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Frequency of Prevalence of *Klebsiella pneumoniae* in Clinical Samples and the Evaluation of the Role of Efflux Pump in Determining Antibiotic Resistance

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Abstract

In this study 193 *Klebsiella pneumoniae* were isolated from urinary tract infections, ulcers, sputum and blood. Initially, Mac agar medium was used to isolate the bacterium, and for each suspected isolate, pink and aqueous colonies were stained and biochemical tests of catalase, oxidase, TSI, IMVIC Test and urase were performed. Confirmation of the isolates using 16SrRNA sequencing Some isolates are evaluated. Then all isolates evaluated for sensitivity to antibiotics such as Ampicillin, Amoxicillin clavulanate, Piperacillin, Cefoxitin, Cefuroxime Imipenem, Tetracyclines, Nitrofurantoin, Polymyxin B Colistin, they use disk diffusion test. In the process, the presence of the acr efflux pump gene is confirmed by using an specific primer namely Acr primer, and finally, using phenylalanine-arginine- beta naphthylamide inhibitor, the relationship between antibiotic resistance and efflux pump function is evaluated. Overall, 50.2% of the collected samples contained *Klebsiella*. Thus, 193 of 384 clinical specimens contained *Klebsiella*. Of the 193 positive samples, the groin lesions had the highest percentage and the abscess had lowest percentage of *Klebsiella* infection, although *Klebsiella* was significantly separated from the throat, sputum, catheter and foley. Antibiotics, cefazolin, ceftazidime, ciprofloxacin, Chloramphenicol, tetracycline had higher antibacterial activity. Results were analyzed by Whonet 5 and SPSS software.

Keywords: *Klebsiella pneumoniae*, *Enterobacteriaceae*, efflux pump, drug resistance

Introduction

Klebsiella, a gram-negative bacterium, belongs to the family Enterobacteriaceae. This bacterium was isolated by Carl Friedlander from the lung of a patient with pneumonia and is called Bacillus Friedlander, a severe and deadly pneumonia agent. Like other immobilized *Klebsiella* species and produces a polysaccharide capsule (Mphba, 2005).

Morphology and cultivation characteristics

The members of the genus *Klebsiella* are shorter and thicker than other members of the Enterobacteriaceae family and are about 1–2 μm long and 0.8–0.8 μm wide. They are seen as placenta or single and sometimes resemble diplobacillus pneumococcus. It produces a large capsule in environments that contain carbohydrates and no nitrogen. *Klebsiella* strains have type 1, 2 and 3 fimbriae. This bacterium disappears for 30 minutes at 55C (Mphba, 2005; Hsueh *et al.*, 1977).

These organisms are positive for urease, lysine decarboxylase and negative for tests of ornithine decarboxylase, arginine dehydrolase, and indole production. The VP test is positive. This organism is acidic in the TSI medium, without hydrogen sulfide gas production as well as pyrolysis. Growth of this bacterium, although optional anaerobic, is weak under absolute anaerobic conditions. Lack of ability to lysis red blood cells on blood agar medium. The optimum growth temperature of this organism is 37 ° C (Forbes *et al.*, 2007).

Almost all grow in citrate and molar Hinton containing KCNs and their G + C is 52-58%. These organisms are part of the natural microflora of the intestine, stomach, and human respiratory tract, and about one-third of them are carriers of this bacterium. These bacteria are one of the most important causes of nosocomial infections and cause a wide range of infections, including septicemia, pneumonia, urinary tract infection, meningitis, and purulent abscesses (especially liver abscesses) (Mphba, 2005).

Klebsiella pneumoniae clinical isolates are divided into four groups based on nucleotide changes in the gyrA, parC and rpoB genes, including KP I, A-KP II, KP II-B, KP III (Alves *et al.*, 2006).

Klebsiella is the only genus in the Enterobacteriaceae family that has the ability to stabilize nitrogen in the air and convert it to ammonia and amino acids. Another

metabolic product of this bacterium is 1,3-propanediol, which is produced under microaerophilic conditions (Huang *et al.*, 2002).

Pathogenic factors

Five factors have been proposed for *Klebsiella* pathogenesis, including capsular antigen (as the most important virulence factor in *Klebsiella pneumoniae*), adhesives, lipopolysaccharide, siderophore, *Klebsiella pneumoniae* (Struve *et al.*, 2009; Campos *et al.*, 2004).

Typing methods of *Klebsiella pneumoniae*

Klebsiella pneumoniae typing methods are either based on phenotypic characteristics called phenotyping or based on genotypic characteristics called genotyping. Phenotyping, which includes serotyping, biotyping, bacteriocin typing, phagotyping, and antibiograms, can generally be said to lack complete differentiation, reproducibility, and typing. Genotyping examines the differences and similarities of DNA sequences that are called Mass-Sequencing Technologies if they look at specific parts of DNA, and Whole Genome Sequencing, if they look at the entire DNA sequence. Some Fingerprinting methods are currently considered as the most suitable methods for genotyping microorganisms for epidemiological purposes. These include PFGE, ribotyping and some PCR-based typing techniques such as RAPD, AFLP, VNTR. All of these methods use an electric field to separate DNA fragments (Shakil *et al.*, 2008).

