TURKISH REPUBLIC
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF BIOLOGY

MULTILOCUS SEQUENCE TYPING OF CARBAPENEM RESISTANT *KLEBSIELLA PNEUMONIAE* ISOLATES COLLECTED FROM TURKEY AND IRAQ

Prepared By
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MSc Thesis

October, 2020
KAYSERİ

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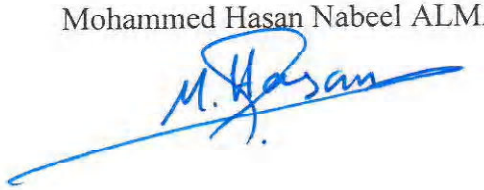
SCIENTIFIC ETHICS SUITABILITY

I declare that all informations in this work were obtained in accordance with academic and ethical rules. All results and material that not been at the essence of this work are also transferred and expressed by giving reference as required by these rules and behavior,

Mohammed Hasan Nabeel ALMASHAT



SUITABILITY FOR GUIDE

The MSc thesis entitled MULTILOCUS SEQUENCE TYPING OF CARBAPENEM RESISTANT *Klebsiella pneumoniae* ISOLATES COLLECTED FROM TURKEY AND IRAQ has been prepared in accordance with Erciyes University Graduate School of Natural and Applied Sciences Thesis Preparation and Writing Guide.

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ACCEPTANCE AND APPROVAL PAGE

This study entitled "**MULTILOCOS SEQUENCE TYPING OF CARBAPENEM RESISTANT** *Klebsiella pneumoniae* **ISOLATES COLLECTED FROM TURKEY AND IRAQ**" prepared by Mohammed Hasan Nabeel ALMASHAT under the supervision of Prof. Dr. Mikail AKBULUT was accepted by the jury as MSc. Thesis in Biology department.

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APPROVAL

That the acceptance of this thesis has been approved by the decision of the Institute's Board of Directors with the 27./10./2020 date and 2020/1589 numbered decision.

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Prof. Dr. Mehmet AKKURT

Director of the Institute

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MULTILOCUS SEQUENCE TYPING OF CARBAPENEM RESISTANT *Klebsiella pneumoniae* ISOLATES OBTAINED FROM TURKEY AND IRAQ

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Erciyes University, Graduate School of Natural and Applied Sciences

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Thesis Supervisors: Prof. Dr. Mikail AKBULUT

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ABSTRACT

In this study 72 putative *Klebsiella pneumoniae* (Kp) isolates were collected from Kayseri-Turkey and Baghdad-Iraq and MIC values determined for meropenem resistance among them 50 meropenem resistant (25 from Turkey and 25 from Iraq) isolates were typed by using high discriminatory power methods such as PFGE, 7 housekeeping Multilocus Sequence Typing and wzi gene typing. Majority of the isolates (%32) were obtained from the urine samples and the rest of isolates from different sources such as blood culture (%18), sputum (%12), wound (%14), skin swab (%10), respiratory system (%8) and ear swab (%6). With 52% frequency, Sequence type (ST) 15 was the most frequent sequence type among the sequence type of studied isolates. ST 307 was the second most frequent Sequence type with %6 frequency. Among the isolates, the frequency of each of ST16, ST 101 and ST 2943 was %4. The frequency of each of ST 14, ST 2096, ST 4634, ST 530, ST 469, ST 377, ST147 and ST 37 was found to be %2. However, %6 of isolates were determined as novel STs, and %8 of isolates were un-typeable. Based on wzi gene all isolates were typed but some of them had cross reaction in K-type and for some isolates only allelic number was determined. In PFGE profiles, the level of similarity among isolates were 80% and above. The phylogenetic relationship of isolates were also determined based on rpoB, wzi gene, MLST and PFGE profiles. Being the most frequent ST, ST 15 was expected to be more problematic in Middle East countries considering their resistance against most antibiotics in general and specifically to carbapenems.

Keywords: *Klebsiella pneumoniae*, PFGE, MLST, wzi, Carbapenem Resistance.

TÜRKİYE VE IRAKTAN ELDE EDİLEN KARBAPENEM DİRENÇLİ *Klebsiella pneumoniae* İZOLATLARININ ÇOK LOKUSLU DİZİ TİPLENDİRMESİ (MLST) İLE TİPLENDİRİLMESİ

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Erciyes Üniversitesi Fen Bilimleri Enstitüsü
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ÖZET

Bu çalışmada Kayseri-Türkiye ve Bağdat-Irak'tan 72 adet *Klebsiella pneumoniae* (Kp) izolatı toplanmış ve meropenem direnci için MIC değerleri belirlenmiştir. Bu izolatlardan 50 tanesi PFGE, 7 housekeeping MLST ve wzi geni gibi ayırma gücü yüksek olan farklı tiplendirme yöntemleriyle tiplendirilmiştir. İzolatların büyük çoğunluğu (% 32) idrar örneklerinden, geri kalanı ise kan (% 18), balgam (% 12), yara (% 14), deri sürüntüsü (% 10), solunum sistemi (% 8) ve kulak sürüntüsü (% 6) gibi farklı kaynaklardan elde edilmiştir. Çalışılan izolatlar arasında %52 frekansla ST15 en fazla görülen dizi tipi olmuştur. İkinci en fazla bulunan dizi tipi % 6 frekansla ST 307 olmuştur. ST16, ST 101 ve ST 2943 dizi tiplerinin her biri % 4 oranında bulunurken ST 14, ST 2096, ST 4634, ST. 530, ST 469, ST 377, ST147 ve ST 37'nin her biri% 2 oranında tespit edilmiştir. Ayrıca, izolatların % 6 sı yeni tipe sahipken % 8'i tiplendirilememiştir. Tüm izolatlar Wzi geni için tiplendirilmiş olup, bazılarının K-tipinde çapraz reaksiyona sahip olduğu belirlenmiş ve bazılarında ise wzi geni için sadece allel numarası belirlenmiştir. İzolatların PFGE profilleri % 80 ve üzerinde benzerlik göstermiştir. Ayrıca izolatlar arasındaki filogenetik ilişkiyi belirlemek için rpoB ve wzi genleri, 7 housekeeping genleri ve PFGE kullanılmıştır. En yüksek frekansta bulunması ve karbapenem dahil çok sayıda antibiyotiğe dirençli olması nedeniyle ST 15 dizi tipine sahip suşların Ortadoğu ülkelerinde problem olacağı beklenilmektedir.

Anahtar Kelimeler: *Klebsiella pneumoniae*, PFGE, MLST, wzi, Karbapenem direnci

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LIST OF SYMBOLS AND/OR ABBREVIATION

UTI	: Urinary Tract Infections
PBP	: Penicillin-binding protein
OMP	: Outer-membrane protein
ESBL	: Extended-spectrum β-lactamase
MIC	: Minimum inhibitory concentration
PFGE	: Pulsed-field Gel Electrophoresis
MLST	: Multi-Locus Sequence Typing
PCR	: Polymerase Chain Reaction
CLSI	: Clinical and Laboratory Standards Institute
ST	: Sequence Type
CRE	: Carbapenem-Resistant Enterobacteriaceae
BIGSdb	: Bacterial Isolate Genome Sequence Database
LPS	: Lipopolysaccharide
AMR	: Antimicrobial Resistant genes
CPS	: Capsule Polysaccharide
CSF	: Cerebrospinal fluid
AST	: Antimicrobial Susceptibility Test
E-test	: Epsilometer test
rpoB	: Beta-subunit of RNA polymerase
gapA	: Glyceraldehyde 3-phosphate dehydrogenase
mdh	: Malate dehydrogenase
pgi	: Phosphoglucose isomerase
phoE	: Phosphorine E
infB	: Translation initiation factor 2

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INTRODUCTION

In recent years, *Klebsiella pneumoniae* has been recognized among one of the most multidrug-resistant gram- negative bacteria. Those isolates were especially resistant to those antibiotics considered as the last choice, such as carbapenems. For this reason, we studied *K. pnemoniae* isolates to investigate sequence type and relatedness between two groups of isolates which were collected from Turkey/Kayseri and Iraq/Baghdad. Two typing methods, sequence-based MLST (Multi Locus Sequence Typing) and gel-based PFGE (Pulsed Field Gel Electrophoresis), were used to characterize those isolates. By using these methods, we will be able to distinguish and understand the epidemiological characteristics of CRKP (Carbapenem resistant *K. Pnemoniae*) infections distributed throughout Turkey and Iraq. In recent years, CRKP was reported in many countries. Molecular epidemiology data, based on multiple-locus sequence typing (MLST), indicated the presence of several *Klebsiella pneumoniae* Sequence Types (ST) worldwide [1]. Various reports suggested that ST was related to drug resistance of strains. *Klebsiella pneumoniae* ST258 type reported to be the most common carbapenem-resistant ST type in the United States and Greece [2], [3]. A Russian study also suggested that the ST340 type was related to the *Klebsiella pneumoniae* that produces NDM-1 carbapenem [4]. In China, resistance monitoring showed that the number of carbapenem-resistant *Enterobacteriaceae* (CRE) increased year by year, of which CRKP occupied the most critical proportion (64.2%). In A report published in 2007, it was discovered that CRKP have produced KPC-type 2 enzyme in China, Zhejiang province [5]. Since then, strains produce a KPC-type 2 enzyme that has been continuously detected and reported [6]. In recent years, a few different typing methods were suggested for typing of the same pathogen groups in different laboratories and to standardize those methods. However, even with a standardized method, it is often difficult to compare the data between laboratories and

are often unsuitable for evolutionary, phylogenetic, or population genetic studies[7]. The use of MLST as a standard method for typing bacterial pathogens has already provided a possible solution. It is ideal for global epidemiology, as it is required, a method that retains the ability to distinguish a vast number of genotypes but uses the genetic variation that accumulates relatively slowly, and the data are portable and unambiguous. It has also been shown that although MLST only typically examines seven core loci, which is a small portion of genome (approx. 0.1% of the core genome of a species), the genetic relationships inferred by MLST data are confirmed by analysis of complete genome sequences[8]. The sequence data obtained from MLST can then be analyzed using numerous packages available through several websites[9].

The problem of multidrug-resistant bacteria is a global issue. CRKP is threatening the lives of millions of people, if the situation is not remedied. By using MLST, we will be able to study the evolution of bacteria in Turkey and Iraq and compare them with the versions in the global database.

Discovery of new ST and their inclusion to the database and make the data accessible to all researchers contribute to prevent the spread of drug resistant *K. pnemoniae* causing infections in hundreds or thousands of people all around the world.

Given the emergence of *K. pneumoniae* as a major clinical pathogen with high degrees of genomic plasticity and diversity, we aimed to study carbapenem resistant clinical isolates of *K. pneumoniae* isolated from Turkish and Iraqi patients to determine epidemiological diversity present in this bacteria from two countries by using various typing methods such as MLST and sequencing of the wzi gene. PFGE was also used to characterize their genetic diversity and asses their clonality.

CHAPTER 1

GENERAL INFORMATION

1.1.MLST

The ability to distinguish pathogenic strains with high-precision is essential in the epidemiological surveillance, analysis, and the structure and mechanism of microbial society. More important is the development of strategies to control public health. Many molecular typing approaches have been suggested to help such general goals that can diagnose isolates from all over the world called international epidemiology and the local epidemic. The sequence mutability inside particular genes can be exploited in molecular typing schemes to assess the bacterial relatedness[10]. However, in 1998 a standard golden method was reached; it is Multi-locus sequence typing abbreviated as MLST. MLST examines the difference in DNA sequence fragments (450-500bp) in genes responsible for primary cellular functions, which are usually seven genes. For each gene, there is a different sequence in a species that represents distinct alleles. As a result, each isolate has alleles in the seven loci that determine the allelic profile or expressed as sequence type (ST). Additionally, each isolate is signified by a sequence of seven integer numbers. These numbers are consistent and match the alleles at the seven loci of the housekeeping genes. There are billions of distinct sequence types to be distinguished using just seven loci; this is due to the sufficient variation in the housekeeping genes of most bacterial species, which resulted in several alleles per locus[11]. One of the essential qualities that make genes certified in genotyping is their slow variability. In other words, they are not exposed to changes or mutations in the short term. Therefore, the use of housekeeping genes for typing, which have a relatively slow contrast rate compared to the hyper-variable genes that usually develop at an accelerated rate and whose function

usually encodes surface-linked proteins that interact with a host organism, or pathogenic bacteria are associated with virulence[8].

Moreover, housekeeping genes are transmitted vertically, which can be invested in detecting the genetic relationship between strains without paying attention to environmental factors and the host's impact. Maiden and his colleagues described the first multilocus sequencing scheme by selecting seven loci of the genes of housekeeping for the pathogenic bacteria *Neisseria meningitides* isolated from patients. They used the Sanger sequencing method to obtain the DNA sequences of the selected genes and then analyzed it to obtain the sequence type and phylogenetic map. The next step was to make the sequences and the resulting information available on the Internet for easy access by all laboratories around the world and to make use of genetic information as much as possible without the risk of direct dealing with these types of bacteria that are very dangerous[12].

1.1.1.MLST Types

There are a few types of MLST methods, depending on the number of genes used to study the evolution and epidemiology of bacteria, in which the number of genes increases the accuracy and resolution of the results. For example, the analysis of 16s rRNA genes as a single locus is not practical and poorly differentiated some genus like Neisseria, because of low-resolution distortions[11] as well as, by using two loci, the *Calymmatobacterium granulomatis* was reclassified by sequencing phoE genes and 16S rRNA genes to be categorized as *Klebsiella granulomatis* [13]. Therefore, there are several numbers of loci to make it easier to confirm the classification of bacteria and study their epidemiology on a global level.

1.1.1.1.MLST 7 housekeeping genes

Multilocus sequence typing is based on housekeeping genes. It provided a means to reliably identify relationships between relevant organisms and was proposed as the basis for a new approach to the identification of microbial species[14] by sequencing of core metabolic gene that are typically not exposed to the quick evolution[8], but it does not always supply appropriate accuracy between closely related bacteria[15]. Moreover, due

to the diversity of bacterial metabolism throughout the field, and even among closely related organisms, each MLST scheme should be developed for a particular group of related bacteria. Therefore, MLST schemes are usually limited to the same genus of bacteria, and even within a particular genus, many distinct MLST schemes may be needed[12]. However, MLST is a highly accurate method but has not got the discriminatory power to satisfy the need to distinguish between outbreak and non-outbreak isolates. There were additions to this method to be more accurate[16].

1.1.1.2.Ribosomal MLST

Ribosomal MLST (rMLST) is a method for indexing the variance between the 53 genes that encode the subunits of the bacterial ribosome protein (rps) to integrate microbial lineages and typing. As with MLST, rMLST uses coordinated reference chains to efficiently and quickly identify ribosomal gene variables [17]. The ribosomal protein subunit (rps) genes have the advantage of being widely present but unequally variable for bacterial typing and taxonomy. Moreover, even if some rps genes have prevented their presence in the core gene[18], this heterogeneity allows for high levels of differentiation between closely linked isolates. Besides, the rps genes are considered stable in horizontal gene transfer due to the distribution of the ribosome genes along with the genetic material. An allelic profile of rMLST or ribosomal sequence type (rST) may accurately summarize the interactions among bacterial genomes, offering a functional structure for universal bacterial phylogeny and typing, as illustrated by the use of rMLST to describe groupings of species including strain types throughout the Neisseria genus as a model for MLST[19], [20]. Moreover, the genus Campylobacter had a problem with the lineage structure, and after using rMLST it was resolved[21].

