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The Frequency of *Klebsiella pneumoniae* Strains Producing Extended-Spectrum Beta-Lactamases (ESBL) with Phenotypic and Genotypic Methods in Hospitals of Ardebil

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***Klebsiella pneumonia* is an opportunistic pathogen which causing important nosocomial infection such as urinary tract infections, pneumonia, septicemia and soft tissue infections. Studies have shown that today in the world *K. pneumoniae* strains with multiple antibiotic- resistance are growing quickly and continuous use of antibiotics and selective pressure caused by these factors cause resistance to these category of antibiotics in bacteria.In this study fromApril, 2014 to November, 2014120 isolated of *Klebsiella pneumoniae* were isolated from hospitalized patients in Ardabil. Antibiotic susceptibility pattern of isolates toward isolates, at first in term of producing Beta-lactamase wide range were screenedaccording to CLSI recommendations and through disk agar diffusion method. Then Candida isolated producing Beta-lactamase wide range was confirmed with combined disk method in term of producing ESBL. Positive isolates in term of producing ESBL by PCR for the presence of genes blaTEM, blaSHV, blaCTX-M-1, blaCTX-M-2, blaCTX-M-8 and blaCTX-M-9 were examined. A total of 120 isolates of *Klebsiella pneumonia*, 79 isolates had reduced susceptibility to the antibiotic of screening phase that 61 isolates (50.8%) were positive for ESBL production. The frequency of genes blaTEM, blaSHV, bla CTX-M-1 was in order 34 isolates (55.7%), 24 isolates (39.3%), 47 isolates (77.04%) and in none of the isolates genes of blaCTX- M-2, blaCTX-M-8 and blaCTX-M-9 was observed. According to high prevalence of isolatesproducing wide range beta-lactamases in the studied hospitals, primary identification of resistant isolates and following them in order to prevent their prevalence is more important. Also applying appropriate treatment strategies and proper and logical prescription of anti-biotic by doctors is also important to control them.**

Key word: *Klebsiella pneumoniae* strains, Phenotypic, genotypic, extended-spectrum beta-lactamases.

Klebsiella pneumonia is the most important pathogenic species in the Klebsiella genus. In recent years, Klebsiella species are considered as the most important pathogens in hospital infections[1] *Klebsiella pneumonia*e is also acquired pathogenic potential pathogens of society[2]. Gram-negative bacteria are resistance to antibiotics due to ESBL production which is related to increase of death, Prolongation of hospital stay, and increasing hospital costs[7]. In human Klebsiella species are on skin, throat or gastrointestinal tract. The bacterium is settled in sterile wound of urine and may be considered as normal flora of the small intestine and bile ducts[3]. *Klebsiella* transfer from a patient to a patient through contaminated medical devices, contaminated hands of hospital personnel and blood product, while the areas causing infection by *Klebsiella* species are surgical wounds, the peritoneum, and the place of entering catheter, urinary tract, gastrointestinal tract and biliary tract[4]. Hospital acquired pneumonia is a severe disease with high mortality associated with fast invasion, high fever, Hemoptysis and observable abscess on chest radiography. The rate

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of death is about 25 to 50%. The society acquired bacteremia is usually occurring in urinary tract infections, vascular catheter infection and inflammation of the bile ducts[5]. The death rate among patients with immunosuppression or diabetes is about 50-100%[6]. In addition, Klebsiella species, particularly *Klebsiella pneumonia*e has been shown that intra-abdominal infections caused by the production of toxins which are sensitive and resistant to heat[7].

Main objective

The main objective was to determine the frequency of resistant strains of *Klebsiella pneumonia*e producing extended-spectrum beta lactamase (ESBLs) through Phenotypic and genotypic methods in hospitals of Ardebil.

Specific objectives

1. Determine the frequency of resistant strains of *Klebsiella pneumonia*e producing extended-spectrum beta lactamase (ESBLs) through phenotypic and genotypic methods in hospitals in Ardabil (Razi, Kowsar, Qods, Bu-Ali and Rajai).
2. Determine the frequency distribution of strains producing extended-spectrum beta lactamase (ESBLs) with separation of different hospital departments
3. Determine the frequency distribution of strains producing extended-spectrum beta lactamase (ESBLs) with separation of type of clinical sample.