Pathogenicity of *Klebsiella pneumoniae*

The strains of *Klebsiella pneumoniae* that cause pneumonia are usually capsular serotypes 1 and 2. The cases are in people of middle age or older who have underlying medical problems, such as chronic bronchopulmonary disease, diabetes, or alcoholism. *Klebsiella pneumonia* in about 25 to 75% of patients produces a thick bloody sputum that does not smell disgusting. In this pneumonia, necrosis and abscess are more likely than other bacterial pneumonia and blood cultures are positive in 25% of patients. *Klebsiella pneumoniae* strains are particularly resistant to many drugs and generally cause more severe disease than streptococcal pneumonia. Even with proper treatment, the mortality rate of *Klebsiella* pneumonia is 40 to 60 percent. *Klebsiella pneumoniae* is one of the causes of urinary tract

infections, wound infections, bacteremia and meningitis (Cortés *et al.*, 2002). Pneumonia caused by *Klebsiella* spp often causes necrotic destruction of alveolar spaces, produce cavity formation and pale bloody sputum. The organism's ability to cause disease due to decreased host defense is due to complex and prolonged surgical procedures as well as increased use of different drugs (Lin *et al.*, 2004). In humans *Klebsiella* species are found on the skin, pharynx or digestive tract. The bacterium is found in sterile wounds, urine, and may be considered normal flora of the small intestine and bile ducts (Campos *et al.*, 2004). *Klebsiella* is transmitted from one patient to another through contaminated medical devices, contaminated hands of hospital staff, and blood products, while areas causing infection by *Klebsiella* spp., Surgical wounds, peritoneum, catheter insertion sites, Urinary ducts, gastrointestinal and biliary tracts (Wilson's, 2006). *Klebsiella pneumoniae* is the cause of urinary tract infections, neonatal arthritis, meningitis, wound infections, nosocomial pneumonia, bacteremia, septicemia, and soft tissue infections (Lin *et al.*, 2004). Hospital-acquired pneumonia is a severe disease with high mortality associated with rapid invasion, high fever, bloody sputum and visible abscesses on chest radiographs. The mortality rate is about 25 to 50% (Campos *et al.*, 2004). *Klebsiella pneumoniae* cause syndrome which is the reason for septicemia with liver abscesses, which brings about 10 to 40% of deaths. K1 capsular serotype is the dominant serotype of *Klebsiella pneumoniae* in the development of hepatic abscesses (Williams *et al.*, 1983). Various underlying diseases including diabetes, alcohol overuse, bile duct disease, malignancies, liver cirrhosis, kidney disease, intra-abdominal infections, history of steroids, history of abdominal surgery and history of antibiotic use (Roberts *et al.*, 1989), all are risk factors for liver abscess infection due to the K1 capsular serotype in *Klebsiella pneumoniae* (Cbat, 1989). *Klebsiella pneumoniae* as a common pathogen causing bacterial endophthalmitis of intrinsic origin associated with liver abscess in Asia. *Klebsiella pneumoniae* K1 capsular serotype has the ability to cause central nervous system infection as a complication of purulent liver abscesses in patients. *Klebsiella pneumoniae* is also associated with chronic diarrhea in HIV-positive people (Hbjak *et al.*, 1987).

Antibiotic Resistance Mechanisms in *Klebsiella*

Antibiotics are natural products or synthetic organic compounds that usually inhibit or destroy certain bacteria at low concentrations. Antibiotics are divided into 5 categories in terms of function. 1) Cell wall synthesis inhibitor 2) Protein synthesis inhibitor 3) Interference with nucleic acid synthesis 4) Inhibition of metabolic pathway 5) Degradation of membrane structure.

Resistance to antibiotics

Resistance to antibiotics is applied in two ways. 1. Genetic dependent 2. Non-genetic dependent. Genetically dependent resistance is either dependent on the chromosome or the plasmid (Izard *et al.,* 1981).

Drug resistance dependent on *Klebsiella* efflux pump

Nowadays one of the mechanisms of drug resistance in bacteria is the existence of efflux pump in bacteria. efflux pumps are transfer protein like toxins and antibiotics from the cell to the environment (Izard *et al.,* 1981). In recent years, *Klebsiella* drug-resistant strains have been widely observed, and resistance to quinolones is generally one of the most important object. On the other hand, the mechanism of resistance to quinolone antibiotics reports the presence of efflux pumps in these bacteria (Bagley *et al.,* 1981). A common feature of all efflux systems is the ability to remove a variety of antibacterial substances such as antibiotics, biocides, dyes, cleaners, fatty acids and organic solvents (Ferragut *et al.,* 1983). Efflux pumps reduce the concentration of drug inside the cell and create resistant strains of bacteria by increasing the lowest inhibitory concentration (MIC). In bacteria, there are five families of efflux pumps that are divided into two groups according to the energy acquisition method for substrate transport: first-class pumps that use ATP energy for transport and only include ABC pumps or (cascade-dependent proteins to ATP). Second-class pumps use the gradient energy of the proton concentration to transport the substrates (Sakazaki *et al.,* 1989). The five major classes of efflux pumps are the MFS or Major facilitator superfamily, the SMR or Small multidrug resistant family, the RND or Resistant nodulation division family, the MATE or Multidrug and toxic efflux family, and the ABC or ATP binding cassette family (Gavini *et al.,* 1983). Efflux pumps are either single or triple. One-part efflux pumps direct the protein out of the bacterial cell after protein absorption, while three-part efflux pumps excrete it directly after the drug is absorbed (Ferragut *et al.,* 1983; Drancourt *et al.,* 2001; Alves *et al.,* 2006).

Multidrug resistance (MDR) is a bacterial pathogenic ability to withstand deadly doses of structurally diverse drugs capable of eradicating non-resistant strains. MDR is recognized as a major threat to human health by the World Health Organization. Of the four general mechanisms that cause antibiotic resistance, including target transformation, drug inactivation, permeability reduction, and increased efflux activity, efflux pump removal, which acts as an important MDR mechanism.