1.1.1.3. Core genome MLST

MLST types failing to detect severe discontinuities between clonal groups (CGs) because of the significant loss of resolution. However, the low nucleotide sequence variance among gram-negative bacterial isolates, especially those with hypervirulence or multidrug resistance characteristics, makes it more difficult to characterize clone borders[22], [23]. By the developing of core genome MLST based on 634 genes that

showed apparent discontinuities genotype space and showed that high-risk CGs are significantly different from their nearest genotypes neighbours. This results in stark comparison to the genotypic scale obtained through the use of MLST[24]. Also, to address large-scale epidemiological questions, it was necessary to escalate genotyping on a large scale. Therefore there were several experiments to use an increasing number of loci and note the accuracy of the results and the resolution[20]. So, there was an addition to the Core genome 634 MLST scheme to be based on 694 loci. The 694 genes cgMLST structure has been identified by the fusion of 53 ribosomal genes MLST[17], seven housekeeping genes MLST and the 634 delicate core genome MLST genes. This structure Contains around one hundred time additional sequence data than that achieved from the 7-gene MLST data[25].

1.1.1.4.wgMLST

To obtain the highest accuracy, this can be realized using the entire genome resolution by examining the Whole Genome MLST approach. This approach relies mainly on the particular isolate to compare with all other isolated loci in the same species. This means that it applies to monogenic pathogens (single-clone pathogen) with closed genomes or variables closely related to more diverse organisms. This analysis can include the entire genome if the parallel locations of the genetic regions are identified. However, Groups of WGS data from various bacterial isolates quickly identify groups of isolates, and these groups can then be investigated with greater accuracy with the wgMLST[26]. The allelic differences in nucleotide sequences are defined in segmented characters from several thousand gene loci, a process that has proven to be very useful for tracking strains and investigating the outbreak of many bacterial pathogens. In 2012, it was established that 24% of the *C. jejuni* that were isolated from humans belonged to chicken slaughter groups using very preferential wgMLST associated with the temporal relationships between patient disease and chicken slaughter. The results demonstrated the power of whole-genome analysis in rebuilding the infection-linked poultry slaughter pathway for locally acquired human campylobacteriosis. This approach has been able to identify successive *C. jejuni* related epidemics on farms and differentiate them from unrelated isolates. However, the results showed that wgMLST's highly discriminatory intensity supports its use as a precise tool for comparing temporarily connected isolates

[27]. Another example, investigating the clonal groups of two outbreaks happened in Switzerland 2015 and 2013 by *Klebsiella pneumoniae* producing carbapenemase.

Nonetheless, very few single nucleotide variants between strains were identified in the wgMLST analysis, regardless of their relationship. In comparison, plasmid analysis showed a high degree of heterogeneity mostly due to mutations, as evidenced by the loss of plasmid regions between the relevant strains. WgMLST was able to recognize the two strain clusters at the whole genome level (along with plasmids) in both outbreaks[28].

1.1.2.MLST Database

In 1998, the MLST database was created to organize all the sequence data used in this method that enables the exchange of molecular typing data for global epidemiology across the Internet. The predominant advantage of MLST over other molecular typing methods is that sequence data is already portable between laboratories, allowing one global database to expand for each species on a global web site [11]. These years required a database that has features compatible with the volume of data that flows day after day to be kept, arranged, selected, interpreted, analysed, and accessible. Indeed, these goals were achieved to study pathogenic bacteria and some microorganisms by working with the Bacterial Isolate Genome Sequence Database (BIGSdb) software is running the PubMLST.org databases [29]. The increase in data at PubMLST.org came after the evolution of the sequencing methods obtained by working with NGS (Next Generation Sequencing) to obtain whole-genome sequences (WGS) at a lower cost and higher accuracy [30]. However, with the increase in WGS data on a large scale, work began in 2008 on a platform designed to process genetic data flexibly using any number of loci and typing systems. Since then, the BIGSdb database was used to host databases on PubMLST as well as used for databases hosted at the Pasteur Institute like *Klebsiella pneumoniae*, *K. qusipneumoniae*, and *K. variicola* database. It has been under continuous development ever since. In 2016, the databases hosted on mlst.net were moved to PubMLST, which resulted in hosting most of the MLST charts now using the same platform (the main exceptions are *Salmonella* and *Escherichia coli*, hosted on Enterobase, although these charts are reflected in PubMLST) [7]. These databases are varied among many standard seven housekeeping genes that have tools for identifying

and analyzing species. The highest-accuracy associated ribosomal 53 rMLST genes [17], in addition to cgMLST have significance at several levels such as genotyping and clonal complex in addition to genealogical relationships [7]. Moreover, PubMLST.org website has more than a hundred species of microorganisms, most of them are bacteria. Also, there are some schemes for eukaryotes like *Aspergillus fumigatus*, *Candida albicans* and *Trichomonas vaginalis*, and other databases for Bacteriophage MLST, *Borrelia burgdorferi* MLSA, NucleBact (Nuclease Bacteriocin) database and Plasmid MLST [Jolley, K., 2018. PubMLST Website. (Web page: https://pubmlst.org/databases/),(Date accessed: January, 2020)].

Furthermore, the citation of the BIGSdb platform was 1360 times (January 2020, source Google Scholar). In addition, per month, there is around 15000 visitors and more than a million page views [7]. Generally, the use of molecular technologies in typing has contributed a high gain in discriminatory power relative to traditional methods. Molecular typing techniques used the most today for *K. pneumoniae* include macro-restriction profile analysis by PFGE (pulsed-field gel electrophoresis) and multilocus sequence typing (MLST)[31]. In the coming days – with the accessibility to full-genome sequencing – molecular epidemiology could develop into "genomic epidemiology," achieving the endpoint of resolution and carrying out real-time examinations of outbreaks[32].

1.2.PFGE

It is a unique electrophoretic technique for separating large bacterial DNA fragments after treatment with a specific endonuclease enzyme (XbaI in *K. pneumoniae*). This technique can also be described as "macrorestriction profile" refers to the pattern of fragmentation revealed by PFGE. The relativity of isolates can be assessed by comparing such profiles. PFGE 's macro-restriction profile analysis is most useful in local outbreak investigations since it has relatively high discriminatory power, and the interlaboratory reproducibility of results is good.

1.3.*Klebsiella spp*

Klebsiella is a genus in the *Enterobacteriaceae* family, gram-negative bacteria. In 1882, Carl Friedlander was the first describer of encapsulated bacilli when he isolated the bacterium from the lungs of who had died of pneumonia and named Friedlander's bacillus. In 1885, it was named Trevisan honourable for the German microbiologist Edwin Klebs[33].

According to National Centre for Biotechnology Information Taxonomy browser website(https://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?mode=Tree&id=570&lvl=3&keep=1&srchmode=1&unlock), fifteen species are belonging to this genus[13], [34]. They are in the following taxonomy.

Kingdom: *Bacteria*

Phylum: *Proteobacteria*

Class: *Gammaproteobacteria*

Order: *Enterobacterales*

Family: *Enterobacteriaceae*

Genus: *Klebsiella*

+-*Klebsiella africanensis*

+-*Klebsiella grimontii*

+-*Klebsiella kielensis*

+-*Klebsiella quasivariicola*

+-*Klebsiella steroids*

+-*Klebsiella quasipneumoniae*

| +-*Klebsiella quasipneumoniae* subsp. *quasipneumoniae*

| \-*Klebsiella quasipneumoniae* subsp. *similipneumoniae*

+-*Klebsiella michiganensis*

+-*Klebsiella variicola*

+-*Klebsiella senegalensis*

+-*Klebsiella milletis*

+-*Klebsiella cf. planticola* B43

+-*Klebsiella granulomatis*

+-*Klebsiella pneumoniae*

| +-*Klebsiella pneumoniae* subsp. *pneumoniae*

| +-*Klebsiella pneumoniae* subsp. *rhinoscleromatis*

| \-*Klebsiella pneumoniae* subsp. *ozaenae*

+-*Klebsiella oxytoca*

\-*Klebsiella aerogenes*

Most members of this genus are non-motile, capsulated, and non-spore-forming. *Klebsiella* species are determined in all places in nature. This is conceptualized to be due to awesome sub-lineages growing particular niche adaptations, with related biochemical adaptations which make them better suitable for a precise environment[35]. This bacterial genus is endemic in water and soil and is also found in the gut of animals, including humans as normal flora in the mouth, nose, and intestine[36]. Morphologically, it is likely to be shorter and thicker than others in the *Enterobacteriaceae* family. The rod-shaped cells generally measure from 0.3 to 1.5 µm wide, ranging from 0.5 to 5.0 µm long. They can be observed as single cells, in pairs, linked end to end or in chains. One of the essential characteristics of the genus *Klebsiella* is the ability to grow in routine laboratories without special conditions. For example, the ideal pH is 7.2, and the growth temperature is 35° to 37 °C. Moreover, these bacteria are aerobic and grow in a conventional bacteriological medium like Eosin Methylene Blue agar and MacConkey agar[37], [38].

The members of the genus *Klebsiella* typically express two types of antigens on the cell surface. Firstly, O antigen is associated with lipopolysaccharide (LPS), which has nine variants. The second is a K - antigen, a polysaccharide capsule of more than 80 variants. Both are cooperating in the disease's pathogenesis and form the basis for serogrouping [39], [40].

The mucous colonies produced by all types of genus *Klebsiella* make diagnosis easier and through the presence of this mucous layer used for serologic identification of this

particular genus, but with the development of molecular typing techniques became more accurate identification [33], [40], [41].

Klebsiella organisms can be the reason for various disease states, including pneumonia, brain infections, sepsis, meningitis, diarrhea, and costs (complicated skin and soft-tissue infections). It also played a role in the pathogenesis of ankylosing spondylitis[42] and other spondyloarthropathies[43].

Most human *Klebsiella* infections are caused by *K. pneumoniae*, followed by *K. oxytoca*. The infections caused by those species are common in older patients, and there are subsidiary diseases such as cancer[44].

1.3.1 *Klebsiella pneumoniae*

K. pneumoniae is omnipresent in the environment, and it is found naturally in the mucous membranes of mammals and its spread in water and soil. It can colonize living beings such as animals, plants, and people[13], [36], [45]. *K. pneumoniae* is a significant repository for various Antimicrobial Resistant genes (AMR) and has been the primary source of emergency nosocomial infections 3% to 8%. It is the cause of clinic procured diseases and neonatal sepsis worldwide for a long time. The bacterium is viewed as a selfish pathogen that can be conveyed asymptomatically in the nose, throat, intestinal tract, and skin. Nonetheless, it can likewise cause an extended of contagions in hospitalized patients, most ordinarily pneumoniae, urinary tract infections (UTI), bloodstream infection, wound, or soft tissue[46], [47]. Most of the contaminations brought about by *K. pneumoniae* happens for the most part in hospitalized, immunocompromised patients experiencing maladies, for example, diabetes mellitus or constant respiratory check[39] and are related to high paces of horribleness and mortality[48]. However, since the end of the 1980s, *K. pneumoniae* diseases causing pyogenic liver sore (PLA) in any case trustworthy people developed in some Asian nations, and cases are progressively revealed [49], [50]. Pathogenicity of *K. pneumoniae* relies upon different, diverse virulence factors, including the capsular serotype, lipopolysaccharide, iron-searching frameworks (siderophores), and adhesions[39]. Among these harmful factors, the capsule is believed to be the most significant [49]. It is made out of complex acidic polysaccharides. It ensures the bacterium for an infection from phagocytosis [51] and imaging a new component for confirmation of Aps

(antimicrobial peptides) based on bacterial capsule polysaccharide (CPS)[52]. The CPS of *K. pneumoniae* are categorized into 77 diverse capsular serotypes, it is referred to as K. Among these serotypes there are high virulence ones, which are often associated with severe infections such as K1, K2, K4 and K5[49]. Also, in the pyogenic liver abscess, K1, and K2 were the leading cause[53].

To obtain the K types of *K. pneumoniae* bacteria, it is possible to compare the sequence of nitrogenous bases and focus on polymorphisms of one or more of the six cps locus genes (wzi, orf2, wza, wzb, galF and wzc) that involved in the association of the CPS with the outer membrane of bacteria [54], [55]. Moreover, hypervirulent strains isolated from East Asia often show a Hypermucoviscosity phenotype associated with the abnormal production of the outer polysaccharide. It mainly composed of a complicated network of fine fibers emanating from the CPS[56]. Hypermucoviscosity phenotype is formed by genes, including magA, rmpA, and the cps groups itself [57]. Additionally, the mutation only in the magA gene makes the hypermucoviscosity unrecognized and has the impact of phagocytosis, further considering the isolate noninvasive to mice [58].

Due to the impressive combination of virulence and phenotype, accompanying multidrug resistance, *K. pneumoniae* isolates which caused widespread prevalence among patients leads to the spread of nosocomial infections, especially in newborn units[39], [59].

1.3.2. Biochemical characteristics

Biochemical characteristics used for the identification of *K. pneumoniae* include negative indole-test, production of lysine decarboxylase (but not ornithine decarboxylase), fermentation of specific sugars (e.g., D-glucose, lactose, sucrose, Larabinose, and maltose) and sugar alcohols (e.g., D-mannitol). Furthermore, *K. penumoniae* is non-motile and usually produces a prominent acidic polysaccharide-based capsule [60]. Biochemical characteristics are still being used for species identification of bacteria isolated from clinical samples. However, biochemical identification is increasingly performed by automated systems, such as Vitek2 (bioMèrieux, Marcy l'Etoile, France) or Phoenix (BD Diagnostics, Sparks, USA). Moreover, new identification approaches, such as MALDI-TOF mass spectrometry, have been used in many laboratories with great success.

1.4. Antibiotics

Microorganisms have existed on our planet since ancient times. The activity of some of them with the release of deadly toxins to other organisms has enabled scientists to use these compounds to treat some bacterial diseases, which are called **Antibiotics**. The peculiarity of its work is either by inhibiting bacterial growth or by killing the bacteria permanently.

In cases where immunization is either not an option or fails to prevent disease, treatment with antimicrobial drugs is the primary weapon against infection. Antimicrobial drugs either kill or inhibit the growth of microorganisms in the host (in vivo). They are classified based on their molecular structure, their mechanism of action, and the spectrum of antimicrobial activity.

The first antibiotic discovered by Alexander Fleming in 1928 was penicillin, but it was put into therapeutic uses in 1940 (Tan & Tatsumura, 2015). Usually, the source of antibiotics is from soil microbes like fungi and bacteria. Those microorganisms were naturally exposed to these biologically active products during evolution. It was considering billions of years of co-evolution or development of antibiotic production and antibiotic-resistant organisms. The use of this agent was rapidly associated with the resistance of bacteria against each other. Later it was observed that when an antibiotic was introduced for use, the bacteria exhibited sensitivity and resistance to it.

1.4.1. Antibiotics Targets

Antibiotic selectivity may be due to its selection of the receptors to which it will bind. The antibiotic may depend on the pathogen's biochemical events but not the host. Also, according to the mechanism of action and the targets of antibiotic, it can be classified into four groups:

- Lysis of bacterial cells by blocking the synthesis of cell wall when the antibiotic binds to penicillin-binding proteins (PBPs) (e.g., β-lactams).
- Blocking of DNA or RNA synthesis by binding to enzymes like DNA gyrase and stops DNA replication (e.g., rifampicin and quinolones)

- Cytoplasmic membrane synthesis blocking by acting like a detergents and disrupts the osmotic properties of the organism (e.g., polymyxins).
- Block the transcription and translation of genetic material by binding to the 30S subunit of ribosomes, and block the binding of tRNA (e.g., tetracyclines that treat STD and acne).

Generally, antibiotic targets include cell membranes, cell walls, and specific metabolism processes. For example, Daptomycin is a lipopeptide produced by Streptomyces that specifically binds to the residues of phosphatidyl glycerol from the bacterial cytoplasmic membrane leading to pore formation and depolarization of the membrane, which ultimately leads to cell death. Some antibiotics target the outer membrane of the Gram-negative cell. For example, polymyxin is a cyclic peptide that explicitly targets its long hydrophobic tails of LPS layer and ultimately disrupts the membrane structure, causing leakage and cell death [61]. Moreover, more antibiotics target the cell wall of the bacterial cell, these kinds of antibiotics generally mentioned as β-lactams.

