International Journal of Psychosocial Rehabilitation, Vol. 24, Issue 05, 2020
ISSN: 1475-7192

Multi Locus Sequence Typing of Multi Drug Resistance Klebsiella Pneumoniae Isolated from different Clinical Samples

Saba J. Jawad. Al – Zubaidi and Hadi R. Rasheed. Al – Taai

Abstract--- *Klebsiella pneumoniae is an important opportunistic Pathogen, that commonly causes nosocomial infection The problem of antimicrobial resistance is highlighted by a recent increase of antibiotic resistant A multi locus sequence typing (MLST) scheme was developed for K. pneumoniae . The current study included collection of two hundred and seventy eight samples from Patients in Baqquba Teaching Hospital in Diyala during the period from December 2018 to May 2019. The samples included urine, sputum, swab from wounds, burns and blood. All isolates were diagnosed depending on microscopic, biochemical tests and confirmed by VITEK2 compact system. It was found (39.4%; n=69) was K.pneumoniae and (26%; n=18) of isolates were multi drug resistance K. pneumonia. MDR K. pneumoniae were showed high resistance against different types of antibiotics it was as follows: β – Lactam groups (AMP 100%, AMC 73.36%, PIP 81.16%, ATM 72.46%, FEP 71.01%, CAZ 62.32%, CRO56.5%, IPM27.53%, MEM26.19%). Aminogly consides groups (AK 47.82%, TOB 43.47%, GM 36.23%). Quinolones groups (LEV 31.82%, OFX 28.98%, CIP 24.63%) and Cephalosporin groups (SXT 65.22%). The technique used in this study to determine genetic diversity of MDR K. pneumoniae was MLST (Multi locus sequence typing).Seven house Keeping genes (ropB, gapA, mdh, pgi, phoE, infB, tonB)were taken from website Institute Pasteur. Eighteen isolates have been for PCR amplification reaction. Nucleotide Variances were seen and specific alleles for each locus were designated. The alleles profile for each isolate was then used to determine sequence type (ST). New seven isolates, new one isolates and seven housekeeping genes have been published in (NCBI). The MLST approach provides unambiguous data useful for the epidemiology of K. pneumoniae. To the best of our Knowledge, this was the first study that involved on MLST analysis of clinical K. pneumoniae isolates from hospital in Diyala, Iraq.*

Keywords--- *Klebsiella Pneumonia, Multi Drug Resistance, Housekeeping Genes, Multi Locus Sequencing.*

I. INTRODUCTION

Klebsiella pneumoniae commonly is a Gram negative opportunistic pathogenic bacterium that causes nosocomial infections in urinary tract, respiratory treat and blood and causes septicemia, pneumonia, bacteremia, meningitis, burn and wounds infection. (Gorrie *et al*., 2017). Furthermore, it was the most medically important species of the genus *Klebsiella*. In recent years, *Klebsiella* have become important pathogens in nosocomial infection it was also a potential community–acquired pathogen. (Levinson, 2016). Antibiotic therapies are widely used for treating infection diseases. Nowadays, antibiotic–resistant bacteria are a great concern of worldwide Public health. (Dsouza *et al*., 2017). The problem of antimicrobial resistance is highlighted by a recent increase of

Saba J. Jawad. Al – Zubaidi, College of Education for Pure Sciences, Diyala University.
Hadi R. Rasheed. Al – Taai, College of Sciences, Diyala University.

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

antibiotic resistant. Antimicrobial resistance is commonly related to the spread of transmissible plasmids and the acquisition of resistance genes that normally occur by horizontal gene transfer, which may also carry virulence determinants. (Derakhsban *et al*., 2016). For pathogen survival, the acquisition of resistance and virulent traits is necessary and some reports suggest that such may have an essential role in the pathogenesis of *K.pneumoniae* infection, Capsule, lipopolysaccharide(LPS), fimbriae and siderophores are virulence factors that contribute to the pathogenicity of *K .pneumoniae* (Paczosa and Messas 2016). There are many of bacterial typing systems actually in use that greatly vary in the effort required. The cost, the accuracy and ability to identify between bacterial strains. (Reza *et al*., 2014).The genotyping is important to identify casser out breaks due to *K.pneumoniae* and to further track soure and spreading of infection. the major genotyping methods of *K.pneumoniae* include multi locus sequence typing (MLST), pulsed field gel electrophoresis (PFGE), multiple–locus variable number tandem repeat analysis (MLVA) restriction fragment length polymorphism(RFLP). (Turton *et al*., 2010).