The efflux pump can not only remove a wide range of antibiotics due to its multi-substrate property, but also provide additional resistance mechanisms that reduce intracellular antibiotic concentration and increase mutation accumulation. The

over-expression of multi-drug efflux pumps is increasingly indicative of clinical drug resistance. On the other hand, the evidence gathered indicates that efflux pumps have physiological function in bacteria and that their expression is in tune with different types of environmental and physiological signals (Bsgfg, 2006).

Mechanism of drug secretion by efflux pump

Efflux pumps are prominent in terms of their high drug efficacy and broad substrate properties, due to their multidrug resistance. The fact that all bacterial genomes contain the efflux pump gene, particularly in stressful situations, development, pathogenesis and contagion, their expression is closely monitored by a number of local and global transcription regulators. It has been suggested that drug efflux pumps have physiological function. The evidence gathered suggests that efflux pumps actually play a general role in detoxification in different physiological processes of bacteria.

Although efflux genes are distributed throughout bacterial genomes, with the exception of a few native efflux systems, most of them are under strict control by different transcriptional regulators and their role is to facilitate the adaptation of bacteria to specific stimuli.

Given the capacity of efflux pumps for a wide range of indirect structural chemicals, it is predictable that over-expression of efflux pumps may lead to unintended withdrawal of metabolites or other signaling molecules, resulting in deleterious effects, affects cellular physiology.

Therefore, expression of efflux pumps is usually well regulated and expressed only at low levels under normal and normal growth conditions. Although the composition and performance of MDR, efflux pumps are relatively conserved in different species, their regulatory mechanism is significantly different.

Given the high importance of *Klebsiella pneumoniae* multidrug resistance in the treatment of infectious diseases as well as the strong role of oqxAB efflux pumps in the development of this type of resistance, we aimed to investigate the frequency of efflux pump-dependent antibiotic resistance in isolates. Clinical *Klebsiella pneumoniae* isolated from patients referred to Shiraz hospitals.

Literature review

In a study by Ziqing Deng and colleagues in 2014 on the efflux pump, they concluded that the efflux pump can not only remove a wide range of antibiotics due to its Poly-substrate property, but also gain mechanisms. Additional resistance is also found, which reduces intracellular antibiotic concentration and increases

mutation accumulation. Overexpression of multi-drug efflux pumps is increasingly associated with clinical drug resistance. On the other hand, the evidence gathered indicates that efflux pumps have physiological function in bacteria and that their expression is in tune with different types of environmental and physiological signals (Sun *et al.,* 2014).

In 2007 According to study by the Department of Microbiology and Immunology at Kingston University, the efflux pump is found in almost all bacterial species, and the genes of this type of proteins are put on chromosomes or plasmids. Membrane scattered regions, energy sources and substrates, bacterial efflux pumps are divided into five families: RND family, large facilitator family (MFS), ATP family (adenosine triphosphate), family (ABC), small multiple family (SMR) (Poole, 2007; Piddock, 2006).

In a 2013 study by Jadwiga and Anna, apart from the RND family found only in gram-negative bacteria, the output systems of the other four families MFS, ABC, SMR and MATE are widely used in both bacteria. Gram positive and negative are distributed (Handzlik *et al.,* 2013).

In a 2004 study by Nikaido, Efflux pumps, which are single-component transducers or multicomponent systems, include not only an inner membrane receptor but also an outer membrane channel and a pre-plasmatic adapter protein similar to the efflux pumps such as RND (Li & Nikaido, 2004).

According to a 2006 study by the University of Birmingham, RND family pumps are widely used, due to tripartiite compounds that directly release various drugs from the cytosol or periplasmic space out of the bacterial cells. It is known with significant antibiotic resistance, such as AcrB in Escherichia coli, Salmonella typhimurium, and MexB in Pseudomonas aeruginosa. In gram-positive bacteria, efflux pumps are clinical members of the MFS family (Piddock, 2006).

In 2013 at Rakuno Gakuen University in Sapporo, Japan on 50 clinical specimens obtained on *E.coli*, isolated from human clinical specimens and canine feces, show a strong correlation in overexpression of AcrAB pumps with It is highly resistant to fluoroquinolone (Sato *et al.,* 2013).

In another study conducted by Dr. Pakzad et al. (2013) at the University of Ilam on the *Klebsiella pneumoniae* strain, 52 of the 40 patients who were hospitalized at Shahid Motahari Hospital in Tehran, all 40 isolates of ciprofloxacin, tetracycline, Ceftazidime and gentamicin showed high levels of AcrAB efflux pump, especially in ciprofloxacin-resistant strains (Pakzad *et al.,* 2013).

Also, a 2012 study by Wayne State University in the United States reported a relationship between over-expression of the efflux pump with appropriate clinical MDR in Gram-positive bacteria. Among the several hundred clinical specimens of S. aureus isolated by Christos et al., It was found that overexpression of the pump gene was geographically widespread. These strains were predominantly resistant to methicillin and their resistance was correlated with norA and mepA expression (Kosmidis *et al.*, 2013).

According to the findings, at the University of Birmingham in 2006, the Efflux pump is prominent in terms of its high drug delivery efficiency and its broad substrate properties, due to its multidrug resistance (Piddock, 2006).

In 2015, Jing Sun et al., Realized that all bacterial genomes contained the efflux pump gene, Espicially under stressful conditions, development, pathogenicity, and contagion, their expression being closely monitored by a number of Local and global transcriptional regulators have suggested that medicinal efflux pumps have physiological function. Evidence was collected that efflux pumps actually play a general detoxification role in different physiological processes of bacteria (Zou *et al.*, 2015).