1.4.2. β-lactams

Biochemically, ordinarily, it has a standard feature, which is the third carbon molecule, with a nitrogen ring known as a beta-lactams ring (as shown in figure 1.1.), which is highly reactive. Because of dissimilarities in their side chains, it is possible to classify lactams into the mentioned main groups: penicillins, cephalosporins, monobactams, and carbapenems[37].

Therefore, beta-lactams antibiotic is considered as the most prescribed antibiotics and drug classes with many clinical indications. Because of their variety, broad activity ranges, and low toxicity, β-lactams are the world's most prescribed antibiotics[62]. They target the synthesis of cell walls and act by covalent bonds ratification to PBPs.

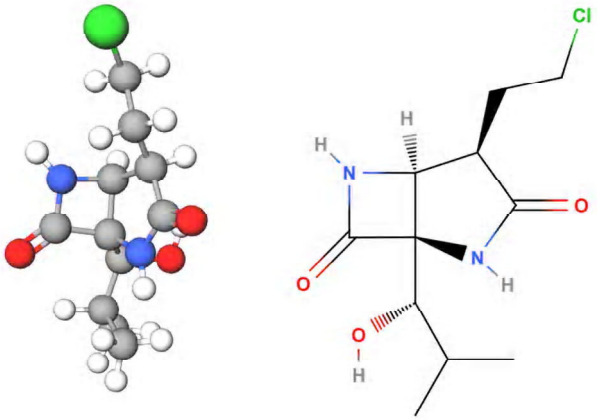


Figure 1.1. A and B are 3D and 2D model of Beta-lactam compound, respectively. (NCBI. PubChem Database. CID=11680493, https://pubchem.ncbi.nlm.nih.gov/compound/Proteasome-Inhibitor-VIII_-beta-Lactam-3 (accessed on Apr. 20, 2020) [63].

β-lactams differ in their side chains and based on side chain they may be classified into these groups: penicillins, monobactams, cephalosporins, and carbapenems[62].

1.4.3. Carbapenems

Carbapenems, members of the β-lactam class of antibiotics that damage bacteria by binding to penicillin-binding proteins and inhibiting cell wall synthesis, were first used in the United States in the 1980s to treat resistant bacteria. The β-lactam antibiotics are structurally related to these drugs. Imipenem, the first drug, has strong efficacy against various gram-negative rods, gram-positive bacteria, and anaerobes. It prevents β-lactamases but is inactivated in renal tubules by dehydropeptidases. As a consequence, it is given along with a peptidase inhibitor, cilastatin.

Imipenem penetrates deep into body tissues and fluids like Cerebrospinal fluid (CSF). The drug is given intravenously every 6–8 hours and in reduced renal insufficiency dosages. Imipenem can be recommended for infections caused by other-drug-resistant pathogens. Imipenem could have greater gram-positive coverage as opposed to the other carbapenems. Meropenem and doripenem protect from Gram-negatives best.

Imipenem's adverse effects include vomiting, diarrhea, skin rashes, and infusion site reactions. Excessive levels can cause seizures in patients with renal failure. Patients with penicillin allergies may be allergic to imipenem, too. Moreover, in pharmacology and antimicrobial activity spectrum, meropenem is identical to imipenem. However, dipeptidases do not inactivate it and are less likely to induce seizures than imipenem.

Ertapenem has a long half-life, perfect for prescribing once daily. It is useful for treating complicated infections that do not include pathogens in hospitals. It has low activity against the species *Enterococcus* and *P. aeruginosa* and other gram-negative rods that do not ferment glucose.

The most recent carbapenem approved for use in the United States is doripenem. The group of sulfamoylaminoethyl–pyrrolidinylthio in its lateral chain at position 2 increases its activity against glucose-fermenting gram-negative rods. Reportedly, this drug has a high affinity for species-specific PBPs. In *P. aeruginosa*, for example, doripenem has an affinity to PBP3. Doripenem is reported to be more active against *P. aeruginosa* than imipenem but equal in action to meropenem. None of the carbapenems have anti-*Stenotrophomonas maltophilia* activities.

In a recent European study, up to 7 percent of all *Klebsiella pneumoniae* bloodstream isolates reported carbapenem resistance. This troubling rise in antimicrobial resistance prescribes the creation of a logical antibiotic approach. Also, it has been documented that carbapenem-resistant infections have a mortality rate of between 40 and 80%[64]. However, the transmission of carbapenem resistance is typically plasmid-related, although chromosomal transmission has also been well established. Specific genes have been reported including *Klebsiella pneumoniae* carbapenemase or bla_{KPC} (worldwide spread), New Delhi metallo-β-lactamase or bla_{NDM-1} (now prevalent in South-East Asia), and others such as OXA-48, VIM (usually found in Europe, Asia and South America)[65].

Prevention and treatment of carbapenem-resistant *Enterobacteriaceae* infections should be a high priority at hospitals and long-term health facilities due to the associated mortality. In both inpatient and outpatient environments, antimicrobial stewardship can be a critical factor in preventing emerging local resistance. Nevertheless, the regulation of infections is essential for the management of cases imported from other facilities. A judgmental use of carbapenems is also key to preventing the production of Local

resistance; these agents should be reserved for high-risk patients with MDR infections. Once a carbapenem-resistant *Enterobacteriaceae* infection is documented, consultation with infectious diseases is recommended to help manage infections.

1.4.4. Antimicrobial Susceptibility Test (AST)

Antibiotic resistance is a health issue worldwide. Methods testing synergies to determine the resistance mechanisms of microbes and varying antibiotic antagonisms are fundamental. Antibiotic resistance is disseminated not only speedily across hospital environments, but in culture as well. However, different methods were developed for investigating these resistance mechanisms, including the broth dilution and the Epsilometer test (E-test)[66]. The E-test method is simple, easy to reproduce, and can be used to explore any synergies possible combination therapy. The process of broth dilution can also be evaluated to forecast bactericidal activity. The broth or agar dilution methods can be used to quantitatively calculate an antimicrobial agent's in vitro activity against a given bacterial isolate. To carry out the experiments, a series of tubes or plates are prepared with a broth or agar medium to which different antimicrobial agent concentrations are applied. A standardized suspension of the research organism is then inoculated on the tubes or surfaces. The tests will be checked after incubation at 35 ± 2 °C for 16 h, and the MIC is determined. The final result is significantly impacted by technique, which must be closely monitored to produce reproducible outcomes.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Several chemicals and kits are used throughout the experiments. Kits, tools, chemicals, and bacterial samples are purchased from different resources and different manufactures (Table 2.1). Sterilized tips and tubes were used for sample preparation, antibiotic susceptibility tests, and PCR work.

Table 2.1. List of chemicals and kits

Name of chemicals and kits	Manufacturer	Aim of use
DNA Polymerase	EasyTaq® Beijing, China	PCR
DNA Polymerase	abm®Taq Canada	PCR
5x Master Mix Ready to Load	FIREPol® Tartu, Estonia	PCR
6x DNA Loading Dye Buffer Blue	SOLIS BIODYNE® Tartu, Estonia	Sample gel loading
6x DNA Loading Dye Buffer	TRANS® Beijing, China	Sample gel Loading
dNTP mix 10µmol	BIOLINE® London, UK	PCR

100bp DNA Ladder	**Atlas Star Tartu, Estonia**	**For precise sizing of dsDNA fragments from 100 to 1000 bp**
100bp DNA Ladder	**Geneaid® Taiwan**	**For precise sizing of dsDNA fragments from 100 to 1000 bp**
Distilled Water DNase/RNase Free	**invitrogen® UK**	**PCR reaction**
$MgCl_2$	**Thermo Fisher Scientific® USA**	**PCR Reaction**
RedSafe	**Abm Canada**	**Detecting DNA fragments**
Meropenem	**Sigma, USA**	**Meropenem susceptibility**
XbaII	**Thermo Fisher Scientific® USA**	**Reestriction enzyme for PFGE**
Low Melting Temperature agarose	**Amresco®**	**PFGE**
14mL Polypropylene Round-Bottom Tube	**FALCON® USA**	**PFGE**
CHEF DNA Size Marker- *S.cerevisiae*	**BioRad USA**	**Used as size for pulsed-field gel electrophoresis**
Ribonuclease A	**vivantis®**	**Degrades single stranded RNA**
Phenol/chloroform/Isoamlalcohol	**Roti®**	**Separation of DNA from other cellular components**
Proteinase K	**SIGMA® USA**	**Digestion of proteins**
TRIS	**MERCK® Germany**	**TBE, TAE and TE preparation**
BORIC ACID	**SIGMA® USA**	**TBE Buffer preparation**
Acetic acid	**SIGMA-ALDRICH® Germany**	**TAE Buffer preparation**
Ethanol	**SIGMA-ALDRICH® Germany**	**Precipitates DNA from solution**
Sodium dodecyl Sulfate	**MERCK® Germany**	**Solubilization of cell membrane lipids**
Plates (Chocolate/Blood agar plates)	**BD BBLTM StackerTM Germany**	**Growing** *Klebsiella pneumonia*

LB broth(1x)	**gibco® USA**	**For high yield growth needed for DNA extraction**
Sequencing company	**Ankara/Turkey**	
Primers prepared in sentebiolab®	**Ankara/Turkey**	**For amplifying target genes**
1.5mL Graduated Microtubes	**SSIbio USA**	**DNA Extraction, PCR mixture preparation**
PCR strips	**USA**	**PCR**

- **10X TAE**

48.4g of Tris Base and 3.72g of EDTA (disodium salt) was dissolved in sterile distilled water, and 11.4 ml of Glacial acetic acid was added and completed to a final volume of 1 litre. The buffer was diluted 10 times and 1X TAE buffer was used for running gel.

- **10X TBE buffer**

108g of Tris base, 55g of Boric Acid and 7.5 g of EDTA was dissolved in 800 ml of sterile distilled water and completed to 1 liter. The pH was adjusted to 8.0 and the solution was sterilized. The buffer was diluted 10 times and 1X TBE buffer was used for running gel.

2.2. Methods

2.2.1. Collection of bacterial isolates from Turkey and Iraq

72 Samples were collected from August 2016 to September 2019 from clinical sources in, Baghdad, Iraq and Kayseri, Turkey. All 72 samples were stored in glycerol-containing microbial bank tubes. Certain characteristics of the samples have been confirmed for suitability to our research purpose, the most important of which is their resistance to one or more of the carbapenems, depending on the initial diagnosis by the Vitek II device.

2.2.2. Determination of minimum antibiotic inhibitory concentrations (MICs) by Microdilution Method (Antimicrobial Susceptibility Test)

Minimum inhibitory concentrations (MICs) are considered as a 'gold standard' for assessing the sensitivity of pathogens to antimicrobial agents in accordance with Clinical and Laboratory Standards Institute [67].

Meropenem which is one of the most efficient among the Carbapenem group antibiotics was used to test clinical isolates used as research materials in this study.

2.2.2.1. Preparation of antibiotic solution for MIC tests

To reach desired antibiotic concentration, the following formula and calculation was carried out;

Concentration = 1280µg/ml

Potency = 980µg/ml

Volume =? ml

$$Weight(mg) = \frac{Volume(mL) * Concentration(\mu g / mL)}{Potency(\mu g / mg)}$$

$$Volume(mL) = \frac{Weight(mg) * Potency(\mu g / mg)}{Concentration(\mu g / mL)}$$

$$Volume(mL) = \frac{10 * 980}{1280} = 7.656mL$$

Therefore, 10 mg of meropenem powder was dissolved in 7.656 mL of distilled water and kept in -20 until use. The stock solution was diluted 10 times in Muller Hinton broth to reach working concentration (128µg/ml). 1ml of meropeneme stock solution

was diluted in 9ml of Muller Hinton Broth to reach final concentration of 128µg/ml of meropeneme.

2.2.2.2. Inoculum Preparation for Dilution Tests

All isolates were cultured on Blood agar for 18-24 hours. Fresh colony was picked and diluted to 1 to 2 × 10^8 colony-forming units (CFU)/mL in 2.5mL of Sterile Saline to reach 0.5 McFarland standard. 1mL from suspension was transferred to 9.9 mL of Sterile Saline to reach 2 × 10^6 CFU/mL. Then, 0.5mL was transferred from last dilution to 4.5mL of Muller Hinton Broth to reach 2 × 10^5 CFU/mL. The suspension was ready to use in micro dilution plate.

2.2.2.3. Microplate preparation

The microplate with 96 wells was used for MIC test. The steps of procedure as follows (figure 2.1);

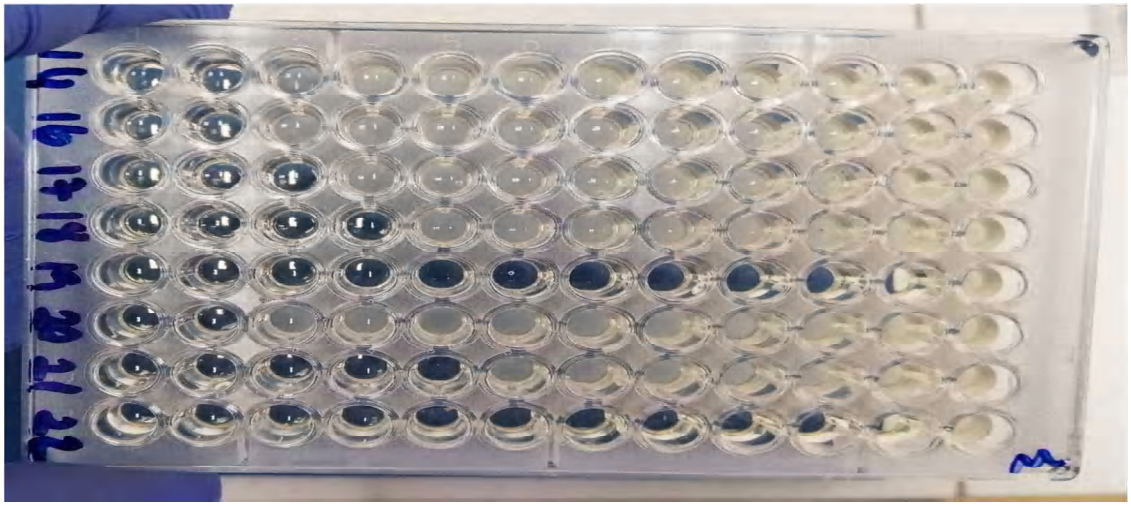
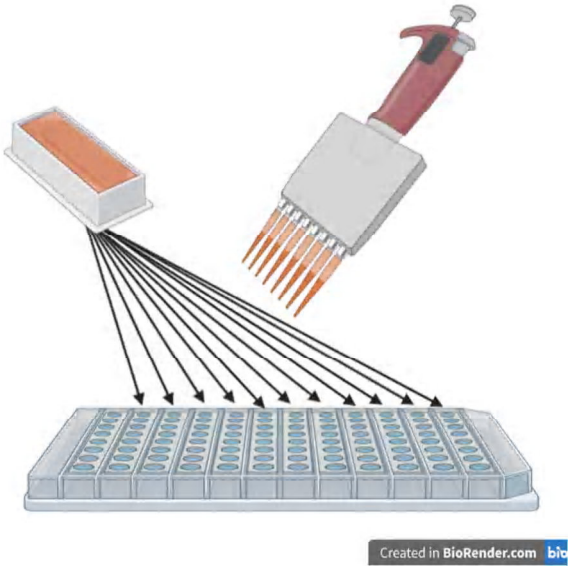
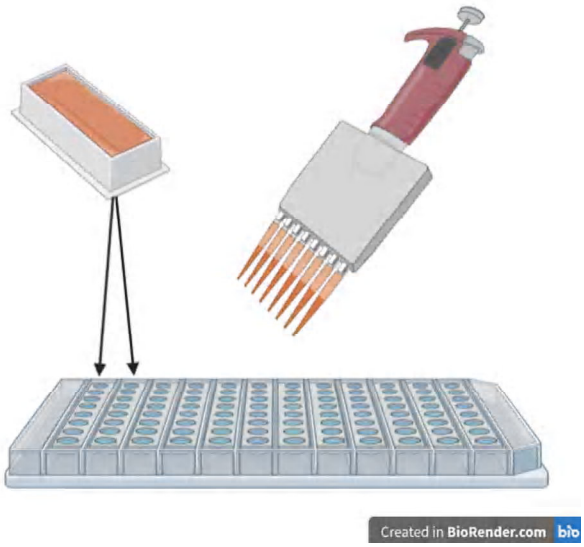