In a study Mousavian and et al (2011) in Ahvaz from 420 collected isolates from Enterobacteriaceae, 84 subjects (20%) were *Klebsiella pneumonia* that 45.4% produced extended-spectrum beta lactamase (ESBLs) with phenotypic method, 48.5% had the TEM gene and 23% had SHV alone. 28.5% had TEM and SHV genes concurrently. None of the isolates had CTX-M gene. Also 20.5% of isolated lacked all three examined genes[8]. In a study conducted by Bali and et als (2010) in Turkey, from 17 isolates of *Klebsiella pneumonia* from 15 (88.23%) were producing ESBLs in phenotypic method[9].

In a study conducted by Liu and et al (2009) in china, from 425 collected Enterobacteriaceae isolates 110 isolates of *Klebsiella pneumonia*e was identified. In phenotypic study of 47 isolates (42.72%) were ESBLs positive that in comparison other isolates of the Enterobacteriaceae had the highest resistance in this species. 20 isolates (42.5%) had CTX-M-1 and 14 isolates (29.78%/) had CTX-M-9[10]

In a study by Nasehi and et al (2010) in Tehran, 80 collected *Klebsiella pneumonia*e isolates were examined through combined disk method that 77 isolate (96%) had ESBLs. In Genotypic method isolates had in order 26%, 18%, and 24.5% had SHV, TEM and CTX-M genes[11]

In a study conducted by Oliveira et al. 2010 in Brazil, in phenotypic method of combined disk 71.9% of isolates had ESBLs that from 64 isolates 50(78.1%) were producer of extended-spectrum beta lactamase of SHV type[12]

Research Method

This was a descriptive study and 120 samples of *Klebsiella pneumonia*e isolates, from different departments of Ardebil hospitals collected during April, 2014 to November, 2014.

Bacterial isolates were collected from clinical samples of urine, tracheal, wound and blood. Clinical samples in hospitals laboratory were cultured in both medium based on Macconkey *agar and* Blood *agar, after growth, the cells were passaged.*

After 24 hour incubation at 37 ° C, the cells through microbiological and biochemical tests to identify the species of *Klebsiella pneumonia*e were studied.

Diagnostic phenotypic tests

Biochemical tests for the identification of *Klebsiella pneumonia*e were used as follows:

1. Culturing on on Macconkey agar medium
2. Gram staining and microscopic examination
3. Oxidase test
4. Culturing TSI medium
5. Indole Test (SIM medium)
6. Methyl red Test (culturing on MR-VP medium)
7. VP-Voges-. Proskauer. Test (culturing on MR-VP medium)
8. Citrate Test (culturing on citrate Simon medium)
9. Urea test (culturing on urea agar medium)
10. Motion test (culturing on SIM medium)

After identification of isolates, in order to keep bacteria for long time, first we culture them into the vial containing theTrypticase Soy Broth (TSB Broth) after incubation at 37 ° C, if the bacteria

grew one or two drops of 20% sterile glycerol is added and then they were stored in the freezer -70 ° C until performing tests.

Phenotypic study of presence of extended-spectrum beta-lactamases (ESBL) was done in in two stages:

ESBL antibiotic screening test

To test the International Institute for Laboratory Standards (CLSI) was used as follows:

1. First, the Mueller Hinton agar medium was prepared and the pH was adjusted 7.2 to 7.4. In order to control the infection these plates were incubated for 24 hour at 35 ° C.
2. In the next stage to do the test, containers of antibiotics disks such as cefotaxime, ceftriaxone, ceftazidime, cefpodoxime and aztreonam were transferred from -20 ° C freezer (long term care) into 4 ° C refrigerator (short term storage)