According to a study done at the Department of Microbial Biotechnology in Madrid in 2004, although efflux genes are distributed everywhere in bacterial genomes, with the exception of a few autophagy systems, most of them are under strict control by different transcriptional regulators. And their role is to facilitate the adaptation of bacteria to specific stimuli (Alonso *et al.*, 2004).

According to a 2009 study by the School of Microbiology and Infectious Diseases at the University of Saka, Japan, given the efflux pump's capacity for a wide range of indirect structural chemicals, it is predictable that over-expression of the efflux pumps may be Lead to the unwanted secretion of metabolites or other signaling molecules, which in turn has deleterious effects on cellular physiology. Therefore, expression of efflux pumps is usually well regulated and expressed only at low levels under normal growth conditions. Although the composition and performance of MDR efflux pumps are relatively conserved in different species, their regulatory mechanism is significantly different (Nishino *et al.*, 2009).

Methodology

In this study, SPSS software version 20 “Statistical Package for the Social Sciences” and the statistical program, Student's t-distribution and Chi *Square* were used to analyze the data.

Collection of clinical specimens

The following statistical formula was used to determine sample size:

$$n = \frac{z^2 pq}{d^2}$$

where in;

z: The value of the standard normal variable is 95% and is 1.96

p: A proportion of people in the community who had the desired attribute were considered to be 0.5.

q: A proportion of people in the community who did not have the desired attribute were considered to be 0.5.

d: And the error value is equal to 0.05.

Due to the above relation, sample size is calculated as follows: 384

A total of 384 specimens, from those who had clinical symptoms, the sampling was done randomly included pharyngitis patients swabs, sinus secretions of patients with chronic sinusitis, secretion of middle ear infections patients, pulmonary secretions of patients with respiratory failure who were admitted to the intensive care unit, and samples of urinary tract infections, in Dena and Namazi hospital were collected. The sampling period lasted from 3 months from May 2019 to July 2019

Phenotypic isolation and identification of clinical specimens

Klebsiella pneumoniae isolates are Gram-negative bacilli, oxidase negative and lactose positive which in acidic and yellow environment in TSI medium acidic and yellow which are associated with gas production. In terms of endolysis and MR test negative and VP reaction and citrate test positive. These organisms are positive for the lysine decarboxylase test and are generally immobilized and their urea hydrolysis test is also positive.

Preservance Preservation of *Klebsiella* strains

After identifying the isolates for long-term storage, the bacteria were first cultured in vials containing TSB medium and incubated at 37 ° C with 20% sterile glycerol

and then incubated until the tests were performed. The culture was stored in a -70 ° C freezer (BMB, 2012).

Confirmation of the identification of *Klebsiella* by molecular method

To confirm the phenotypic identification, 5 isolates were selected by chance and evaluated using 16SrRNA primer.

Determination of susceptibility of *Klebsiella* isolates to antibiotics by disk diffusion method

The test was performed using the Kirby Bauer standard method according to the International Laboratory Standards Institute (CLSI) guidelines. Muller Hinton Agar medium, antibiotic discs with standard table were used for this test. Muller Hinton agar medium was prepared and its pH was adjusted from 7.2 to 7.4. The plates were incubated at 35 ° C for 24 h to control infection. Containers containing antibiotic discs of ampicillin, cefazolin, piperacillin, tobramycin and imipenem were then transferred from a -20 ° C freezer (long-term storage) to a 4-degree refrigerator (short-term storage) (MAST UK antibiotic discs were purchased). A few minutes before the test, the disc containers were kept in the laboratory to reach room temperature. Next, a standard microbial suspension was prepared for the test. Since the strains that were not cultured for more than 24 hours were used for the preparation of the suspension, the samples were cultured on simple gel medium the day before antibiogram. They were then incubated at 35 ° C for 24 h. Transfer some of the colon to a tube containing 2 ml of sterile physiological serum, and after mixing with the mixer, the resulting suspension turbidity was adjusted to half-McFarland's turbidity. The sterile cotton swab was then immersed in the prepared bacterial suspension and then squeezed onto the lateral wall of the test tube to drain off the excess fluid, then cultured on a Muller Hinton agar medium by rotating the culture angle, for three times. 15 minutes after the inoculation of the antibiotic discs that were brought to room temperature, they were placed on a plate at a distance of at least 2.25 cm from each other as well as from the edge of the plate. Plates were incubated at 35 ° C for 24 hours. Then, using the ruler, the diameter of the growth zone around each disk was measured and the corresponding results were recorded in the prepared forms.

Table 1. Features of *Klebsiella*

Antibiotics *Klebsiella* Antibiogram	Sensitive to	Resistance to	Intermediate to
Cefazolin	13%	88%	-
Ceftriaxon	23%	77%	-
Cefixime	22%	78%	_

Nalidixic acid	18%	82%	-
Tobramycin	33%	50%	17%
Pipracillin	_	100%	_
Cefepime	63%	38%	-
Imipenem	100%	-	-
Meropenem	45%	54.00%	_
Chloramphenicol	81%	18%	-
Ampicillin	-	100%	-
Nitrofurantoin	66%	33%	_
Cefotaxim	26.08%	74%	-

DNA extraction for genotypic identification and determination of efflux pump genes

DNA extraction was performed in this study using the Total Extraction Kit, a product of Sinaagen. The basis of DNA extraction using the kit was that the high concentration of salt binds DNA to the matrix with a silica filter. Reversibly, under conditions of low salt concentration, such as in the wash solution or Tris-HCL 10 mM, the DNA is extracted from the silica filter and inserted into the above solutions. The ributinase enzyme in this kit digests the RNA and protein together with the DNA, making the resulting DNA readily available for polymerase chain reaction or PCR.