Figure 2.1. AST plate

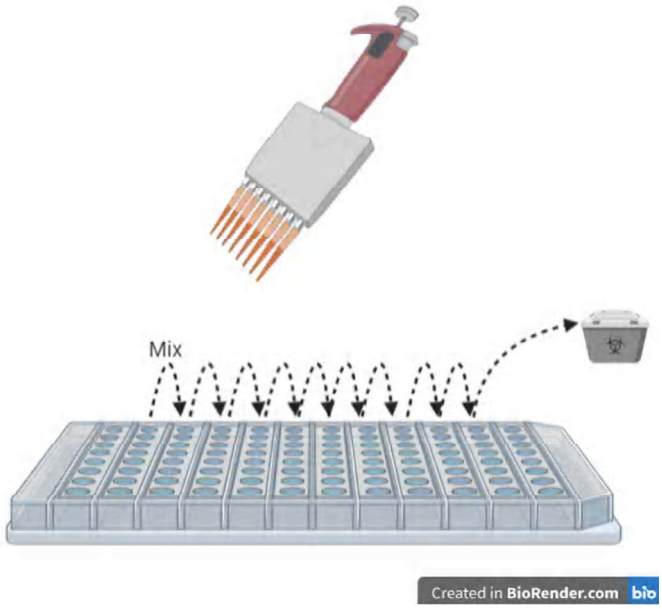
1. 100µL from Mueller Hinton broth was transferred to each hole except first column



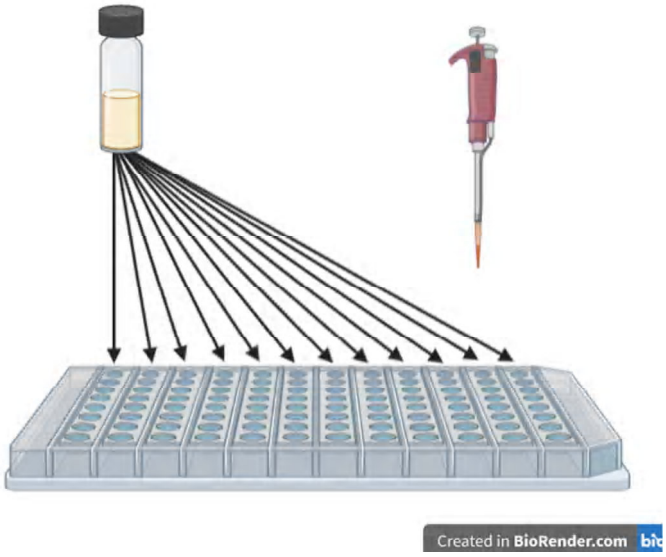
2. 100µL from meropeneme stock solution (128µg/mL) was transferred to first and second column.



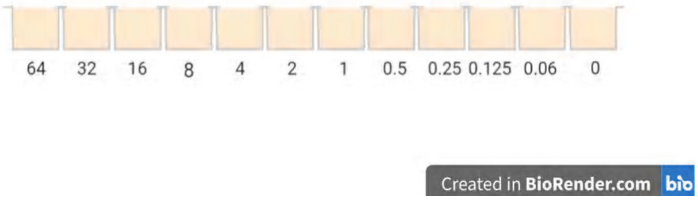
3. From second column mixing was started by using multichannel pipette and 100µL was transferred to the next column until column 11 and throw final 100µL to the waste to keep column 12 without meropeneme to use it as a positive control for the inoculum.



4. 100μL inoculum suspension (2 × 105 CFU/mL) was transferred to each hole.



5. Final concentration of meropenem in each well was shown as follows



6. All plates were incubated at 36°C for 18-24 hr and observed by naked eyes based on the appearance of turbidity.

2.2.3. DNA Extraction

DNA extraction was carried out as described previously by [68]. DNA samples were diluted ten times for PCR assay.

2.2.4. Polymerase Chain Reaction (PCR)

To perform MLST of *K. pneumoniae*, we amplified seven housekeeping genes as recommended by Institute Pasteur. This platform hosts MLST databases and whole genome typing schemes that are used for genotyping bacterial isolates. These include reference nomenclatures of microbial strains and are intended primarily for the molecular epidemiology of essential public health pathogens, the identification of virulence and antimicrobial resistance genes, and population biology research. The primer sequences of seven Housekeeping genes are given in Table 2.2

Table 2.2. Primers used in this study

Primer	Sequence	Annealing Temperature
gapA	**F: gapA173: TGAAATATGACTCCACTCACGG** **R: gapA181: CTTCAGAAGCGGCTTTGATGGCTT**	**52°C**
infB	**F: infB1F: CTCGCTGCTGGACTATATTCG** **R: infB1R: CGCTTTCAGCTCAAGAACTTC**	**48°C**
Mdh	**F: mdh130: CCCAACTCGCTTCAGGTTCAG** **R: mdh867: CCGTTTTTCCCCAGCAGCAG**	**56°C**
Pgi	**F: pgi1F: GAGAAAAACCTGCCTGTACTGCTGGC**	**58°C**

	R: pgi1R: CGCGCCACGCTTTATAGCGGTTAAT	
phoE	**F: phoE604.1: ACCTACCGCAACACCGACTTCTTCGG** **R:phoE604.2: TGATCAGAACTGGTAGGTGAT**	**51°C**
rpoB	**F: Vic3: GGCGAAATGGCWGAGAACCA** **R: Vic2: GAGTCTTCGAAGTTGTAACC**	**49°C**
tonB	**F: tonB1F: CTTTATACCTCGGTACATCAGGTT** **R: tonB2R: ATTCGCCGGCTGRGCRGAGAG**	**53°C**
Wzi	**wzi_for2: GTGCCGCGAGCGCTTTCTATCTTGGTA TTCC** **wzi_rev: GAGAGC CAC TGG TTC CAG AAC TTG ACCGC**	**55°C**

*

2.2.4.1. Optimization of PCR conditions

PCR conditions were optimized to obtain single and specific band for each gene. 5x FIREPol® Master Mix Ready to Load was used for optimization of PCR conditions. In a sterile microfuge tube, 4µL of master mix, 1µL Forward primer (10 µM stock), 1µL Reverse primer (10 µM stock) and 2µL DNA template were mixed. For optimization, various concentrations of MgCl2 was used. Final volume was adjusted to 20µL by adding distilled water. Gradient PCR was also used to optimize annealing temperature for some of the primers giving nonspecific and faint bands.

2.2.4.2. PCR amplification of gene fragments (amplicons) for sequencing

Master mix was prepared in a sterile microcentrifuge tube by adding 4µL 10x Taq polymerase buffer, 2µL Forward primer (10 µM stock), 2µL Reverse primer (10 µM stock), 2µL DNA template, different MgCl2 concentrations (2 to 3mM), 0.8µL dNTP mix (200 µM), 1 unit of Taq polymerase. Finally, the volume adjusted to 40µL by adding Distilled water. Then, thermocycler operated by following program (Table 2.3).

Table 2.3. The PCR cycle of the housekeeping and wzi genes

Primer	Stages		Cycles
gapA **infB** **mdh** **pgi** **phoE** **ropB** **tonB**	**Initial Denaturation**	**94°C for 2 min**	**1**
	Denaturation **Annealing** **Extension**	**94°C for 20 sec** **48-58°C for 30 sec** **72 °C for 30 sec**	**35**
	Extension	**72 °C for 5 min**	**1**
Wzi	**Initial Denaturation**	**94°C for 2 min**	**1**
	Denaturation **Annealing** **Extension**	**94°C for 30 sec** **55°C for 40 sec** **72 °C for 30 sec**	**30**
	Extension	**72 °C for 5 min**	**1**

2.3. Amplicon Sequencing

All amplicons were sequenced by Illumina Miseq platform. 2x150bp paired end reads and assembled by Assembler (CLC workbench). The sequencing was carried out by (SuGenomik Biyoteknoloji) Ankara/Turkey.

2..Sequence Data Analysis of Housekeeping genes and Wzi gene

The housekeeping genes and wzi gene were analyzed in Sequences and profiles database/Institute Pasture (https://bigsdb.pasteur.fr/cgi-

bin/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef) to determine Sequence type and allelic profile and K-type for each isolate.

2.5. PFGE

For *K. pneumoniae*, we used standard operating protocol for Pulse Net[69]. The DNA of the bacteria was cut by XbaI restriction enzyme and the following protocol was used.

XbaI enzyme recognition site:

5' T ↓C T A G A 3'

3' A G A T C ↑T 5'

1. Inoculate 50 isolates on blood agar and incubate for 20-24 hours at 35-36 °C.

2. Suspend bacterial colonies in cell suspension buffer (100 mM Tris, 100 mM EDTA, pH 8.0) (2.0 mL) at a concentration of 5-6 McFarland.

3. Mix the cell suspension (200.0 µL) with 20 mg/mL Proteinase K (10.0 µL), then add 1% Amresco Low Milting agarose1 (200.0 µL), mix briefly and transfer to PFGE plug moulds.

4. After solidification, add 0.5mL of lysis I (50 mM Tris, 50 mM EDTA, pH 8.0, Lysosome 2.5mg/mL and proteinase K 1.5mg/mL) to all plugs that kept in sterile falcon Polypropylene Round-Bottom Tubes and incubated for 1 hour with shaking.

5. Discard all solution, add 400µg/mL proteinase K with lysis II (50 mM Tris, 50 mM EDTA, pH 8.0, 1% Sarcosyl) to the plugs and incubated for 2 hours.

6. Discard all solution, then wash plugs 3 times with pre-heated dH2O (50 °C, 4 ml) for 10-15 minutes at 50 °C, then 3 times with pre-heated TE-buffer (50 °C, 4 ml) for 10 – 15 minutes at 50 °C.

7. Store washed plugs in TE-buffer at 4 °C.

8. Cut 1.5 mm pieces of the plugs and incubate at 37°C for 15 minutes in 1:10 diluted XbaI2 restriction buffer (200.0 µl).

9. Incubate plugs at 37 °C for 5 hours in XbaI restriction enzyme2 mix (table 3.18).

10. Prepare a 1.1 % Pulsed Field Certified Agarose gel.

11. Fill the electrophoresis chamber with 0.5 x TBE buffer (1.9 L). Calibrate pump to pump one liter per minute, and set temperature to 12 °C.

12. Mount the plugs on the gel comb and fix with agarose. Pour the gel, remove the comb and add lambda ladder2.

13. Seal wells with agarose and run the gel with the settings presented in table 2.4.

14. Dye gel with ethidium bromide solution for 30 minutes.

15. Depict gel.

Table 2.4.: Parameters of running PFGE gel.

Parameter	Value
Pulse Time	**5 – 30 s**
Total run time	**20 H**
Voltage	**6.0 v/cm (200 V)**
Buffer temperature	**12°C**
Buffer	**0.5 x TBE**

CHAPTER 3

RESULTS

3.1. Identification of Isolates

All 72 isolates were diagnosed by biochemical test and Vitek II Systems for *Klebsiella pneumoniae*.

Initial cultures contained mixed Gram-negative bacteria and those cultures were purified and a pure single colony was obtained. A total of 72 isolates were identified as non-fermenting Gram-negative *Klebsiella spp*. Phenotypic and automated identification is not nearly as accurate as molecular identification. Further genotypic identification was done thorough MLST by amplicon sequencing of 7 housekeeping genes and PFGE to ensure the isolates are *Klebsiella pneumoniae*.

3.2. Automated and phenotypic susceptibility testing

Routine susceptibility testing in the all laboratories in Turkey and Iraq are done by disk diffusion in accordance with CLSI guidelines. Susceptibility is tested furthermore using the automated machines as stated. The isolates were resistant to imipenem, meropenem, ertapenem or more than 1 of these antibiotics. Moreover, these isolates were found to be resistant to those antibiotics when Antibiotic Susceptibility testing by microdilution method was applied to approve phenotypic resistance to meropenem as shown (table 3.1). However, 44 isolates that meropenem resistant and 6 isolates which are not resistant to meropenem was studied (4:3.1).

Table 3.1. MICs of all isolates.

#	MIC Mero	Vitek Mero	Other Carbapenems	Source	Country	Isolation year
1	32	>16	IMP R	Blood culture	TR	2018
2	>64	>16	IMP R	Urine	TR	2018
3	32	>16	IMP 2 Inter	Urine	TR	2018
4	>64	>16	IMP >16 R	Blood culture	TR	2018
5	32	>16	IMP 8 Inter	Urine	TR	2018
6	16	>16	ERT 8 R	Blood culture	TR	2018
7	16	4 Inter	IMP 8 Inter	skin swab	TR	2018
8	16	>16	IMP 2 Inter	Blood culrure	TR	2018
9	16	>16	IMP 8 Inter	respiratory system	TR	2018
11	4	4 Inter	ERT >8 R	Urine	TR	2018
12	16	>16	ERT >8 R	Urine	TR	2018
13	>64	>16	IMP >16 R	respiratory system	TR	2018
14	32	>16	ERT >8 R	Blood culture	TR	2018
15	32	>16	ERT >8 R	Skin swab	TR	2018
16	32	>16	IMP >16 R	respiratory system	TR	2018
17	16	X		Blood culture	IQ	2016
18	8	X		Ear swab	IQ	2016
19	0.06	X		Sputum	IQ	2016
20	32	X		Wound	IQ	2016
21	4	X		Urine	IQ	2016
22	0.06	X		Sputum	IQ	2016
23	32	X		Sputum	IQ	2016
24	4	X		Urine	IQ	2016
25	8	X		Sputum	IQ	2016
26	4	X		Blood culture	IQ	2016
27	8	X		Ear Swab	IQ	2016
28	0.06	X		Sputum	IQ	2016
29	4	X		Wound	IQ	2016
30	32	X		Ear Swab	IQ	2016
31	8	X		Sputum	IQ	2016
32	16	X		Blood culture	IQ	2016
33	32	X		Sputum	IQ	2016
34	32	X		Blood culture	IQ	2016
35	32	X		Wound	IQ	2016
36	32	X		Wound	IQ	2019
37	0.06	X		Sputum	IQ	2019
38	0.06	X		Blood culture	IQ	2019
39	32	X		Wound	IQ	2019
40	32	X		Urine	IQ	2019
41	0.25	X		Urine	IQ	2019

42	0.5	0.5	ERT 1 Inter	Urine	TR	2019
43	32	>16	ERT>8 R	Skin Swab	TR	2019
44	32	>16	IMP 8 Inter	Skin Swab	TR	2019
45	64	>16	ERT >8 R	Skin Swab	TR	2019
46	32	>16	IMP R	Respiratory System	TR	2019
47	32	>16	IMP 8 Inter	Skin Swab	TR	2019
48	16	X	X	Wound Swab	TR	2019
49	>64	>16	IMP >16 R	Skine Swab	TR	2019
50	32	>16	IMP 8 Inter	Blood culture	TR	2019
51	64	>1	IMP >16 R	Sputum	TR	2019
52	0.06	<0.25	IMP 0.5 S	Seminal fluid	IQ	Isolation year
53	16	2 Inter	IMP 2 In ERT 1 In	Urine	IQ	2018
54	4	8 R	IMP >16 R	Urine	IQ	2018
55	0.06	2 Inter	ERT 1 Inter	Urine	IQ	2018
56	0.06	Inter	X	Urine	IQ	2018
57	0.06	X	ERT 1 R IMP Inter	Urine	IQ	2018
58	4	1 S	ERT <0.5 IMP Inter	Urine	IQ	2018
59	0.06	X	IMP +	Seminal fluid	IQ	2018
60	0.125	X	ERT <0.5 S	Respiratory System	IQ	2018
61	4	X	X	respiratory system	IQ	2018
62	0.06	X	X	Urine	IQ	2018
63	0.06	X	X	Urine	IQ	2018
64	0.125	X	X	respiratory system	IQ	2018
65	0.06	X	X	Blood culture	IQ	2018
66	0.06	X	X	Skin swab	IQ	2018
67	0.06	X	X	respiratory system	IQ	2018
68	0.06	X	X	Blood culture	IQ	2016
69	0.06	X	X	Ear swab	IQ	2016
70	0.06	X	X	Sputum	IQ	2016
71	0.06	X	X	Wound	IQ	2016
72	0.06	X	X	Urine	IQ	2016
73	32	>8 R	IMP 8 R	Sputum	TR	2016
74	0.06	X	Negative Control	E.coli ATCC® 25922™	TR	X
75	8	X	Positive control	K. pneumoniae ATCC® BAA-1705™	TR	X

3.3. Sources of Isolates

The isolates were obtained from various sources. The highest frequency of the isolates was collected from Urine samples and the lowest from Wound (figure 3.1.).

