

The length of hospitalization and the rising cost of care during infection with resistant organisms, especially MDR organisms, is a global challenge (1,15). The severity of gram-negative infections is usually higher than gram-positive infections, such as bacteremia (16-18). In such situations, an immediate antimicrobial prescription is needed. However, the chance of empirical therapy, which covers most cases, is decreasing because of antimicrobial limitations (19). In the present study, 4125 urine, 34 sputum, and 1 tracheal aspiration tube samples were submitted to Payvand Clinical and Specialty Laboratory for direct examination and microbial culture. Various gram-positive and gram-negative bacteria, as well as yeasts, were isolated. Exclusively the gram-negative bacteria were included for further study in the current investigation. The AST was performed simultaneously with isolation using VITEK 2 system and special cards as mentioned above.

Based on the AST results, 46% of *E. coli* isolates and 12.82% of *K. pneumoniae* isolates were ESBL-positive. Furthermore, 31 isolates of gram-negative bacteria were confirmed as XDR, while no PDR was detected. Zhou *et al.* in 2019 reported that *E. coli* and *K. pneumoniae* might be the main gram-negative XDR bacilli (20). Similarly, *E. coli* and *K. pneumoniae* were the most frequently isolated gram-negative bacteria in the present investigation. The MDR and XDR gram-negative prosthetic joint infections were evaluated by Papadoulous *et al.* (21, 22). In their study, the prevalent gram bacilli were *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *Enterobacter* spp. However, in the current research, the detected gram-negative bacteria were more variable than the latter study. *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Enterobacter* spp. were similarly reported as the most frequent isolated bacteria from different clinical samples.

In the study performed by Mirzae *et al.*, 3248 clinical samples were collected from the Burns Center of the Northeast of Iran. They observed that 309 cases were culture-positive, with 75 and 234 specimens being positive for *P. aeruginosa* and *A. baumannii*, respectively. Most samples were from the Burn Intensive Care Unit (ICU) (60.5%) and Burn Wards (20.4%). Moreover, they reported that 16.5% and 15.53% of *P. aeruginosa* isolates and 74.75% and 73.13% of *A. baumannii* isolates were MDR and XDR, respectively. Finally, they recommended improving the prevention criteria to inhibit the spreading of XDR bacteria.

It should be noted that sampling in both studies was performed only in one center. The frequency and variation of microbial isolates in the present study were higher than the mentioned research. According to the findings of AST, 52% and 100% of *P. aeruginosa* isolates were imipenem-resistant in the study conducted by Mirzaie *et al.* and the current study, respectively. In addition, 62.7% and 100% of *P. aeruginosa* isolates, as well as 97.4% and 100% of *A. baumannii* isolates, were ciprofloxacin-resistant in the study by Mirzaei and our study, respectively. However, the frequency of *P. aeruginosa* and *A. baumannii* isolates in the current study was lower than the results of Mirzaei *et al.* Both of these investigations revealed a high resistant rate to imipenem and ciprofloxacin as common antibiotics. Susceptibility to colistin was not assessed in their study, which is one of their limitations (23).

According to Magiorakos A-P *et al.* study to investigate MDR and PDR bacteria, all or almost all suggested antibiotics in the CLSI protocol should be tested. Similarly, in the current study, all CLSI suggested antibiotics for each isolated bacteria were tested but no PDR (pan drug resistant)bacteria was detected (24).

Different studies showed that the rate of infections with gram-negative bacteria, including *Enterobacteriaceae*, *A. baumannii, P. aeruginosa*, and *S. maltophilia* is increasing in China and other countries (24-27). Furthermore, gram-negative bacteria were the most frequent bacteria in the ICU, neonatal ICU

(NICU), and Cardiac Care Unit of Saudi Arabia hospitals. It was shown that *A. baumannii* was the most prevalent isolated gram-negative bacteria in this region followed by *K. pneumonia*, *E. coli*, and *S. maltophilia* **(25-28)**. The emergence of XDR bacteria is a global challenge because of limitations in the treatment of these pathogens **(25-31)**. However, *E. coli* and *K. pneumoniae* were the most frequently isolated resistant bacteria from a teaching hospital in Sri Lanka and a tertiary care hospital in Nepal **(29, 31)**.

Finally, to understand the accurate frequency of XDR and PDR organisms, multicenter sampling is recommended in future studies. Sampling from only one clinical laboratory was the main limitation of this study. Moreover, we investigated XDR bacteria only among gram-negative bacteria. As a result, a similar evaluation of gram-positive resistant bacteria is also recommended. Such local assessments may determine whether any modifications to treatment guidelines are necessary.

5. Conclusion

The rate of XDR bacteria was high in the investigated laboratory in this study. Therefore, accurate screening based on a standard protocol, antimicrobial stewardship, and surveillance is recommended in different medical centers of Iran. In addition, to decrease antimicrobial resistance, the monitoring of MDR and XDR organisms in all clinical laboratories is recommended.

Authors contribution

This study was done in Payvand clinical and Specialty laboratory (a private clinical laboratory), Tehran-Iran, under scientific supervision and management of Dr. Behzad Poopak. The idea and study design was done by Dr. Mojdeh Hakemi-Vala (Ph.D In medical bacteriology), Dr. Hadi Rezaei (Ph.D In medical bacteriology) and Dr. Behzad Poopak (Ph.D in hematology, Doctorate in Clinical Laboratory Sciences). Routine sampling was done based on the physician's request and standard protocols. The practical parts including: bacterial isolation, identification and processing of Vitek 2 system was done by MS. Sepideh Ghasemshahi and Mr. Mohammad Ahmadpour under supervision of Dr. Hadi Rezaei, head of department of microbiology, Payvand Clinical and Specialty laboratory. All demographic and practical data registration including age, gender, background diseases and bacterial reports was done by Mrs. Aazam Booskabadi. Data analysis and draft preparation of the recent paper was done by Dr. Mojdeh Hakemi-Vala.

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