The steps of DNA extraction

For this test, 5 ml of the bacterial suspension containing 1.5 108 108 bacteria per ml were centrifuged at 300 rpm for 5 minutes. The solution was then discarded and the precipitate was washed with PBS. This operation was repeated twice. Subsequently, the addition of 10µl of periclase buffer and 10µl of ributinase was added and slowly vortexed for a few seconds. The test tubes were then incubated at 55 ° C for 30 min. From the above sample, 10µl was added to a 1.5 sterile Ependorf tube. Then 400µl of Laisse buffer solution was added and vortexed for 20 seconds at high speed. Afterwards, 300µl of the solution was added to the tube and vortexed at high speed for 20 seconds. After completion of this step, the sample was transferred to the spin column and centrifuged at 1 g for 1 minute. The spin column was transferred to a new collecting tube, and 400µL of the wash buffer was added and centrifuged for 1 minute at 13,000 rpm. This operation was repeated twice.

The spin column was again inserted into a new collection tube and this time 30µL of buffer was added and then incubated at 65 ° C for 3–5 minutes. Finally, the sample was centrifuged at 13,000 rpm for 1 minute to wash the DNA.

Determination of extracted DNA purity:

Optical absorption of the DNA extracted at 260 and 280 nm was measured by a nanodrop machine. If the resulting number is equal to or greater than 1.8 ng / µl, the extraction steps are well performed.

The primers used in the present study

16SrRNA-F5′-AGA GTT TGA TCM TGG CTC AG-3′

16SrRNA-R 5′-CGG TTA CCT TGT TAC GAC TT-3′

OqxA-F 5′-GCGTCTCGGGATACATTGAT-3′

OqxA-R 5′-GGCGAGGTTTTGATAGTGGA-3′

OqxB-F 5′-CTGGGCTTCTCGCTGAATAC-3′

OqxB-R 5′-CAGGTACACCGCAAACACTG-3′

PCR reaction was performed using positive and negative controls. The standard strain of *Klebsiella pneumoniae* ATCC 700603 was used for positive control and the distilled water without DNA was used for negative control.

Polymerase Chain Reaction and Test Procedures

In order to increase the efficiency, accuracy and ease of carrying out the polymerase chain reaction, the Master Kit made by Synagen was used. This kit contains all the ingredients needed to carry out the reaction, with the exception of DNA template and primers. Polymerase Chain Reaction was performed in a volume of 12.5 µL and in 0.2 ml sterile microtubes.

The following equation can be used to calculate the melting temperature or Annealing Temperature:

Formula for calculating Ta: Ta = 0.3 x Tm(primer) + 0.7 Tm (product) – 14.9

1. Tm(primer) = Melting temperature of the primers

2. Tm(product) = Melting temperature of the product

Primer Tm calculation: Tm = 4(G + C) + 2(A + T) =°C

It should be noted that all primers used in this study were extracted from validated sources. Therefore, no gradient temperature program was required to perform PCR.

Electrophoresis

In this method, the products of the polymerase chain reaction were electrophoresed and the bands formed on the gel, the presence or absence of the desired gene in the isolates genome was evaluated.

Solutions and buffers used in electrophoresis:

Buffer {(X50) TAE} (X 502) Tris-acetate EDTA

Use of Whonet software to analyze the effect of antibiotics on clinical isolates

Whonet is a software program designed to inform antimicrobial resistance based on WHO laboratory evaluation of infections.

This software is a tool for evaluating microbial epidemiology, selecting antibiotics, spreading disease and identifying problems in laboratory tests. In the present study Whonet version 5.6 software was used.

Phenotypic Evaluation of efflux Pumps in *Klebsiella pneumoniae* Isolates Using Cartwheel (Ethidium Bromide)

For this purpose ethidium bromide solution was used and 3 different dilutions of 1/2, 1/4 and 1/8 were prepared using sterile distilled water. When working with ethidium bromide solution, safety conditions such as gloves, masks, goggles and hoods were observed. Then 4 g of the powder was dissolved and autoclaved in 200 ml of distilled water, followed by removal of the culture medium to autoclave when the temperature reached 40 ° C using a sample of one cc of each sample. Diluted ethidium bromide dilutions were poured into a 10 cm sterile plate and then added to the culture medium and stirred to mix with ethidium bromide solution, then allowed to completely solidify. *Klebsiella* specimens isolated by sterile loops were drawn as a straight line. Plates were incubated in a 37 ° C incubator for 24 hours after which the results were recorded and documented by UVTEC gel apparatus.

Determination of dependence of antibiotic resistance on efflux pump in *Klebsiella* clinical isolates

At this stage of the experiment, initially isolates are all resistant to ciprofloxacin, tetracycline and chloramphenicol. MIC test or minimum inhibitory concentration were calculated, then the resistant strains were cultured independently in Muller Hinton liquid medium overnight. In 10 tubes containing 1 ml of Muller Hinton's liquid medium, different dilutions of antibiotics and then bacterial suspension were added. Minimum inhibitory concentration was measured after 24 hours with or without turbidity.

Similarly, after the Muller Hinton liquid medium was cooled, 100 mg / lt of PAβN (phenylalanine-arginine beta naphthylamide inhibitor) was added.

Statistics Analysis

In this study, the results were analyzed using SPSS software version 20. Analytical methods in the present study including descriptive analysis (Cross tab) and determination of significant relationships with Parametric and Nonparametric methods including Chi-square were studied. Finally, the significance level of statistical relationships was evaluated at $p < 0.05$.