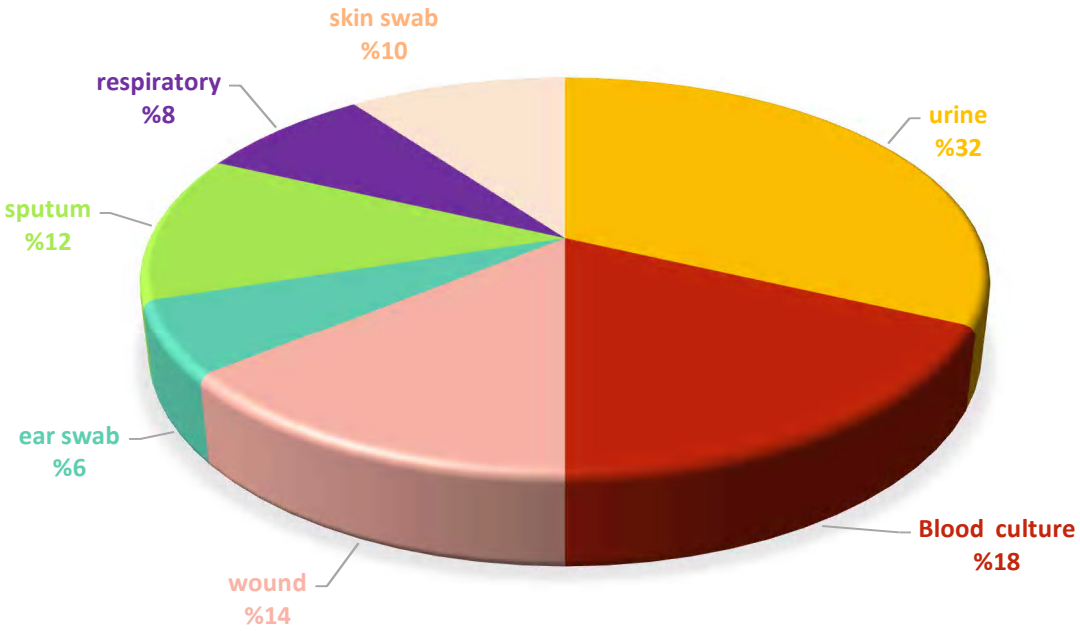


Figure 3.1. The percentage of sources of isolates.

3.4. Amplicon Sequencing of the Seven Housekeeping Genes for MLST and wzi gene

Seven housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB, tonB, wzi) for 44 isolates which are meropenem resistant and 6 non-meropenem resistant were amplified by PCR. The amplicons were sequenced by Next Generation Sequencing by illumina technology. Amplification of 5 Iraqi (24,54,57,58 and 61#) isolates were not successful for seven housekeeping genes in 50 isolates indicating their misdiagnosis by automated vitek system. Furthermore, we replaced them with 5 Iraqi meropenem non-resistant isolates (62, 65, 67, 68 and 70), which are resistant to other carbapenems such as imepenem or ertapenem according VitekII results. Moreover, we sequenced the amplicons of those seven housekeeping genes (gapA, infB, mdh, pgi, phoE, rpoB, tonB, wzi) by Next Generation Sequencing.

3.5. Sequences Data Analysis

The data analysis of fastA files of amplicons were carried out in two websites. First one was Institute Pasture, CGE (Center for Genomic Epidemiology) BIONUMERICS software program (version 8.0; Applied Maths, Sint-Martens-Latem, Belgium) to reach the sequence Types of 50 *K. pneumoniae* isolates. The results were shown in Table 3.2.

Table 3.2. ST and K-type of all isolates.

ID	Sequence Type	Isolation year	City/ Country	Source	Meropenem AST	K-type	gapA	infB	Mdh	pgi	phoE	rpoB	tonB
1	15	2018	Kayseri/Turkey	Blood culture	32	27	1	1	1	1	1	1	1
2	15	2018	Kayseri/Turkey	Urine	>64	27	1	1	1	1	1	1	1
3	2096	2018	Kayseri/Turkey	Urine	32	14.64	1	6	1	1	1	46	1
4	307	2018	Kayseri/Turkey	Blood culture	>64	173*	4	1	2	52	1	1	7
5	15	2018	Kayseri/Turkey	Urine	32	27	1	1	1	1	1	1	1
6	15	2018	Kayseri/Turkey	Blood culture	16	27	1	1	1	1	1	1	1
7	15	2018	Kayseri/Turkey	skin swab	16	27	1	1	1	1	1	1	1
8	?????	2018	Kayseri/Turkey	Blood culture	16	17	2	6	1	5	4	1	704 Novel
9	377	2018	Kayseri/Turkey	respiratory system	16	173*	10	20	2	1	9	11	12
11	15	2018	Kayseri/Turkey	Urine	4	27	1	1	1	1	1	1	1
12	15	2018	Kayseri/Turkey	Urine	16	27	1	1	1	1	1	1	1
13	307	2018	Kayseri/Turkey	respiratory system	>64	173*	4	1	2	52	1	1	7
14	16	2018	Kayseri/Turkey	Blood culture	32	k15k17k50k51k52	2	1	2	1	4	4	4
15	15	2018	Kayseri/Turkey	Skin swab	32	27	1	1	1	1	1	1	1
16	15	2018	Kayseri/Turkey	respiratory system	32	27	1	1	1	1	1	1	1
17		2016	Baghdad/Iraq	Blood culture	16	328*	17	0	0	20	0	0	0
18	15	2016	Baghdad/Iraq	Ear swab	8	27	1	1	1	1	1	1	1
19	530	2016	Baghdad/Iraq	Sputum	<0.06	54	2	39	2	2	1	4	24
20		2016	Baghdad/Iraq	Wound	32	20	4	1	2	37	21	4	36
21	15	2016	Baghdad/Iraq	Urine	4	27	1	1	1	1	1	1	1
23	307	2016	Baghdad/Iraq	Sputum	32	173*	4	1	2	52	1	1	7
25	147	2016	Baghdad/Iraq	Urine	8	K14.K64	3	4	6	1	7	4	38
26		2016	Baghdad/Iraq	Sputum	4	62	4	1	2	1	1	7	Novel 707
27	15	2016	Baghdad/Iraq	Wound	8	27	1	1	1	1	1	1	1
29	37	2016	Baghdad/Iraq	Wound	4	529*	2	9	2	1	13	1	16
30	2943	2016	Baghdad/Iraq	Ear Swab	32	39	2	52	2	1	12	1	34
31	15	2016	Baghdad/Iraq	Sputum	8	27	1	1	1	1	1	1	1
32	15	2016	Baghdad/Iraq	Blood culture	16	27	1	1	1	1	1	1	1
33	15	2016	Baghdad/Iraq	Sputum	32	27	1	1	1	1	1	1	1

ID	Sequence Type	Isolation year	City/ Country	Source	Meropenem AST	K-type	gapA	infB	Mdh	pgi	phoE	rpoB	tonB
34		2016	Baghdad/Iraq	Blood culture	32	328*	17	0	0	20	0	0	0
35		2016	Baghdad/Iraq	Wound	32	328*	17	19	0	20	0	0	0
36	2943	2016	Baghdad/Iraq	Ear Swab	32	39	2	52	2	1	12	1	34
39	15	2016	Baghdad/Iraq	Wound	32	27	1	1	1	1	1	1	1
40	15	2016	Baghdad/Iraq	Urine	32	27	1	1	1	1	1	1	1
43	15	2019	Kayseri/Turkey	Urine	32	27	1	1	1	1	1	1	1
44	15	2019	Kayseri/Turkey	Skin Swab	32	27	1	1	1	1	1	1	1
45	15	2019	Kayseri/Turkey	Skin Swab	64	27	1	1	1	1	1	1	1
46	15	2019	Kayseri/Turkey	respiratory system	32	27	1	1	1	1	1	1	1
47	101	2019	Kayseri/Turkey	Skin Sawb	32	17	2	6	1	5	4	1	6
48	101	2019	Kayseri/Turkey	Wound	16	17	2	6	1	5	4	1	6
49	16	2019	Kayseri/Turkey	Skin Swab	>64	K15K17K50K51K52	2	1	2	1	4	4	4
50	15	2019	Kayseri/Turkey	Blood culture	32	27	1	1	1	1	1	1	1
51	15	2019	Kayseri/Turkey	Sputum	64	27	1	1	1	1	1	1	1
53	469	2019	Baghdad/Iraq	Urine	16	493*	2	1	2	1	10	1	4
62	4634	2019	Baghdad/Iraq	Urine	0.06	122*	2	1	1	97	7	25	25
65	?????	2019	Baghdad/Iraq	Urine	0.06	62	2	0	0	0	7	0	12
67	14	2019	Baghdad/Iraq	Urine	0.06	2	1	6	1	1	1	1	1
68	15	2019	Baghdad/Iraq	Urine	0.06	27	1	1	1	1	1	1	1
70	15	2019	Baghdad/Iraq	Urine	0.06	27	1	1	1	1	1	1	1
73	15	2019	Kayseri/Turkey	Urine	32	27	1	1	1	1	1	1	1

3.6. Data Summary

We used START2 program to obtain the allele and profile frequencies in accordance with the suggestion of PubMLST website. Moreover, we diagnosed 13 different STs that distributed among 43 isolates. For one isolate (20#) allelic combination was determined but ST was not. This isolate may have unique allelic combination. Of the isolates 2 of them (8# and 26#) have novel tonB and they might be novel STs by this new allelic combination. Also, 4 isolates (17#, 34#, 35# and 65#) have unique alleles, so their allelic combination and STs was not determined (table 5.3.3)

3.6.1. Allele Frequencies

Institute Pasture website diagnose only allelic profiles stored in their database. For this reason, some of the alleles was found unique and undiagnosed previously in website. We submitted those unique ones to Pasteur website requesting to add them to their database. Allele frequencies were determined by PubMLST START2 program (table 3.3.)

Table 3.3. Allele frequencies of 50 isolates. The number of alleles for each locus was shown

Allele	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*
1	28	35	32	37	33	37	28
2	12	-	13	1	-	-	-
3	1	-	-	-	-	-	-
4	5	1	-	-	5	5	3
5	-	-	-	3	-	-	-
6	-	5	1	-	-	-	2
7	-	-	-	-	3	1	3
9	-	1	-	-	1	-	-
10	1	-	-	-	1	-	-
11	-	-	-	-	-	1	-
12	-	-	-	-	2	-	2
13	-	-	-	-	1	-	-
16	-	-	-	-	-	-	1
17	3	-	-	-	-	-	-
19	-	1	-	-	-	-	-
20	-	1	-	3	-	-	-
21	-	-	-	-	1	-	-
24	-	-	-	-	-	-	2
25	-	-	-	-	-	1	1
34	-	-	-	-	-	-	2
37	-	-	-	1	-	-	-
38	-	-	-	-	-	-	1
39	-	1	-	-	-	-	-
46	-	-	-	-	-	1	-
52	-	2	-	3	-	-	-
97	-	-	-	1	-	-	-
704	-	-	-	-	-	-	1
707	-	-	-	-	-	-	1
Unique	6	8	3	7	8	6	12

Profile frequencies

ST15 were the highest frequency with 26 isolates among the 46 isolates studied. Of the 26 isolates, 16 of them were isolated from Turkey and 10 from Iraq. Three isolates identified as ST307 and, 2 of them were from Turkey and 1 from Iraq. Only two isolates with ST101 was found among Turkish isolates. Moreover, 2943 ST appeared in 2 Iraqi isolates, and 2096 ST and 377 ST in one Turkish isolate for each sequence type. Also,

14, 4634, 469, 37, 530, 147 STs appeared only once among Iraqi isolates for each sequence type (Table 3.4.).

Table 3.4. Profile frequencies of 50 isolates. Diagnosed and undiagnosed profiles were determined by START2 program.

Complete profiles: 16

ST	*gapA*	*infB*	*mdh*	*pgi*	*phoE*	*rpoB*	*tonB*	*Frequency*	*%*
15	1	1	1	1	1	1	1	26	52
307	4	1	2	52	1	1	7	3	6
16	2	1	2	1	4	4	4	2	4
101	2	6	1	5	4	1	6	2	4
2943	2	52	2	1	12	1	34	2	4
14	1	6	1	1	1	1	1	1	2
2096	1	6	1	1	1	46	1	1	2
4634	2	1	1	97	7	25	25	1	2
469	2	1	2	1	10	1	4	1	2
	2	6	1	5	4	1	704	1	2
37	2	9	2	1	13	1	16	1	2
530	2	39	2	2	1	4	24	1	2
147	3	4	6	1	7	4	38	1	2
	4	1	2	1	1	7	707	1	2
	4	1	2	37	21	4	24	1	2
377	10	20	2	1	9	11	12	1	2

Incomplete profiles: 3

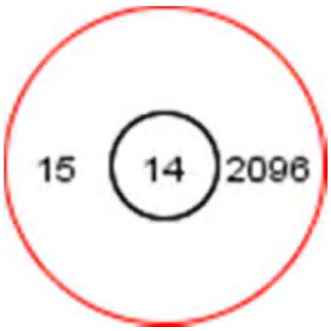
ST	gapA	infB	Mdh	pgi	phoE	rpoB	tonB	Frequences	%
-	17			20				2	4
-	2				7		12	1	2
-	17	19		20				1	2

3.6.2. BURST (Based Upon Related Sequence Types)

The approach looks primarily at the relationships within clonal complexes while ignoring the relationships within different clonal complexes. The implementation can work with four or more loci datasets. We applied our dataset to BURST as shown down in table(3.5.), and we obtained single-locus variants (SLVs) among ST 14 (gapA 1, infB 6, mdh 1, pgi 1, phoE 1, rpoB 1, tonB 1), ST 15 (gapA 1, infB 1, mdh 1, pgi 1, phoE 1, rpoB 1, tonB 1) and ST 2096 (gapA 1, infB 6, mdh 1, pgi 1, phoE 1, rpoB 46, tonB 1). The middle circle contains STs that vary at a single locus. That means they have relationships within clonal complexes. Also, there is relationship within clonal complexes between ST15 (gapA 1, infB 1, mdh 1, pgi 1, phoE 1, rpoB 1, tonB 1) and ST2096 (gapA 1, infB 6, mdh 1, pgi 1, phoE 1, rpoB 46, tonB 1). In DLVs (double-locus variants) as appeared in shape of BURST, the outer isolates contain STs that vary at two loci, but other STs within our isolates did not have relationships within clonal complexes.