Also a few minutes before test, containers of disk were placed in the laboratory to reach room temperature. Antibiotic disks were purchased from the MAST Company England. Then the standard microbial suspensions were prepared for testing. Since for the suspension, the strains that more than 24 hours of their culture passed are used, therefore, samples were cultured on plain agar medium then incubated at 35 ° C for 24 hours. Some of the colonies are transferred to the tube containing 2 ml of sterile saline and after mixing with a mixer, the obtained turbidity of suspension with McFarland half turbidity was adapted. Sterile cotton swab dipped into the prepared bacterial suspension and after pressing the swab into the side wall of the tube to drain excess fluid, on the Hinton agar medium was grass cultured and through changing the culture angel and rotating the swab in all surfaces, we culture three times. 15 minutes after inoculation of suspension, the mentioned antibiotics disks that reached the room

Table 1. Number of isolates with separation of Ardebil hospitals

Hospital	Number of isolate	Percent %
Booali	41	34.1%
Alavi	40	33.3%
Imam Khomeini	39	32.5%
Total	120	100%

Table 2. The isolates resistant to antibiotics in screening stage

Antibiotic	Number of resistant isolate	Percent%
Ceftazidime	72	60%
Cefotaxime	79	65.8%
aztreonam	67	55.8%
Ceftriaxone	71	59.1%
cefpodoxime	78	65%

Table 3. Frequency of *Klebsiella pneumoniae* isolates producing ESBLs in separate departments of the hospital

	Department			
	Special care	Infants	Internal	Total
ESBL positive	4370.4%	1118.2%	711.4%	61
ESBL Negative	1016.9%	1728.8%	3254.2%	59
total	53	28	39	120

Table 4. Frequency of Klebsiella pneumoniae isolates producing ESBLs according to type of clinical samples

	Type of clinical samples				
	Urine	Wound	Trachea	Blood	Total
ESBL positive	2134.4%	1016.3%	2642.6%	46.5%	61
ESBL Negative	4474.5%	35.08%	610.1%	610.1%	59
Total	65	13	32	10	120

Table 5. Frequency of ESBLs-producing genes

genotypes ESBLs	Number of isolates	Total
bla_{TEM}	6	9.8%
bla_{SHV}	3	4.9%
$bla_{CTX\text{-}M\text{-}1}$	14	26.22%
$bla_{TEM} + bla_{SHV}$	5	8.1%
$bla_{TEM} + bla_{CTX\text{-}M\text{-}1}$	15	24.5%
$bla_{SHV} + bla_{CTX\text{-}M\text{-}1}$	8	13.1%
$bla_{TEM} + bla_{SHV} + bla_{CTX\text{-}M\text{-}1}$	8	13.1%
ãÌãæÚ	61	100%

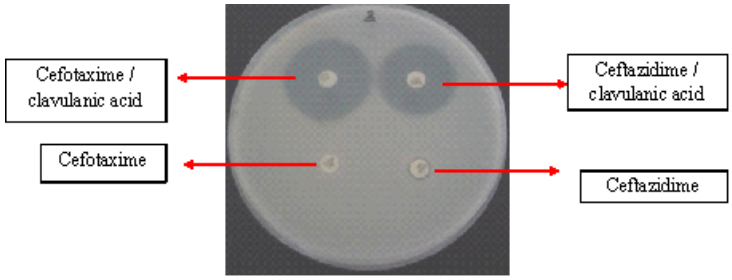

Fig. 1. Confirmatory test of producing ESBLs in combination disk method, desired sample is positive for production of ESBLs

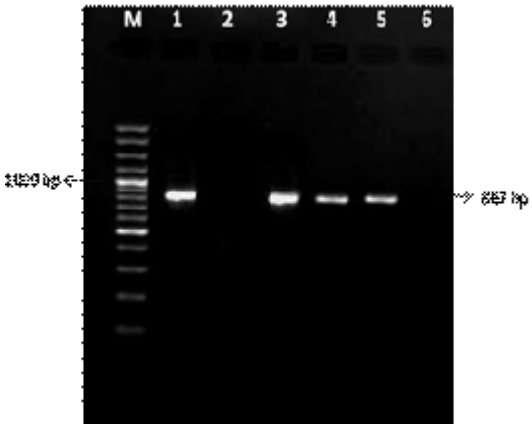

Fig. 2. PCR product of blaTEM gene; M: DNA marker, 1: positive control of E coli ATCC35218. 2: negative control E coli ATCC25922, 4-3: Clinical isolates producing ESBL, 5: negative clinical isolates of ESBL, 6: reaction without DNA template