Data analysis and conclusion

Phenotypic and Genotypic Identification and Determination of *Klebsiella* Occurrence in Clinical Specimens

Overall, 50.2% of the collected samples contained *Klebsiella* by the bacteriological, cultural and biochemical tests. Thus, 193 of 384 clinical specimens contained *Klebsiella*. Of the 193 positive samples, inguinal had the highest percentage and abscess the lowest percentage of Klebsiella infection, although *Klebsiella* was significantly removed from the throat, sputum, and foley catheter. Overall, 18.65% of clinical samples in both Dena and Namazi hospitals contained *Klebsiella*, which, given the ethical issues and commitments that were considered at the time of sampling, indicated the incidence of this bacterium in the listed hospitals separately was avoided. On the other hand, phenotypic identification of *Klebsiella* isolates showed that all isolates belonged to the pneumoniae species. To confirm the phenotypic identification, 5 strains were selected by chance and

evaluated using 16SrRNA primer, all of which confirmed the phenotypic identification.

Table 2. Klebsiella's resistance or sensitive

Clinical Sample	No	*Kl.*	Percentage	Resistance	Sensitive	Determination of antibiotic resistance spectrum and their percentage
Trachea	50	9	18%	-	FM-CPM-CTR-PIP-C	XDR (18) MDR(46)
Urine	63	10	15.87%	CAZ-SXT	CTR-CPM-NA-IMI-C-TE-FM	MDR(20)
Sputum	6	1	16.6%	CZ-PIP	CP-C-SXT-CPM-CTR-TE	-
Nasal Swab	16	2	12.5%	CZ-SXT	PIP-CPM-CP-TE-CTR	-
Inguinal	17	6	35.3%	SXT_CZ-PIP-CP-CPM-CTR	C-TE	MDR(50)
Throat	20	5	25%	CP-CTR-CZ-PIP	SXT-TE-C-CPM	MDR(20)
Abscess	9	1	11.11%	TE-CZ-SXT-C	CTR-CPM CP-PIP	MDR(100)
Foley catheter	12	2	16.6%	PIP-CZ-SXT-CPM-CTR-CP	C-TE	MDR(100)

The results shown in the table show the highest antibiotic resistance in strains isolated from trachea samples and then inguinal and foley catheter and the lowest antibiotic resistance in strains isolated from urine, sputum and nasal swabs. However, the highest sensitivity was evaluated in *Klebsiella* strains isolated from urine and then sputum. On the other hand, all *Klebsiella* strains were susceptible to cefazolin antibiotic and partially and not all *Klebsiella* strains isolated to chloramphenicol, tetracycline and cefipime antibiotics.

As previously described, resistance to at least one agent in three or more groups of antibiotics defined for MDR, resistance to at least one agent in all antibiotic groups except one or two XDR groups, and resistance to all antibiotic agents in all. Groups are considered PDRs. Therefore, the results of the present study showed that only XDR was observed in strains isolated from the trachea, while the rest of the clinical specimens contained the MDR *Klebsiella pneumoniae* strains except sputum and nasal swab samples.

Antibiotic effect analysis by using Whonet software

The results of data analysis using Whonet software showed that *Klebsiella* clinical isolates were the most resistant to cefepamin and ceftriaxon antibiotics and the most susceptible to nitrofurans and chloramphenicol.