Table 3.5. Based Upon Related Sequence Types of 50 isolates (Group definition: Each ST matches at least one other ST in group at 2 or more loci. Groups with central STs will be displayed as an image)

ST	Frequency	SLV	DLV	SAT
15	26	1	1	10
2096	1	1	1	10
307	3	0	0	12
377	1	0	0	12
16	2	0	1	11
530	1	0	0	12
147	1	0	0	12
37	1	0	0	12
2943	2	0	0	12
101	2	0	0	12
469	1	0	1	11
4634	1	0	0	12
14*	1	2	0	10



3.7. Relationships Between Isolates

We created phylogenetic tree based on rpoB (beta-subunit of RNA polymerase) gene which is a ribosomal gene used to identify species. This is one of the housekeeping gene which is included in MLST analysis. This gene is considered as strong as 16sRNA gene for species diagnosis. Phylogenetic tree was also constructed based on seven housekeeping genes to increase the power of differentiation between closely related isolates. Clonal relationships of those isolates were also determined based on PFGE.

3.7.1. rpoB Genes

Minimum spanning tree was constructed based on rpoB gene sequences by using BIONUMERICS software program (version 8.0; Applied Maths, Sint-Martens-Latem, Belgium), and our isolates clustered to 8 groups according to allele sequences of each isolate (figure 3.2.). In the first group, only isolate 2# with ST 2096 and allelic number 46 of gene rpoB was placed. In Second group, isolates with allelic number 1 clustered together, and they were 37 isolates , of which 26 isolates with ST 15, 3 isolates with ST 307, 2 isolates with ST 2943, 2 isolates with ST 101, and one isolates for each ST 469, 37, and 14 was determined. The isolate 8 has novel ST. There is one difference between allelic number 46 and 1 was in position 60, the base quanine in allele 46 changed to Thymine and the identity between both alleles were 99.80 %. Third group, 5 isolates shared allelic number 4 of rpoB, and they were different from the second group in single variant base in position 130, the Guanine in allele 4 changed to Adenine in allele 1 that makes the identity between them 99.80 %. Fourth group, contains only isolate 26# with novel ST and its rpoB allelic number is 7 which differs from third group in single variant base in position 93, the Adenine in allele 4 changed to Guanine in allele 7, and the identity between to alleles was 99.80 %. Fifth group contains only isolate 62 with ST 4634 with rpoB allelic number 25 differs from third group in single base variant in position 339, the Adenine in allele 25 changed to Guanine in allele 4, and the identity between alleles was 99.80 %. Sixth group, contains only isolate 9 with ST 377 and its rpoB allelic number is 11 which is different from third group in double variant bases in position 87 and in 333, in both positions the Thymine in allele 11 changed to Cytosine in allele 4, and the identity between two alleles was 99.60 %.

Seventh group, contains only isolate 65 with undiagnosed ST and rpoB differing from third group in 15 bases variant. Their identity level was 97.01% (Table 3.6.)

Table 3.6. The differences between rpoB alleles of 65 and ropB allele number 4.

Position	rpoB: 4	65# rpoB: ?
P3	C→ T	
P24	G → A	
P33	G → C	
P60	T → C	
P81	G → A	
P96	T → C	
P111	G → T	
P195	T → C	
P220	A → G	
P222	C → A	
P246	T → A	
P249	C → T	
P411	G → C	
P450	C → T	
P495	G → A	

The last group consist of 3 isolates with undiagnosed STs and rpoB allele number. Those isolates might either be novel isolates, or they may be misdiagnosed, they differ from third group in 12 bases variant. The identity between them and rpoB 4 was 98% (Table 3.7.)

Table 3.7. Nucleotide differences between rpoB sequence of Eighth group (17#, 34# and 35#) and ropB allele number 4.

Position	rpoB: 4	17, 34 and 35# rpoB: ?
P3	C→ T	
P24	G → A	
P60	T → C	
P75	G → A	
P105	T → C	
P144	T → C	
P246	T → G	
P411	G → C	
P468	T → A	
P474	T → C	
P483	T → C	
P495	G → T	

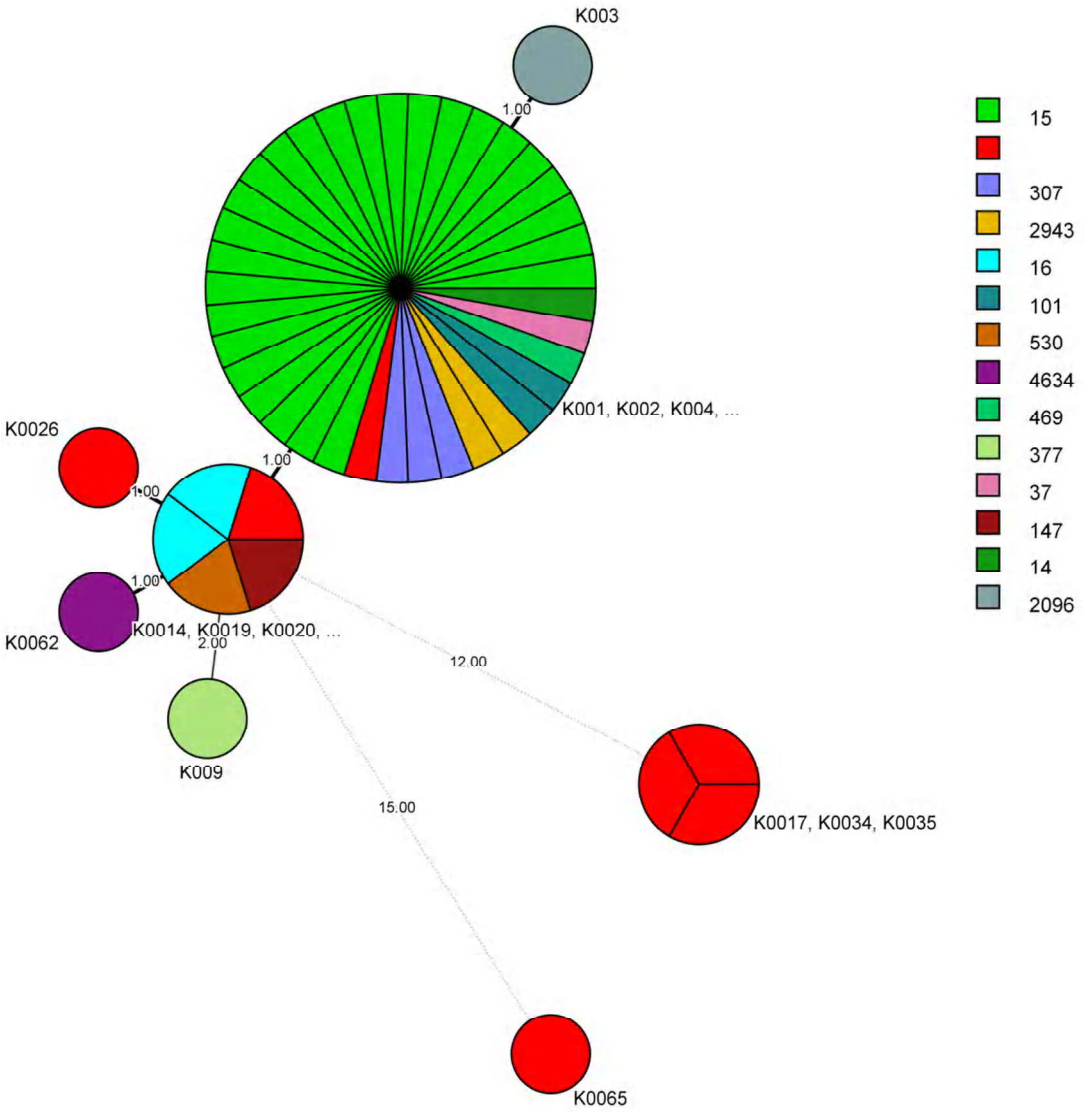


Figure 3.2. Minimum Spanning Tree based on rpoB gene sequences of isolates. (Bionumerics 8.0 Version)

3.7.2. MLST

In MLST analysist 43 of isolates were grouped in 13 sequence types (ST), and 7 isolates did not have a known ST equivalent, they could not be typed. 26 isolates with ST15 were grouped in same MLST type, 3 isolates with ST307, 2 isolates in each ST 101, ST16 and ST2943 and one isolate in each ST14, ST4634 ST530, ST469, ST37, ST147, ST377 and ST2096 (Table 1). Minimum spanning tree obtained from 7 housekeeping gene sequences (figure 3.3.) differentiate the isolates better compared to minimum spanning tree (figure 3.2.) obtained based on rpoB gene sequences. Moreover,

phylogenetic tree based on housekeeping genes differentiate every isolate according its ST and same STs with 100% similarity are grouped together (figure 3.4).

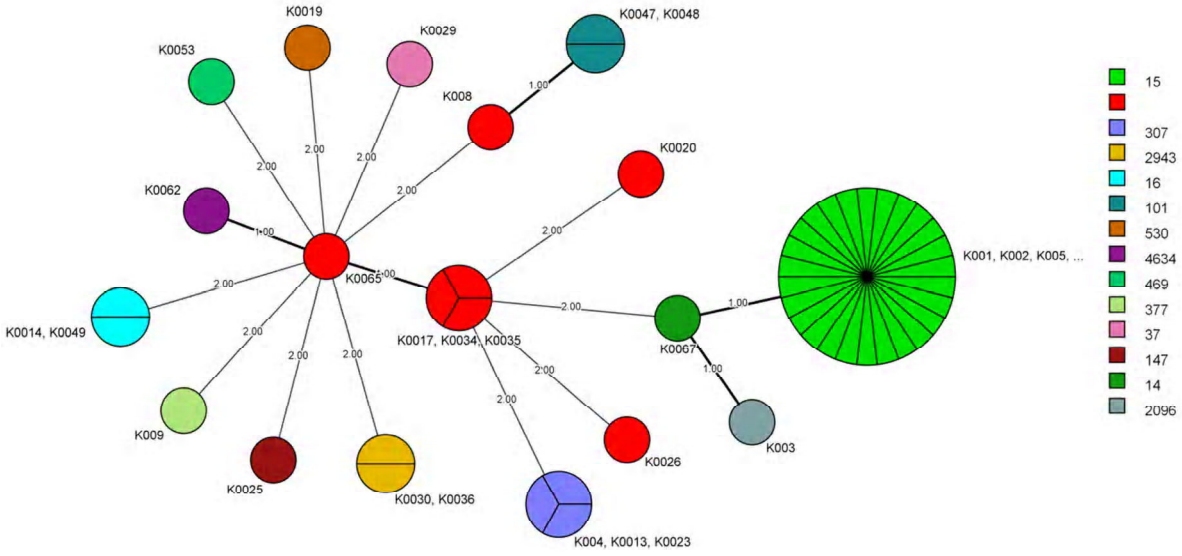


Figure 3.3. Minimum Spanning Tree based on 7 housekeeping genes

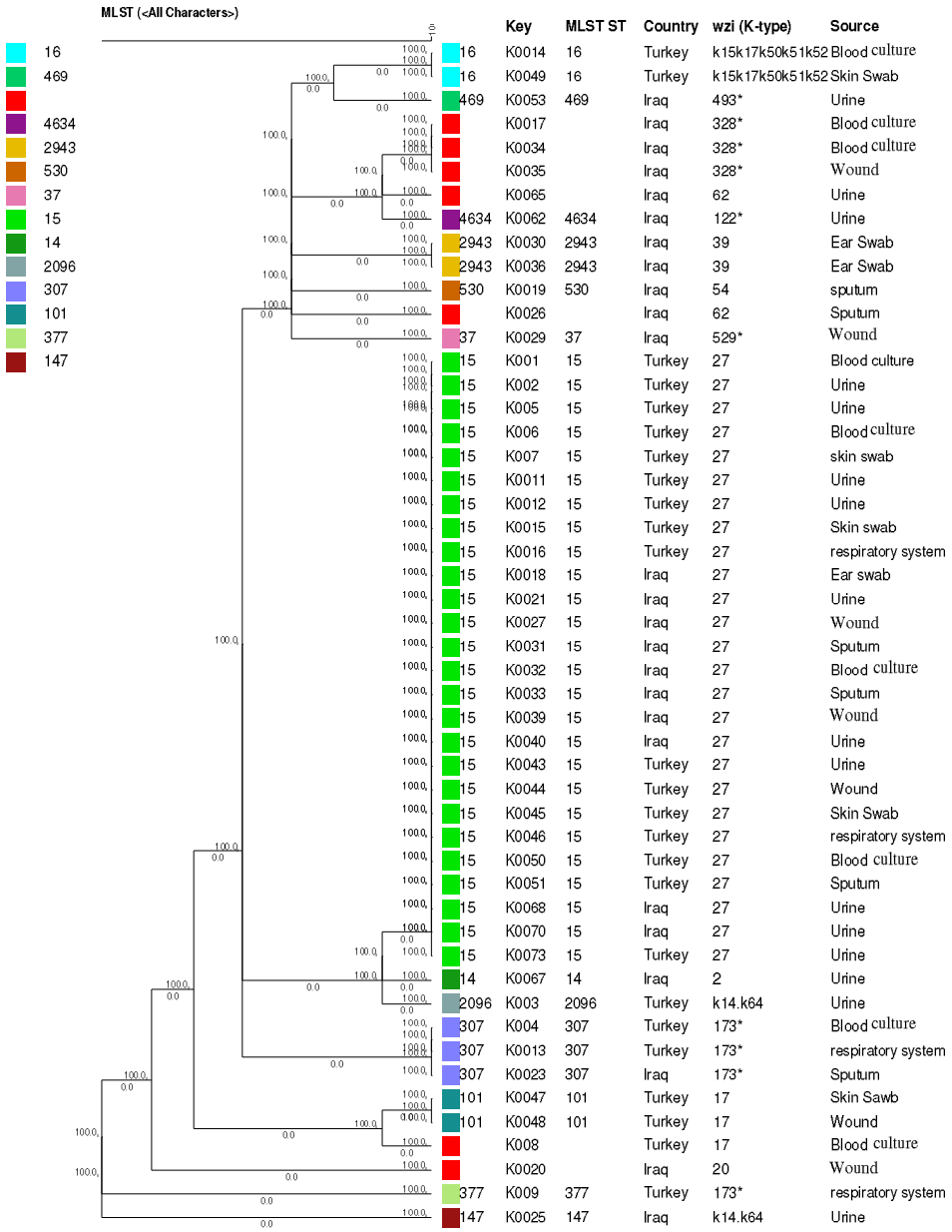


Figure 3.4. Phylogenetic tree based on 7housekeeping genes.

3.7.3. Wzi gene analysis

K-types of isolates were determined based on sequencing of wzi gene which determines the capsular type. Of the studied isolates, 26 of them contained ST 15 and K-type 27 and clustered together and their similarity were 100%. Four isolates contained wzi allele 173, and three of them clustered with 100% similarity. Among those 3 isolates, two of them collected from Kayseri and one from Baghdad. Those three isolates clustered with the fourth isolate that collected from Kayseri in a 99.9% similarity. Moreover, 3 isolates with K-type 17 clustered together in a 100% similarity, and two of them had ST 101 and one had novel ST. All those isolates were collected from Kayseri. Three isolates with undefined ST clustered together in a 100% similarity and they shared the wzi allele 328.

Two isolates shared same ST 2943 and same K-type 39, and they clustered together in a 100% similarity. Those isolates collected from Baghdad.

Two Iraqi isolates (26#, 65#) shared same K-type 62, but they are different in their 7 housekeeping genes combination. Isolate 26# has novel ST and isolates 65# ST undiagnosed, but they are similar in 100% based on K-type sequences.

Two Turkish isolates (14#, 49#) with ST 16 clustered in 99.3% similarity, but they had cross reaction in their K-types (K15K17K50K51K52). Also, two isolates with different ST (One Iraqi isolate with ST 147 and one Turkish isolate with ST 2096) had cross reaction in their K-type (K14.K64) and they are 100 % similar.