K.pneumoniae Multi locus system typing (MLST) is actually a new technique in molecular biology used for typing of various loci. The procedure characterizes isolates of microbial species using DNA sequences of internal fragment of housekeeping genes (Lilian *et al.*, 2012).

This study came to aim to isolate and diagnose *Klebsiella pneumonia* from different clinical sources and detection perform genotyping using (MLST).

II. MATERIAL AND METHOD

Samples Collection

Two hundred and seventy eight sample from different sources (urinary tract, sputum, wounds, burns, blood) were included in this study. They were collected from Baquba Teaching hospital during the period December 2018 to end of May 2019. The clinical samples were collected from different patients attending the hospital. The samples were streaked on blood agar, Mac Conkey agar and Eosin methylene blue (EMB) agar and incubated at 37Ċ for 24hrs. The isolates were show characteristic growth, color, mucoid, hemolysis, and identified as *K.pneumoniae* by manual biochemical tests that were used Gram staining, catalase test, oxidase test, Indol test, Voges–proskaure (UP) test, Methyl red (MR) test, simmons citrate test, urease test and Kligler Iron test. For final confirmation biochemical tests embedded in VITEK2 system (Bio merieux USA).

Sixty nine of *K. pneumoniae* these isolates were tested for their resistance against the following (16) antibiotics: Ampicillin (AMP), Amoxicillin+Clavulanic acid (AMC), Piperacillin (PIP), Azeteronam (ATM), Cefepime (FEP), Ceftazidime (CAZ), Ceftriaxone (CRO), Imppenem (IPM), Meropenem (MEM), Gentamicin (GM), Amikacin (AK), Tobramicin (TOB), Lefofloxacin (LEV), Ofloxacin (OFX), Ciprofloxacin (CIP), Trimethoprimet + Salfamethouxazok (SXT).

Antibiotics Susceptibility Test

Susceptibility test was done according to (National committee for clinical laboratory standards, 2013) using Kirby-Bauer and the turbidity of Mc farland standard to obtain convergent number equal to 1.5 X 10^8 cell/ml (Lalitha, 2004). And VITEK2 compact system (Vitek2 kit sensitive kit) (Biomerieux / USA).

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

Genomic DNA Extraction

Genomic DNA was extracted from an overnight culture using the(ABIO Pure ^{TM} Total DNA ABIO pure, USA) the concentration of the DNA extract and purity was determined by measuring absorbance at Quants florometer.

PCR Primer for MLST to Detection MDR K.pneumoniae

MLST was performed on (18) isolates out of (69) in order to in vestigate which sequence types of MDR were present in Baaquba city. The French MLST scheme was used in this study as the same primers could be used for sequencing all the genes. The isolates were illustrated at seven loci primers for the housekeeping genes used in the French MLST scheme are (*rop*B , *gap*A , *mdh* , *pgi* , *pho*E , *inf*B , *ton*B).All these primers were prepared according to the information of manufacture companies. Diluted stock solution for using in PCR mixture was prepared by adding 10 ML for original stock solution to 90 µl of deionized distal water to yield final concentration (10 pmol /µl) and stored in deep freeze until used in PCR mixture. Information on the methodology used is available in the MLST database for *K.pneumoniae*. The alleles and sequence type(STs)of each isolate study by MLST were determined using the MLST database plat from for *K.pneumoniae*. (http://www.pasture .fr/recherché/genopole/PF8/k.pneumoniae.html).

The determination of the clonal and epidemiological relationships and the formation of clonal complexes were completed by analyzing a genetic similarity diagram constructed with the aid of the pub MLST website.

III. RESULT AND DISCUSSION

The result of this study showed the bacterial colonies which appeared on Mac Conkey agar were grown and pink with mucoid. The biochemical test and VITEK2 system diagnosis of this bacteria. (278) sample showed(62.9% ; n = 175)positive culture and(37.1% ; n= 103) was negative culture. from 175 isolates only(39.4% ; n= 69)was *K.pneumoniae* and (26% ; n=18) isolates were multidrug resistance *K.pneumoniae* and(60.4% ; n= 106)was anther bacteria . these result were agreement with study(Hameed, 2019; Abbas, 2018).

In the present study a total of 69 *K. pneumoniae* isolates were isolated from samples collected from patients from Baquba teaching hospital. Most *K.pneumoniae* isolates were obtained from urine(32.31% ; n= 42), sputum(20.5% ; n= 17), wounds(19.44% ; n= 7), burns(13.33% ; n= 2) and blood(7.14% ; n= 1)and the patients ages ranged from(1–80)years.