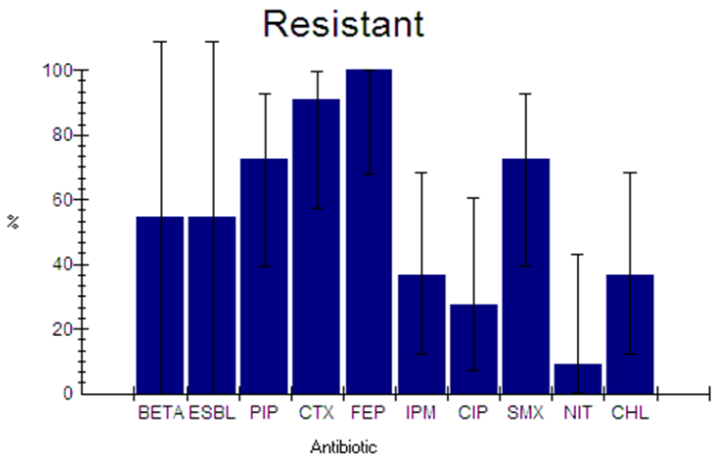

Figure 1. Percentage of resistance of *Klebsiella* isolates to antibiotics

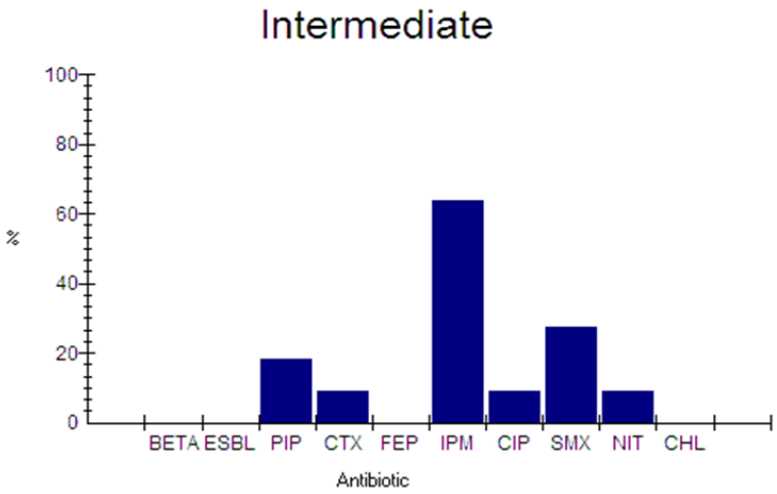

Figure 2. Percentage of *Klebsiella* strains resistance to antibiotics

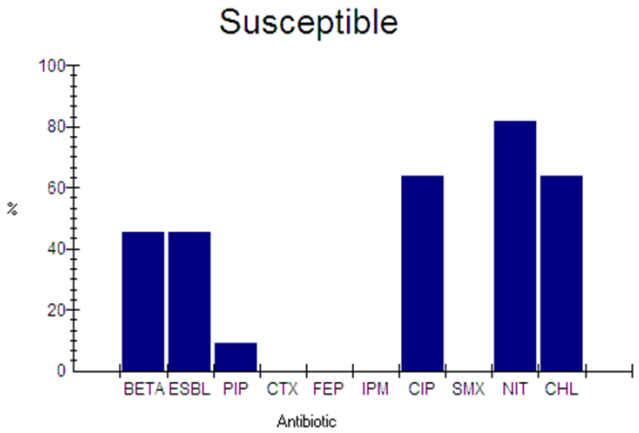

Figure 3. Percentage of susceptibility of *Klebsiella* strains to antibiotics

Genotypic evaluation of Efflux pump in *Klebsiella pneumoniae* isolates using oqxAB genes

Following the results of the Cartwheel test for phenotypic identification of the efflux pump in isolated *Klebsiella*, it was shown that some *Klebsiella* strains were unable to maintain this dye after exposure to ethidium bromide. However, some *Klebsiella* strains were able to maintain this color. Therefore, it was concluded that strains that were not able to maintain dye were likely to contain an efflux pump to remove antibiotics from the cell.

The results of genotypic evaluation of efflux pump in isolated *Klebsiella* showed that all strains in the phenotypic test likely contained efflux pump gene containing oqxAB genes.

The results showed that antibacterial, cefazolin, ceftazidime, ciprofloxacin, chloramphenicol, tetracycline had more antibacterial activity after blocking the efflux pump inhibitor. These antibiotics were antibiotics whose resistance to *Klebsiella pneumoniae* isolates was dependent on the efflux pump as their growth inhibitory region was changed from the original antibiogram. Results were analyzed using Whonet 5 and SPSS v22 software. According to the Whonet software, the horizontal axis in these graphs shows the bacterial growth inhibition diameter and the vertical plot in percent of the isolation growth inhibition diameter.

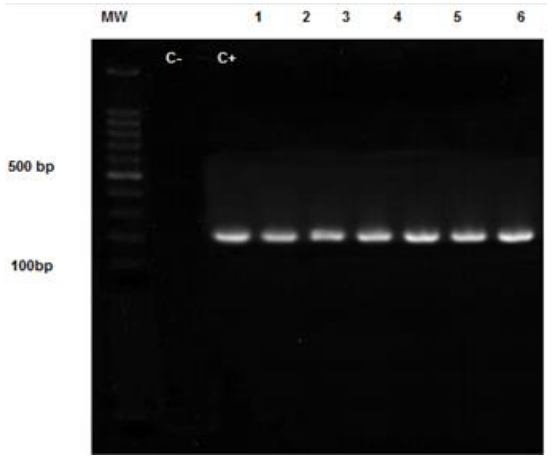

Figure 4. OqxA gene electrophoresis gel band size: 200 pairs

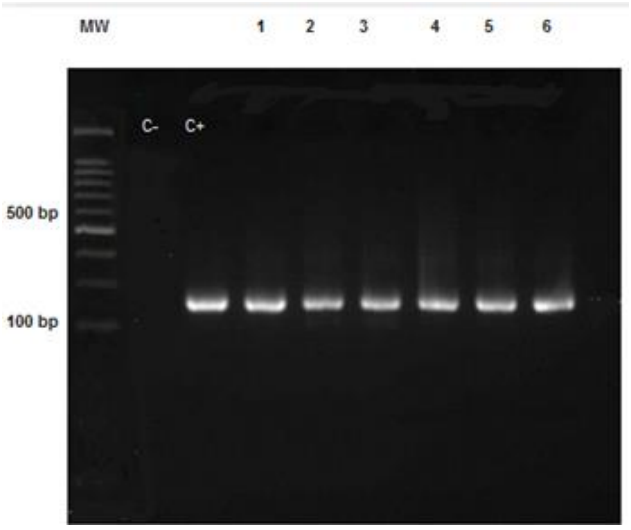

Figure 5. OqxB gene electrophoresis gel band size: 150 pairs

Evaluation of dependence of antibiotic resistance of *Klebsiella* isolates on efflux pump

The results of evaluation of antibiotic resistance of *Klebsiella* isolates to efflux pump showed that addition of efflux pump inhibitor (phenylalanine-arginine- beta-naphthylamide) to culture medium increased susceptibility of *Klebsiella* isolates to ciprofloxacin and tetracycline antibiotics was Resistance to other antibiotics dependent on the efflux pump was not evaluated.

Statistical analysis of data

In the present study, the relationship between resistance of *Klebsiella pneumoniae* to antibiotics and presence and absence of efflux pump genes was examined by chi-squared test. The results showed that there was a significant relationship between the antibiotic resistance of *Klebsiella* isolates to ciprofloxacin and tetracycline antibiotics with P value <0.05. Chi-squared test was used for statistical analysis and the results showed a significant relationship with P value <0.05 between isolates resistant to some antibiotics such as ciprofloxacin and tetracycline and presence of oqxAB genes.