The rest five isolates, three of them have K-type (2, 20 and 54) and two with only wzi alleles (493 and 122). They are unique isolates and they were related to other isolates and have common ancestor at 92% similarity as shown in figure (3.5.).

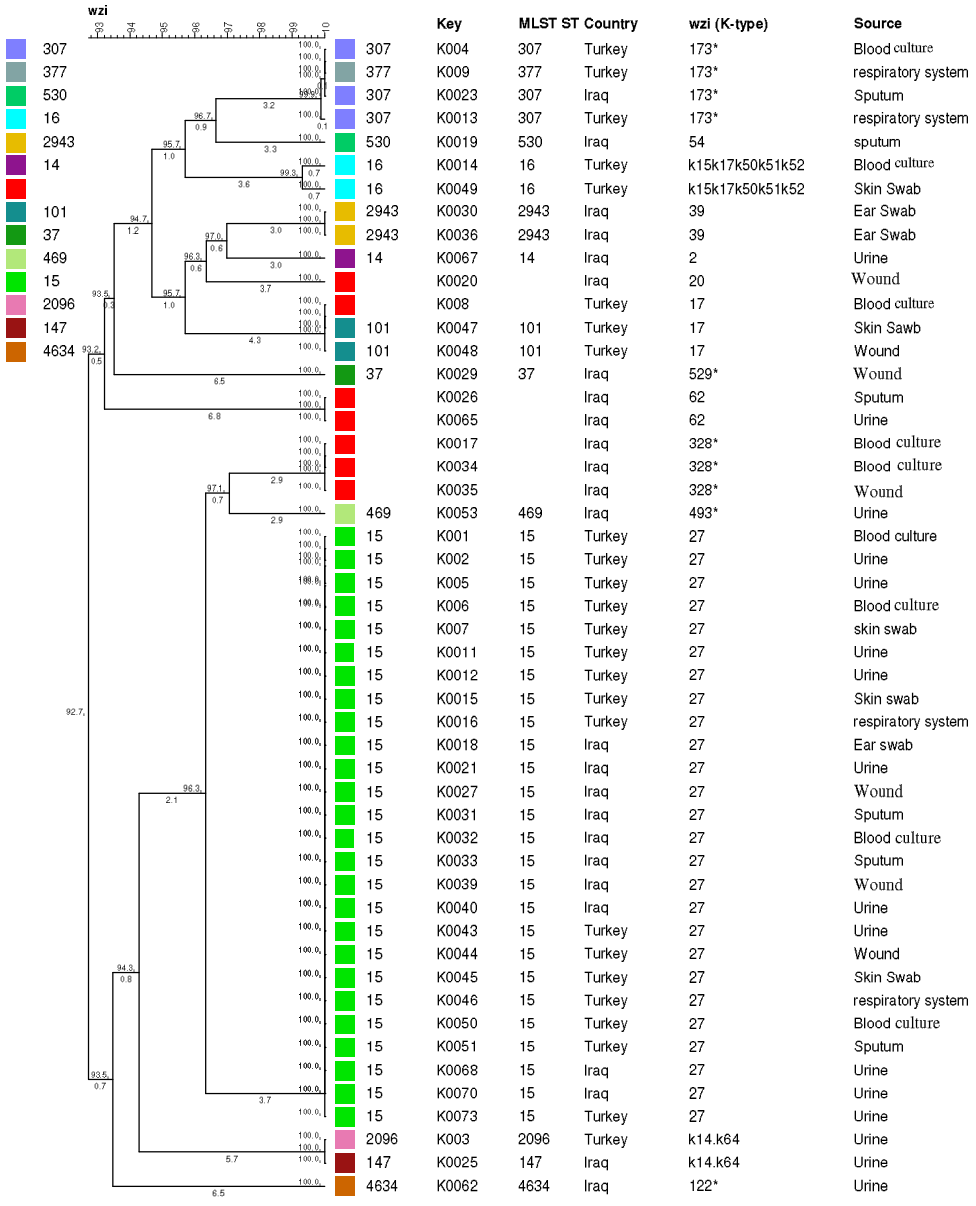


Figure 3.2. Phylogenetic Tree based on wzi genes. This dendrogram built by Bionumerics 8.0 version.

3.7.4. XbaI-PFGE

In the XbaI-PFGE method, restriction enzyme XbaI (Thermo Fisher Scientific) was used to determine restriction fragment polymorphism with the CHEFF DR II Device (Biorad, Nazareth, Belgium) in accordance with Pulsenet CDC KEPA protocol. The relationship between polymorphic DNA fragments was evaluated using the Gel-Compare II software program (version 5.0; Applied Maths, Sint-Martens-Latem,

Belgium). Based on 80% similarity 30 isolates were grouped in 13 clusters and 26 sub-clusters, and the remaining 20 isolates were unrelated, unique isolates. The largest cluster contained 3-members, A, S, and T, θ. This was followed by 2-member clusters C, D, F, H, N, W, X, Y and γ (Figure 6, Table, 8).

3.8. Genotypic surveillance methods

In this study, XbaI-PFGE, MLST and K typing genotypic surveillance methods was used to determine clonal characteristics and genetic relationships of 50 carbapenem-resistant *K. pneumoniae* isolates obtained from clinical samples from two hospitals located in Kayseri, Turkey and Baghdad-Iraq. When single method is considered, the most discriminating genotyping method was found to be XbaI-PFGE method.

Among the 3 isolates clustered in cluster A, 2 isolates with 100% clonal relationship obtained from blood culture and skin swabs (a1) and 1 wound isolate (a2) determined to be 95.7% clonally related and all of which were isolated from Kayseri patients. Having ST101 and K17 types, our blood culture isolates in the a1 sub cluster belongs to different MLST and K types.

Two isolates , one obtained from a skin swab and the other from a urine sample clustered together in cluster D and found to be clonally related at 87%. They have the same K (K27) and MLST type (ST15) and they are Turkish isolates.

Two isolates which are 100% related in the F cluster were isolated from the blood culture and respiratory tract samples of the patients of Kayseri origin. Those isolates had different ST (ST15, ST16) and K (K27, K15K17K50K51K52) types and were clonally related at a rate of 92.9%. Both the MLST types (ST15 and ST16) and the K types (K27 and K15K17K50K51K52) of other 2 isolates in the group H, isolated from the patient samples in Kayseri.

The isolates in the X cluster, which were clonally related 95.7% and isolated from the urine samples of two patients of Kayseri origin, were different in the MLST (ST15 and ST2096) and K types (K27 and K14.64),

Again, among the 2 isolates (y1, y2) obtained from the blood culture and urine samples of the patients of Kayseri origin and found to be 80% related. MLST type of the urine

isolate is ST15, the K type is K27, however the K type of the blood culture isolate is determined as K17, but ST was undetermined,

Θ has 3 sub-clusters and all 3 isolates were isolated from skin swab, blood culture and wound samples belonging to the patients of Kayseri origin and having an association rate of 81.9% with ST15 and K27 types.

In cluster N, there were 2 isolates clustered from the sputum and urine samples of Baghdad-Iraq patients and found to be clonally related at a rate of 88.9%. Sputum isolate was belonging to K26 but it's ST was not determined. The urine isolates were belonging to ST15 and K27. Of the 3 isolates, 2 from the ear specimen obtained from Baghdad-Iraq patients were 100 % clonally related and had the same MLST (ST2943) and K type (K39), but the wound swab isolate was different in terms of both K type (K27) and MLST type (ST15).

Although the similarity rate between the isolates in the 3-membered T cluster including Baghdad-Iraq patient isolates was 90.2%, MLST and K type of none of them could be determined, but they had 328th allele in wzi gene sequence analysis for K typing.

In W cluster, Iraq and Turkey originated two isolates obtained from ear and urine were found to be clonally related at 83.9% and they were belonging to same MLST(ST15) and K type (K27).

In Cluster C, two respiratory tract samples were found to be clonally 87% related and they were belonging to the same ST (ST307) type. One of which belongs to the patients from Kayseri and the other to Baghdad and the K typing does not have any known counterpart. Both two strains have 173 alleles in wzi gene sequence analysis,

When analyzed by XbaI-PFGE two Iraqi isolates clustered in γ with 85.7% clonality. Those isolates were obtained from wound swab and sputum specimen. While ST of wound swab cannot be determined their K type were K20 and the sputum isolate contained ST 15, and its K type was K27.

On the other hand, when the XbaI-PFGE clusters were compared with MLST method in which fewer clusters were detected, XbaI-PFGE method with very high discrimination power randomly distributed all ST groups (Table 3.8).

Table 3.8. Comparison of MLST results with PFGE and K-typing results

ST	Isolates	PFGE cluster and subcluster		K type	Source	Country
ST 15	26	A	a1	K27	Blood culture	Turkey
		D	d1	K27	Urine	Turkey
			d2	K27	Skin swab	Turkey
		F	f1	K27	Respiratory system	Turkey
		H	h2	K27	Sputum	Turkey
		N	n2	K27	Urine	Iraq
		S	s1	K27	Wound	Iraq
		W	w1	K27	Ear swab	Iraq
			w2	K27	Urine	Turkey
		X	x2	K27	Urine	Turkey
		Y	y1	K27	Urine	Turkey
		Γ	γ2	K27	Sputum	Iraq
		Θ	θ1	K27	Skin swab	Turkey
			θ2	K27	Blood culture	Turkey
			θ3	K27	Wound	Turkey
		K		K27	Urine	Iraq
		M		K27	Urine	Turkey
		Q		K27	Urine	Iraq
		R		K27	Urine	Iraq
		U		K27	Urine	Iraq
		V		K27	Sputum	Iraq
		α		K27	Urine	Turkey
		β		K27	Blood culture	Turkey
		ϵ		K27	Blood culture	Turkey
		Σ		K27	Respiratory system	Turkey
		Λ		K27	Skin swab	Turkey
ST307	3	C	c1	173*	Respiratory system	Turkey
			c2	173*	Sputum	Iraq
		Z		173*	Blood culture	Turkey
ST16	2	H	h1	K15,K17,K50,K51,K52	Skin swab	Turkey
		F	f1	K15,K17,K50,K51,K52	Blood culture	Turkey
ST101	2	A	a1	K17	Skin swab	Turkey
			a2	K17	Wound	Turkey
ST2943	2	S	s1	K39	Ear swab	Iraq
ST14	1	E		K2	Urine	Iraq
ST37	1	O	o1	529*	Wound	Iraq
ST 147	1	P	p1	K14.K64	Urine	Iraq
ST377	1	L		173*	Respiratory system	Turkey
ST469	1	I		493*	Urine	Turkey
ST530	1	G		K54	Sputum	Iraq
ST2096	1	X	x1	K14.K64	Urine	Turkey
ST4634	1	B		122*	Urine	Iraq

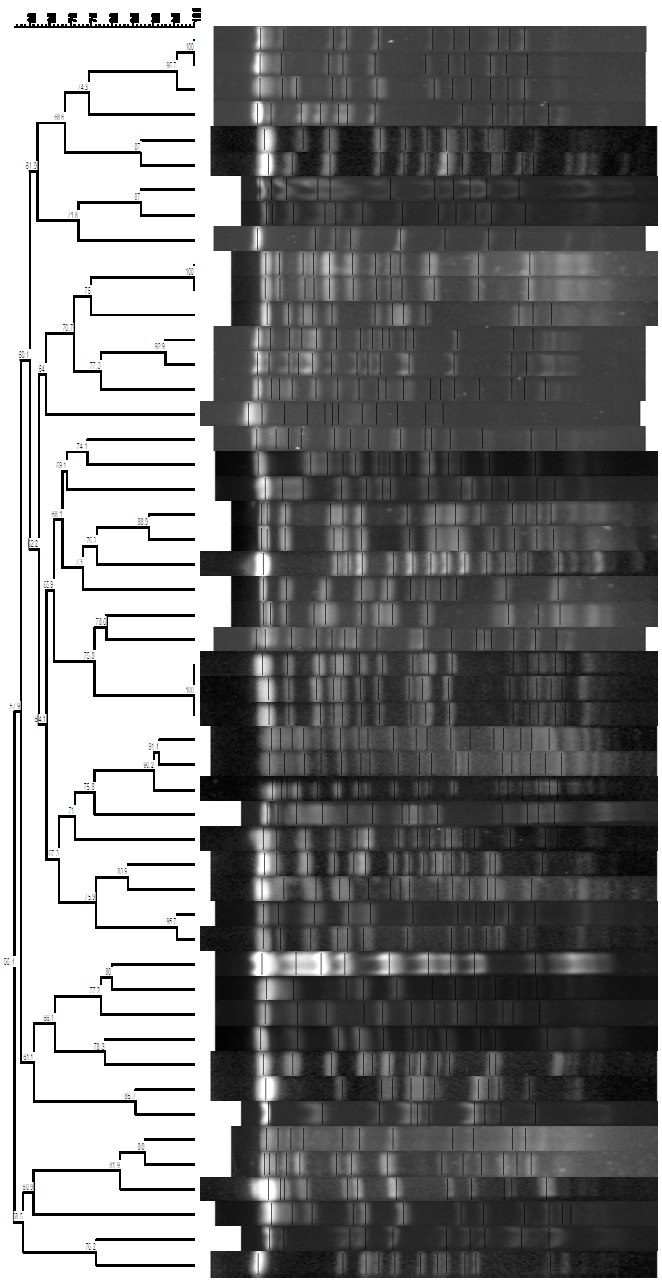
When 26 isolates with ST15 are evaluated according to the results of XbaI-PFGE method 49.4% were found to be clonally related (figure 3.7.). However, based on 80% similarity rate, the ST 15 contained 4 clusters (K, Q, L and O) and 16 unique isolates. When the clusters are ranked according to the number of members, the K and Q clusters were 3-membered, and L and O clusters had 2 members.

K cluster consists of 3 isolates with 81.2% similarity and 2 of which are from Turkey and 1 of them is from Iraq. While Turkish isolates were isolated from urine sample, Iraqi isolates isolate were isolated from ear swab.

Cluster Q consisted of 3 isolates with 81.9% similarity, all isolated from blood culture, wound and skin soft tissue samples of Turkish patients.

L is identified as associated clonal cluster that is isolated from 87% urine and skin swab samples are Turkey-based.

The O cluster consisted of 2 isolates with 81.5% similarity. One of the isolates from urine, from Iraq, and another blood culture sample was isolated from Turkey.



Cluster	Sub-cluster	ST	K-type	Source	Country
A	a1	ST101	K17	Skin Swab	Turkey
A	a1	ST15	K27	Blood culture	Turkey
A	a2	ST101	K17	Wound	Turkey
B	b1	ST4634	122*	Urine	Iraq
C	c1	ST307	173*	Respiratory system	Turkey
C	c2	ST307	173*	Sputum	Iraq
D	d1	ST15	K27	Urine	Turkey
D	d2	ST15	K27	Skin Swab	Turkey
E	e1	ST14	K2	Urine	Iraq
F	f1	ST16	K15k17k50k51k52	Blood culture	Turkey
F	f1	ST15	K27	Respiratory system	Turkey
G	g1	ST530	K54	Sputum	Iraq
H	h1	ST16	K15k17k50k51k52	Skin Swab	Turkey
H	h2	ST15	K27	Sputum	Turkey
I	i1	ST469	493*	Urine	Turkey
J	j1	?	K62	Urine	Iraq
K	k1	ST15	K27	Urine	Iraq
L	l1	ST377	173*	Respiratory system	Turkey
M	m1	ST15	K27	Urine	Turkey
N	n1	?	K62	Sputum	Iraq
N	n2	ST15	K27	Urine	Iraq
O	o1	ST37	529*	Wound	Iraq
P	p1	ST147	K14.K64	Urine	Iraq
Q	q1	ST15	K27	Urine	Iraq
R	r1	ST15	K27	Urine	Iraq
S	s1	ST2943	K39	Ear swab	Iraq
S	s1	ST2943	K39	Ear swab	Iraq
S	s1	ST15	K27	Wound	Iraq
T	t1	?	328*	Blood culture	Iraq
T	t2	?	328*	Blood culture	Iraq
T	t3	?	328*	Wound	Iraq
U	u1	ST15	K27	Urine	Iraq
V	v1	ST15	K27	Sputum	Iraq
W	w1	ST15	K27	Ear swab	Iraq
W	w2	ST15	K27	Urine	Turkey
X	x1	ST2096	K14.K64	Urine	Turkey
X	x2	ST15	K27	Urine	Turkey
Y	y1	ST15	K27	Urine	Turkey
Y	y2	?	K17	Blood culture	Turkey
Z	z1	ST307	173*	Blood culture	Turkey
α	α1	ST15	K27	Urine	Turkey
β	β1	ST15	K27	Blood	Turkey
γ	γ1	?	K20	Wound	Iraq
γ	γ2	ST15	K27	Sputum	Iraq
θ	θ1	ST15	K27	Skin swab	Turkey
θ	θ2	ST15	K27	Blood Turkey	Turkey
θ	θ3	ST15	K27	Wound	Turkey
Σ	Σ1	ST15	K27	Blood culture	Turkey
σ	σ1	ST15	K27	Respiratory system	Turkey
Λ	λ1	ST15	K27	Skin swab	Turkey

Figure 3.6. Phylogenetic tree of isolates based on PFGE. Done by Bionumerics 8.0 ,Version.