The spread of *K.pneumoniae* and pathogenic importance comes through the events of urinary and respiratory tract infection this, may be because these bacteria are from the normal flora that lives in the intestine well it is opportunistic and it's ability to adhesion on surfaces of epithelial cell.

Antibiotic Susceptibility Profile of the MDR K.pneumoniae

Susceptibility of *K.pneumoniae* isolates were detected against(16)types of antibiotics, which are differ in their action. The disc diffusion method recommended by the clinical laboratory standard Institute (CLSI 2018) for phenotypic detection of resistance in all *K.penumoniae* isolates.

These isolates demonstrated high resistance to words some antimicrobial agents that have been tested.

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

K.penumoniae isolates were resistant to β-lactam groups(Ampicillin 100%, Piperacillin 81.16%, Amoxicillin + Clavulanicacid 73.36%, Azeteronam 72.46%, Cefepime 71.01%, Ceftazidime 62.32%, Ceftriaxone 56.51%). Aminoglycosides groups (Amikacin 47.82%, Tobramicin 43.47%, Gentamicin 36.23%) and Quinolones groups (Levofloxacin 31.88%, Ofloxacin28.98%, Ciprofloxacin 24.63%). and Cephalosporin groups (Trimethoprime-sulfamethoxazok 65.22%) Table 1.

Table 1: Resistant of *K.pneumoniae* Isolate to different Antibiotics

Antibiotic groups	***Antibiotics***	***Percentage***	***P (value)***
β - Lactam	Ampicillin	%100	0.08
	Amoxicillin + clavulanic acid	%73.36	0.07
	Piperacillin	%81.16	0.04
	Azeteroname	%72.46	0.05
	Cefepime	%71.01	0.07
	Ceftazidime	%62.32	0.01
	Ceftriaxone	%56.5	0.04
	Imppenem	%27.53	0.04
	Meropenem	%26.19	0.04
Aminoglycosides	Gentamicin	%36.23	0.00
	Amikacin	%47.82	0.03
	Tobramicin	%43.47	0.04
Quinolones	Levofloxacin	%31.88	0.01
	Ofloxacin	%28.98	0.04
	Ciprofloxacin	%24.63	0.09
Cephalosporin	Trimethoprime – sulfame thouxazok	%65.22	0.04

These results agreed with the results of (Al-Obaidi, 2014) and (Abbas, 2018). They found the percentage of *K.pneumoniae* isolates resistance to (Ampicillin 97%; Amoxicillin + clavulanic acid 97.5% and cefepime 74%; Azeteronam 78%; piperacillin 92% ; Ceftazidime and Ceftriaxone 84% for both antibiotics). the ration of resistance to Aminoglycosides antibiotic varied that is agreed with result of (Al-Garawyi, 2016) who found the percentage of *K.pneumoniae* resistance to (Gentamicin 51%; Tobramicin 49%; Amikacin 31.4%) and these results agreed with the results of (El-Badawy *et al*., 2017) which the percentage of(Levofloxacin 38.59%; Ciprofloxacin 44.73%) for Ofloxocin (Moghadasi *et al*., 2016) who found 27% of isolates were resistance to this antibiotic, and for Cephalosporin groups these results agreeds with (Shilpa *et al*., 2016) who found the percentage of *K.pneumoniae* resistance to (Trimethoprim – sulfa methoouxazok 60%).

The different in the resistance ration is due to the deference in the number of isolates and the different working condition and health of the patients as well the offense of antibiotics.

Multi Locus Sequence Typing (MLST)

MLST was performed on all MDR *K.pneumoniae* (18) isolates basis on the position of their resistance patterns. The scheme used the following seven housekeeping genes: (*rop*B, *gap*A , *mdh* , *pgi* , *pho*E , *inf*B , *ton*B).

PCR reactions were performed following the protocols specified at the *K.pneumoniae* MLST website Pasteur institute. For each gene PCR amplification was done using the same reaction mixture Bacterial strain sequence types

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

(STs) were classified on basis of the results of partial sequencing of seven housekeeping genes. Furthermore, the MLST was established as a scalable typing system to locate the diversity and phylogenetic relationship of the isolate (Olivier *et al*., 2015).To the best of our Knowledge, this was the first study that involved an MLST analysis of clinical *K.pneumoniae* isolates from hospital in Diyala. A note of notification must be added that although MLST is considered cost and time exhaustion, but it is a golden standard for all these approaches. (Flaviane *et al*., 2017).