Discussion

Nowadays The high prevalence of *Klebsiella pneumoniae* in the hospital environment today has limited the options available to treat infections caused by this bacterium. However, antibiotic control politics are important along with the implementation of infection control measures. In a 2013 study by Derakhshan et al. In Tehran. Antibiotic susceptibility testing was performed for 116 clinical isolates of *Klebsiella pneumoniae* strain. The results showed that the antibiotic resistance of gentamicin, amikacin is 60% and 37%, which is higher than the resistance of these drugs in the present study (Liu, 2012). In a study conducted by Pakzad et al. (2013) in Tehran. Antibiotic susceptibility testing was performed for 52 *Klebsiella pneumoniae* isolates isolated from burn samples. The results showed that gentamicin resistance was 73% which is higher than the resistance of these drugs in the present study (Pakzad *et al.,* 2013). In a 2011 study by Galani et al in Greece, antibiotic susceptibility testing was performed on 14 *Klebsiella pneumoniae* isolates with high MIC for amikacin, gentamicin, which had a 48.2% and 8.1% frequency of resistance to amikacin and gentamicin, respectively (Galani *et al.,* 2012).

A study by Ma et al. (2008) in Taiwan. Antibiotic susceptibility testing was performed for 235 *Klebsiella pneumoniae* isolates and the gentamicin and amikacin antibiotic resistance were 94.4% and 91.1%, respectively. In a study conducted by Habeeb et al. (2013) in Pakistan, of the 25 isolates of *Klebsiella pneumoniae* producing beta lactamase with a wide range of 60% rmtB gene expression was reported. Results showed that rmtB gene expression in these strains was very high at this hospital in Pakistan. In another study the 55 isolates of *Klebsiella pneumoniae* resistant to several drugs, cefotaxime, ciprofloxacin and amikacin were observed (Yang *et al.,* 2011). A study by Kang et al. (2008) at the Korea Teaching Hospital between 40 *Klebsiella pneumoniae* strains with high levels of aminoglycosides, including amikacin, tobramycin, gentamicin and kanamycin, 28

isolates containing armA and 9 isolates containing rmtB. Multiplex PCR assay was performed for simultaneous detection of 7 aminoglycoside resistance genes in Enterobacteriaceae (Hu *et al.,* 2013). Thirty isolates of *Klebsiella pneumoniae* resistant to gentamicin, tobramycin and amikacin contained rmtB and armA genes (Yang *et al.,* 2011). A study by Wang et al. (2007) in Korea from 7127 Enterobacteriaceae isolates, 463 isolates were highly resistant to amikacin (6.5%). 218 isolates carried the armA and rmtB genes. Of the 218 isolates, 153 strains contained armA and 51 isolates contained rmtB (Kang *et al.,* 2009). A study by Yu et al. (2008) in China of the 337 *Klebsiella pneumoniae* isolates, 39 isolates had very high resistance to gentamicin, amikacin, and tobramycin (MIC≥ 256 μg / ml) (Yu *et al.,* 2009). Wu et al. (2009) in Shanghai determined that among 202 *Klebsiella pneumoniae* isolates, 35 were resistant to gentamicin and amikacin. Feyzabadi et al. (2010) reported that antibiotic susceptibility testing was performed on 89 clinical samples of *Klebsiella pneumoniae*. Results showed that the resistance to gentamicin, amikacin were 34.8% and 16.9%, respectively, which is lower than the drug resistance in the present study. In this study, resistance to amikacin was higher than gentamicin. A 2013 study of *Klebsiella pneumoniae* specimens collected from various locations such as Taiwan-Australia-Argentina-Belgium-Turkey-South Africa found that 100% of the isolated isolates of the OqxAB efflux pump were present (Alexander & Rietschel, 2001). The results of Saadatian Farivar study on *Klebsiella pneumoniae* isolates isolated from Tehran hospitals showed that 96% of samples had OqxAB efflux pump (Nassif & Sansonetti, 1986). In a descriptive study conducted by Hashemi et al. (2014) they concluded that the prevalence of OqxA and OqxB pumps was 50% and in ciprofloxacin-resistant strains a 2/3 times increase in OqxAB efflux pump was observed. Also, studies conducted in 2013 and 2015 showed the presence of both OqxA and OqxB genes was 60.2 percent and in the next study in 2015 the presence of OqxA 56.7 percent and OqxB, 54.6 percent were reported. Beta-lactamase-producing enterobacteria are among the major emerging pathogens in nosocomial infections (Joppa et al., 2006).

There is a high prevalence of beta-lactamase-producing *Klebsiella pneumoniae* in hospitals. As the treatment options available are limited, antibiotic control politics along with the implementation of infection control measures are important.

In another study conducted on the *Klebsiella pneumoniae* strain, 52 patients who were burned and hospitalized at Shahid Motahari Hospital in Tehran showed all 40 isolates resistant to ciprofloxacin, tetracycline, ceftazidime and gentamicin, with high levels of efflux pump. AcrAB was especially expressed in ciprofloxacin-resistant strains (Alves *et al.,* 2006).

Conclusion

The results of the present study showed that resistance to *Klebsiella* bacteria isolated from clinical specimens showed a high level of resistance to antibiotics. However, the mechanism of resistance in these bacteria cannot be largely related to the role of the efflux pump. However, based on the findings, resistance to tetracycline and ciprofloxacin antibiotics is dependent on the efflux pump function.

On the other hand, the results of this study showed that antibiotic resistance in *Klebsiella pneumoniae* strains isolated from clinical specimens was very high, so physicians should be careful in prescribing the drug to be the best option. Use medication for the patient. Antibiogram testing for all isolates at the hospital should also be able to identify antibiotic resistance mechanisms, especially those associated with efflux pumps, and inform physicians. However, alternative drugs to reduce the bacterial resistance of this bacterium should be considered.

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