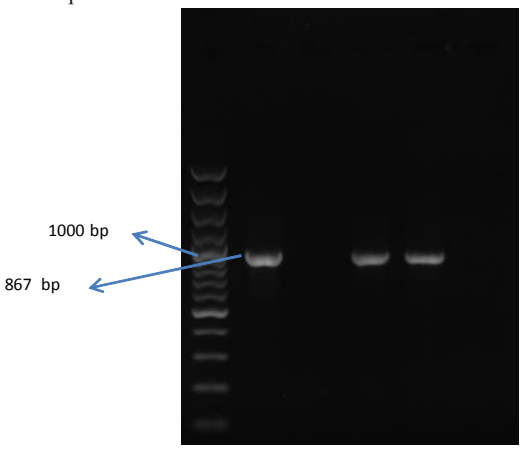

Fig. 3. PCR product blaCTX -M-1; M: DNA marker, 1: control isolates confirmed positive, 2 negative control E coli ATCC25922, 3 and 4 clinical isolates of gene blaCTX -M-1, 5: Control Test (RT-PCR without DNA template)

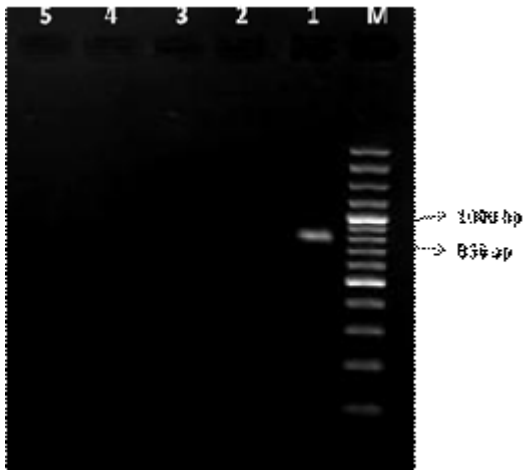

Fig. 4. PCR product blaCTX-M-2; M: DNA marker, 1: control isolates confirmed positive, 2 negative control E coli ATCC25922, 4 and 3 negative clinical isolates of gene blaCTX-M-2, 5: Control Test (RT-PCR without DNA template)

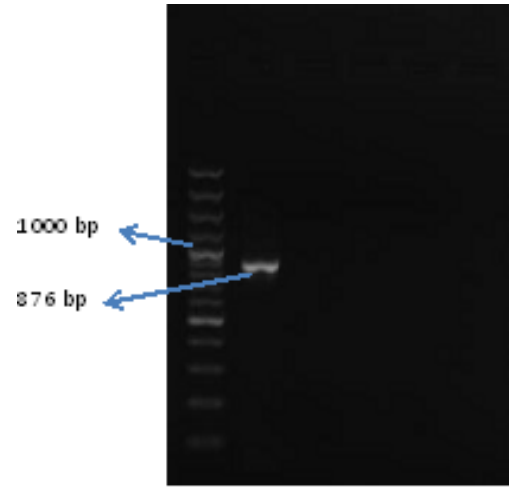

Fig. 5. PCR product blaCTX-M-8; M: DNA marker, 1: control isolates confirmed positive, 2 negative control E coli ATCC25922, 3 and 4 negative clinical isolates, 5: Control Test (RT-PCR without DNA template)

temperature on the plate for at least 2-2.25 cm from each other and from the edge of the plate were placed.

After placing disks, Plates were incubated for 24 hour at 35 ° C. then by a ruler the diameter of the growth inhibition zone around each disk measured results were recorded in prepared forms.

6. According to the CLSI instructions, Isolates that the diameter of the growth inhibition zone of ceftazidime disk 22ml ≥ (30µg), Ceftriaxone disk 27ml ≥ (30µg)

Cefotaxime disk 27ml ≥ (30µg) aztreonam disk 25ml ≥ (30µg) and Cefpodoxime disk 27ml d" (10µg) is as a potential ESBL-producing organism. And ESBL confirmatory test should be conducted for these isolates.