Analysis and Comparing Strains to Database Clustering Using Allelic Profiles

The identify strains in current study with the database that have some minimum level of similarity in their allelic profile to each query strain and to show the relationship of the strain to those recorded in the database using Genius prime 2019 software (http://www.geneious.com)Table 2.

Table 2: MLST Alleles Profile of *K.pneumoniae*

Sample Id	*House Keeping Gene Allele*							*ST (Strain)*
	gapA	*infB*	*mdh*	*Pgi*	*phoE*	*rpoB*	*tonB*	
1	2	1	2	3	27	1	39	219
2	1	1	1	1	1	1	1	15
3	2	9	2	1	13	1	16	37
4	2	6	1	3	8	1	?	New
5	2	6	1	3	8	1	?	New
6	4	1	2	52	1	1	7	307
7	1	1	1	1	1	1	1	15
8	1	6	1	2	1	6	1	3154
9	1	6	1	1	1	1	1	14
10	1	1	1	1	1	1	1	15
11	1	?	?	?	1	82	141	New
12	1	6	1	1	1	4	1	324
13	2	?	246	1	8	18	182	New
14	14	1	2	1	7	4	182	873
15	4	1	1	1	3	4	4	New
16	8	7	2	2	66	4	34	New
17	2	1	62	1	10	4	110	2286
18	1	1	1	1	1	4	1	New

Based on the matrix of pairwise differences between the allelic profiles of the strains. This is no one isolate from Iraq. So that our result considered the first isolates recorded in database of MLST in Pasteur institute in France.

The Table 2 explains appearance (7) new isolates it's (4, 5, 11, 13, 15, 16, 18) as for the rest of the isolates.

The Phylogenetic Tree of K. pneumoniae Strains

The results showed three isolate (2,7,10) belonged to ST15 isolate on number (3) belonged to ST37 and (6) belonged to ST307, (8) belonged to ST3154,(9)belonged to ST14, (12) belonged to ST324, (14) isolate belonged to ST873 and (17) isolate belonged to ST2286 Fig. 1.

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

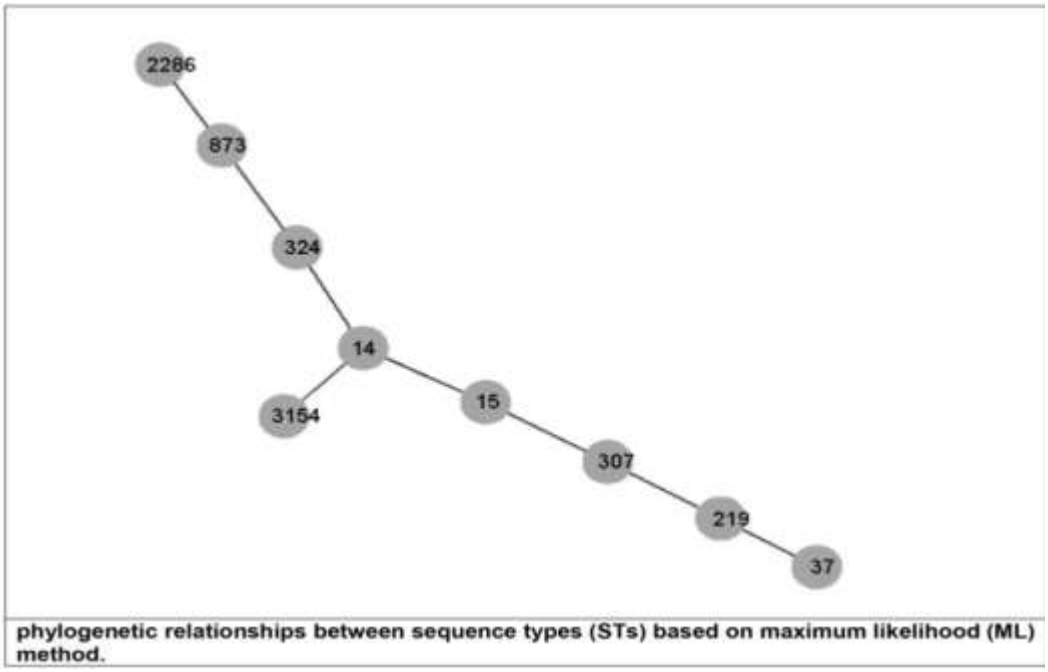

Fig. 1: Phylogenetic Reaction Ships between Sequence Types (STs) based on Maximum Likelihood (ML) Method

And the results showed the minimum spanning tree was constructed by the goe BURST algorithm using phyloviz software V1.1. The allelic profiles were downloaded from the MLST website (http://saureus mlst.net/) which included the *K.pneumoniae* sequence types (STs) Fig.2.