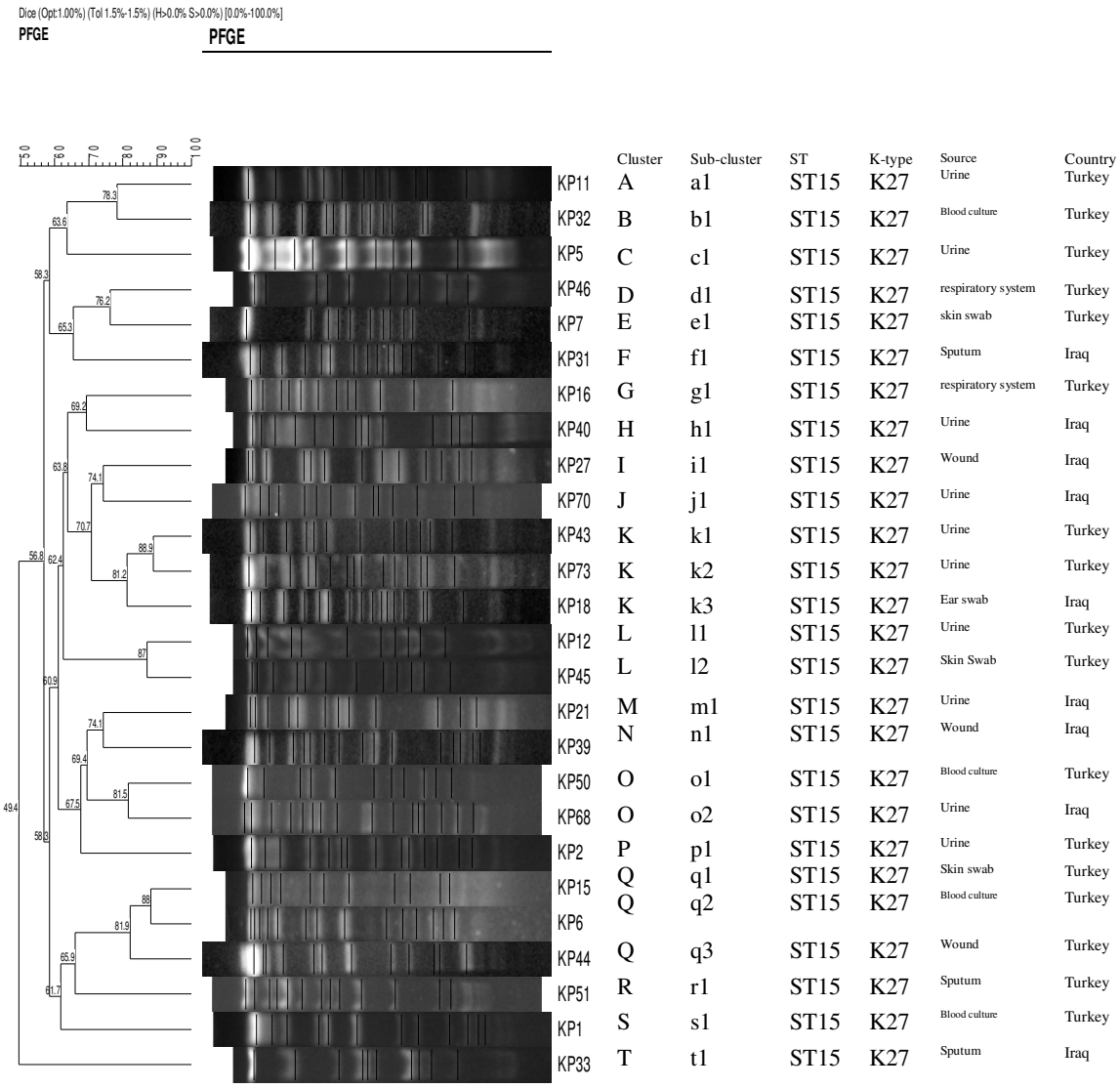


Figure 3.7.Phylogenetic tree of 26 isolates with ST15 and K-type 27 based on PFGE.

CHAPTER 4

DISCUSSION - CONCLUSION AND RECOMMENDATION

4.1. Discussion

In inpatient treatment institutions where modern health services are provided, hospital-acquired multi-drug resistant (MDR) microorganisms continue to be an important threat that causes high morbidity and mortality as well as treatment failures. It is important for hospital staff and patients to be educated in terms of infections that develop depending on the service provided in the hospital. Efforts to prevent the development of resistance by encouraging rational use of antibiotics, and the availability of more effective and broad-spectrum antibiotics produced against resistant strains are important for the control of hospital infections. Genotypic surveillance methods using molecular-based methods also played an important role in the success of infection control programs. Especially in recent years, the increase in the incidence of infections acquired in the society where multi-drug resistant staphylococci, enterococci and gram-negative rods are defined as agent pathogens has shown that microorganisms that we are accustomed to see in hospitals are carried to the streets. On the other hand, outside of the hospital, enteric bacteria such as *E. coli, Klebsiella* and soil-based gram-negative rods such as *A. baumannii* by acquiring resistance genes from resistant bacteria become colonized in the community with food or waterborne contaminants, and colonization can turn into infection in the community or hospital in susceptible individuals. It is predicted that the incidence of healthcare-associated infections (HAI) can be reduced and completely prevented, especially in hospitals, with well-prepared control programs. Phenotypic surveillance methods based on the symptoms and physical findings of patients and / or phenotypic identification of the suspected agent pathogen produce low sensitivity results in the creation of control programs. The surveillance index is based on the determination of the relationships between the strains isolated from different patients by comparing their phenotypic and genotypic characteristics with the strain,

that is, the first isolate identified in relation to infection. As a result of surveillance, information is obtained about whether there is an epidemic, the potential reservoir and vectors of the outbreak, and an action strategy is developed. In this respect, genotype-based accreditable surveillance methods that define the relationship between clinical isolates at clonal level, have high reproducibility, lower cost and give results in a short time are recommended [70], [71].

In this context, contribution of molecular surveillance methods with high discrimination power, such as PFGE, RAPD-PCR, PCR-RFLP, MLST and AFLP where even single point mutations in target genes can be detected, are realistic such as accurate detection of the sources of microorganisms that cause infection, determining the transmission routes and isolation of the personnel responsible for the transmission and contributes to the control of hospital infections by taking precautions [72]–[75].

In recent epidemiological studies, it has been observed that the threat priority among multi-drug resistant strains has shifted from gram-positive cocci to gram-negative rods. As a matter of fact, Pandrug Resistant *A. baumannii* has become an important cause of mortality among patients receiving treatment in intensive care units in recent years. On the other hand, intestinal multi-drug resistant Gram-negative rods have become new threats to public health. In this context, the World Health Organization (WHO) stated that the most important pathogens that will experience treatment failure in hospitals in the next decade will be Carbapenem resistant *E. coli* (CREC), Carbapenem resistant *A. baumannii* (CRAB) and Carbapenem resistant *K. pneumoniae* (CRKP). It makes remarkable warnings reminding that these three Gram-negative rods should be taken into account when developing antibiotic use policies [76]–[79].

The incidence of HAI in hospitals of developed countries, which is 3.6-12%, has been reported to be 5.7-19.1% in developing countries [80]. It is stated that the estimated average incidence of HAI is 15% worldwide, although it varies between units [81].

Compared to phenotypic methods, genotype-based molecular methods have higher discriminatory power and have the advantage of being applicable to many species. Especially the resistance profiles and genetic characteristics of the isolates that cause HAI outbreaks should be known and microbiological genotyping should be done.

Today, when human movements within and between countries are increasing, resistant clones that occur in a patient and / or a hospital can spread in a short time, to other

patients, hospital services, hospitals in the province and region, across the country and among countries, and could be global threat to public health [82].

PFGE which is used to detect clonal relationships; despite its disadvantages such as high installation cost, device dependency, need for experienced staff, it is an ideal surveillance method with its high discrimination power, it provides in local and short-term epidemic analysis, such as the suspicion of an infection epidemic seen in a hospital or even a hospital in a clinic and has been defined as the "gold standard". However, the interpretation of polymorphic fragments which produced by high discrimination power technique, due to the different breaking points that may occur in DNA, even if based on controlled cutting, in long-term epidemic analysis and studies aimed at producing global epidemiological data, makes it impossible to produce and interpret global results. For this reason, instead of a genotypic identification based on the polymorphism of the mutations in the total genome, the low discrimination power based on the polymorphism that may arise as a result of point mutations in fewer and well-preserved structural genes, the number of which varies between 5 and 7 depending on the bacterial species, but is easily interpreted MLST, or as with capsule typing, methods that look at polymorphism in a single gene can be used [83], [84]. However, the most reliable surveillance is surveillance using more than one method, one of which is PFGE.

In this study, we evaluated the *K. pnemoniae* isolates obtained from different hospitals in Baghdad-Iraq and Kayseri Erciyes University Medical Faculty Hospital by using Kirby-Bauer Disk Diffusion method, microdilution and vitek automatized system. We evaluated the genomic characteristics and phylogenetic relationships of 50 isolates, 25 of which were from Kayseri and 25 from Baghdad, which were found to be resistant to carbapenem with the VITEK 2 automated system and the MLST, PFGE and K typing method.

Our findings indicated, the dominant ST in *K. pneumoniae* isolates were ST15 that linked to wzi 27, and we found this profile in 17 Turkish isolates and 9 Iraqi isolates. This is the first study showed this profile in Turkey and Iraq. Previously, Alper Tekeli and his colleagues characterized one carbapenemase producing *K.pneumoniae* with ST15 and wzi 93 were different from our isolates in wzi allele[85]. By Allele sequence comparison analysis, wzi 93 different from wzi 27 in 18 nucleotides variant, but the

similarity was 95.97 %, that means those isolates with wzi 93 and wzi 27 belongs to same clone ST 15, but they were different in wzi allele.

Carbapenemase producing *K. pneumoniae* was linked to ST258 in USA and in some European countries[86]. Nowadays, some studies showed ST15, ST101 and ST147 that linked to Carbapenemes resistant [87], [88] as we detected in our study. Understanding the successful diffusion of other high resistant clonal groups (other than CG258) has not yet been studied very well. According Pasteur Institute, we can get an idea about the dominant STs in recorded ones in some countries around Turkey as Greece, Iran and Syria. Also Russia, because of the huge number of tourists annually in Turkey.

In Greece, the dominant ST was ST11, that recorded 14 times in the database. Also ST307 and ST147, they recorded in one isolate for each one, that match our 3 isolates with ST307 and one isolate with ST147.

In Syria, there is no record for any *Klebsiella pneumoniae* in the database. This indicates the need to study the spread of these bacteria there to find out the prevalence ST and compare it with the ST discovered in Iraq and Turkey and to know the relationship between them, given the presence of Syrian refugees in Turkey and Iraq.

In Iran there are five records of *Klebsiella* bacteria distributed as one isolate for each ST and they are ST147, ST2282, ST2283, ST2284 and ST2285. These isolates match one Iraqi isolate with ST147.

In Russia, there are 335 records. 41.49% of those isolates were ST395 and for STs that match our isolates. 10.45% for ST147, 5.67% for ST307, 2.99% for ST377, 0.9% for ST14 and 0.9% for ST15.

These results indicate that ST147 and ST307 will be the next most popular STs in the mentioned countries. This warning of the need to study these bacteria more broadly and to apply different genotyping methods to obtain a good differentiation between closely related isolates.

It is possible that the genotypes of the microorganisms that we genotyped by targeting limited gene regions such as PFGE, MLST and K typing methods, which are formed by randomly developing sequential mutations, are similar in different regions or clonally related with another expression. For this reason, it is known that genotyping using more

than one method will have a higher discrimination power, but the real genotype determination can be determined 100% accurately by the whole genome sequencing.

In this study, two isolates in Cluster C with 87% similarity and same ST-type and K-type (not shown of the same allele) was isolated one from Turkey and one from Iraq. Similarly, one from Turkey and the other from Iraq was grouped in W cluster with 83.9% clonal relationship with same K-type and S type. The cut regions of the XbaI enzyme used in the PFGE method are not infinite in the mutant genome that develops due to successive mutations, and the changes in the targeted regions in MLST and K typing methods are not infinite. Therefore, isolates belonging to the same clone can be detected in two different countries. The 100% similarity in the ear swab isolates in the S cluster and the same ST and K types are indicative of the distribution of the same clones in similar samples.

4.2. Conclusion

Klebsiella pneumoniae samples included in the study were isolated from clinical material collected over a 2-year period in total. In this respect, the study aims to investigate the clonal distribution of long-term epidemic isolates with three different methods, since spontaneous breaks will occur in the genomes of microorganisms to be evaluated with the PFGE method, even during reproduction and continuation with successive passages, and different sizes of fragments will occur and interpretation differences will be based on Tenover criteria. While the 4-band difference is interpreted as distantly related bacteria, a certain similarity rate is accepted for the relationship in the interpretation analysis made with computer programs. However, in spite of everything, interpretation is extremely difficult in strains isolated from different regions and over a long period of time and stored by passage. This is due to the high discrimination power of the method. MLST types and K types do not change because the targeted gene regions are conserved. ST and K types do not change for a long time compared to PFGE. If the isolation dates of the strains are examined, it can be compared whether they are far or close to each other. For example, in a long-term study, we can say that the ST 15 type or K 27 type is the dominant type in our hospital, but we cannot say that these are hospital infection pathogens originating from same source. We can say that K and ST types can be the dominant type only by clonal selection.

4.3. Our suggestions for future studies

More research is required to determine the prevalence of a clone in Turkey and Iraq. The diversity seen in the current study may be attributed to the centers being tertiary referral hospitals, where the isolates have consequently been taken from patients and diagnosed in hospitals or in private laboratory.

The lack of adequate infection control together with the lack of reporting and the limited funds available for research in developing countries all contribute to the lack of knowledge on the epidemiology of hospital acquired infection. There is an urgent need to fund and support research in developing countries, in order to prevent the clonal dissemination of antibiotic resistant organisms not only in the hospital setting, but in the community.

Antimicrobial stewardship programs aimed to preserve the activity of antibiotics and limiting the rates of resistance are strongly needed in developing countries like Turkey and Iraq, as the inadequate usage of antibiotics in the community and hospitals all contribute to the rise in resistance, and promotes the organism to acquire and express novel resistance mechanisms.

K. pneumoniae is a very interesting pathogen due to its rapid development of resistance and the plasticity of its genome. Further research by using sensitive molecular epidemiology methods, not only in Turkish and Iraqi hospitals, but in other regions of the Middle East might be useful in order to get more information about the problem.

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