Confirmatory test of producing ESBL

In the following, confirmatory test of production of extended-spectrum beta-lactamases with combination disk (CD) through Ceftazidime disks (30 µg) and Ceftazidime (30 µg)/ clavulanic acid **(**10 µg) Cefotaxime (30 µg) and Cefotaxime (30 µg) / clavulanic acid **(**10 µg) was done. Results were interpreted according to CLSI guidelines. Thus if in a isolate the diameter of the growth inhibition zone of combination disk equal to A ≥ 5 ml Compared to diameter of the growth inhibition zone of disk alone in term of producing the ESBL is considered positive.

In confirmatory test, strains of E.coli ATCC 25922 used as negative control and *Klebsiella pneumonia* ATCC 700603 strain was used as a positive control.

Antibiotic susceptibility test for cefepime and imipenem by using Disk Agar Diffusion (DAD) was performed.

Isolation of genes blaTEM, blaSHV and blaCTX-M (CTX-M-1, -2, -8, -9, groups)

In order to determine the frequency of extended-spectrum beta-lactamases (ESBL) genes, blaTEM, blaSHV and blaCTX-M (CTX-M-1, -2, -8, -9, groups) specific primers were used as shown in Table 3, that with desired gene amplification with conditions that will be mentioned and finally the presence or absence of products with agarose gel electrophoresis determined. In PCR test *E.coli* ATCC 25922 strain used as a negative control, and strains of *E.coli* ATCC 27853 (positive control of blaTEM gene), *Klebsiella pneumonia* ATCC 700603(positive control of $bla_{\text{SHV gene)}}$, and confirmed isolate coding $bla_{\text{CTX-M}}$ (CTX-M-1,-2,-8, groups) genes were used as positive control.

Molecular testing processes include

1. DNA extraction
2. The preparation of primers
3. The PCR test
4. Electrophoresis

Research Findings

Phenotypic study of the presence of extended-spectrum beta-lactamases

Antibiotic screening test for ESBLs

In total among 120 isolates of *Klebsiella pneumoniae* after screening test ESBLs, 79 isolate (65.8%) showed reduced sensitivity to the antibiotics used in the screening test of ESBLs. As it is shown in table 8, the highest resistance to cefotaxime was 65.8% and cefpodoxime was 65%.

Confirmatory test of producing ESBLs

As you can see in the Figure 6, based on CLSI instruction and using the combined disk method to confirm the production of ESBLs, 61 isolates (50.8%) were positive for production of ESBLs. Isolates producing ESBLs were often "of patients admitted to the ICU with 43 isolates (70.4%) (Table9) and also often from the trachea samples with 26 isolates (42.6%) and urine with 21 isolates (34.4%) isolated (Table10).

Antibiotic susceptibility test results towards cefepime and imipenem antibiotics were so that 17 isolates (14.2%) had intermediate resistance to imipenem and 47 isolates (39.1%) were resistant to cefepime.

Isolation Results of genes encoding ESBLs

Through performing PCR test on the ESBLs-producing isolates, the frequencies of involved genes in the production of ESBLs were measured (Table 11). As shown in Table 11, CTX-M-1 has allocated the most frequency of the involved genes in the production of ESBLs in isolates producing ESBLs. Also, as shown in Table 12 BlaCTX-M-1 gene frequency in the intensive care unit had higher rate (61.33) than other departments. Figures 7, 8, 9, 10, 11, in order are genes electrophoresis blaTEM, blaSHV, blaCTX-M-1, blaCTX-M-2 and blaCTX-M-8.

CONCLUSION

Because isolates producing ESBLs are resistant to antibiotics available today and can

transfer resistance factors to other bacteria, rapid diagnosis of these isolates in microbiology laboratories is important. Molecular detection and phenotypic detection of isolates provide reliable epidemiological research in order to cope with the isolates. The results of this study suggest that significant amount of *Klebsiella pneumonia*e isolates producing ESBLs alongside similar studies worldwide indicate that organisms causing ESBLS in different departments of the hospital are increasing. Change in strategy of antibiotics use and using proper infection control devices in departments that patients are hospitalized for long periods particularly such as ICU important factor that can partially play role to control the spread of ESBLs-producing organisms.

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