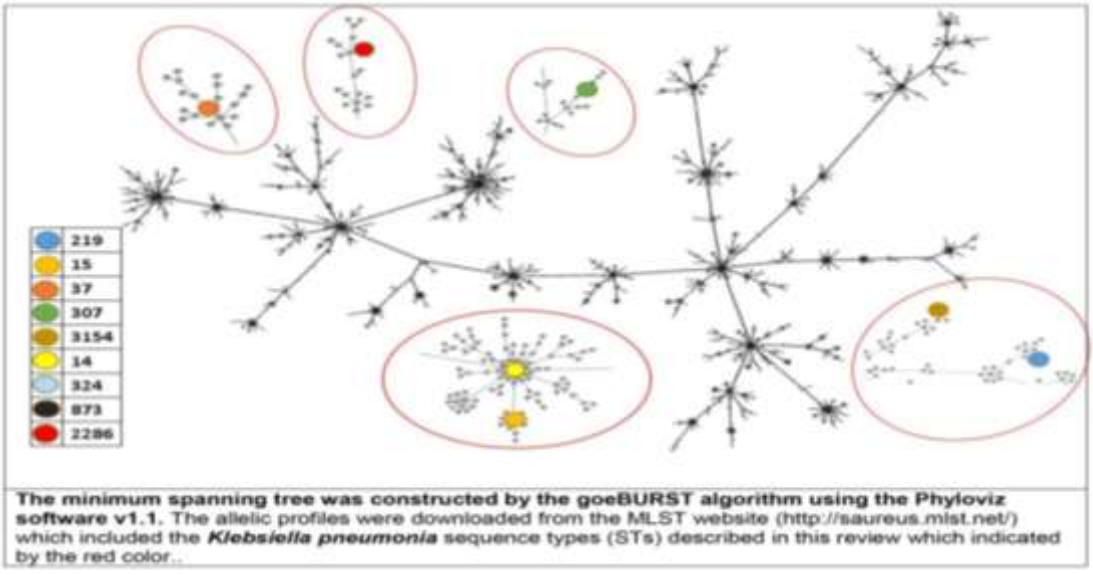

Fig. 2: The Minimum Spanning Tree was Constructed by the Goe BURST Algorithm Using the Phyloviz Software V1. 1.

And Fig.3 Geographical distribution of strain *K.pneumoniae* sequence types (DSTs) retrieved based on the seven gene markers MLST scheme.

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

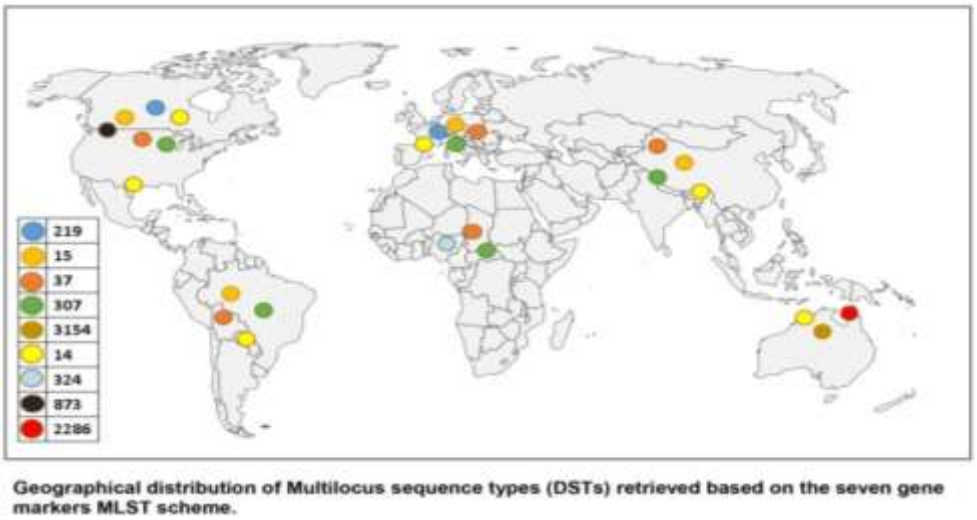

Fig. 3: Geographical Distribution of Multi Locus Sequence Types (DsTs) Retrieved base on the Seven Genes

Comparing K. pneumoniae Strains with Database Using Allelic Profiles

The first isolate number (1) belonged to ST219 its convergence and possibility matching in all loci with the strains in database from the website of MLST. (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?). Fig.4

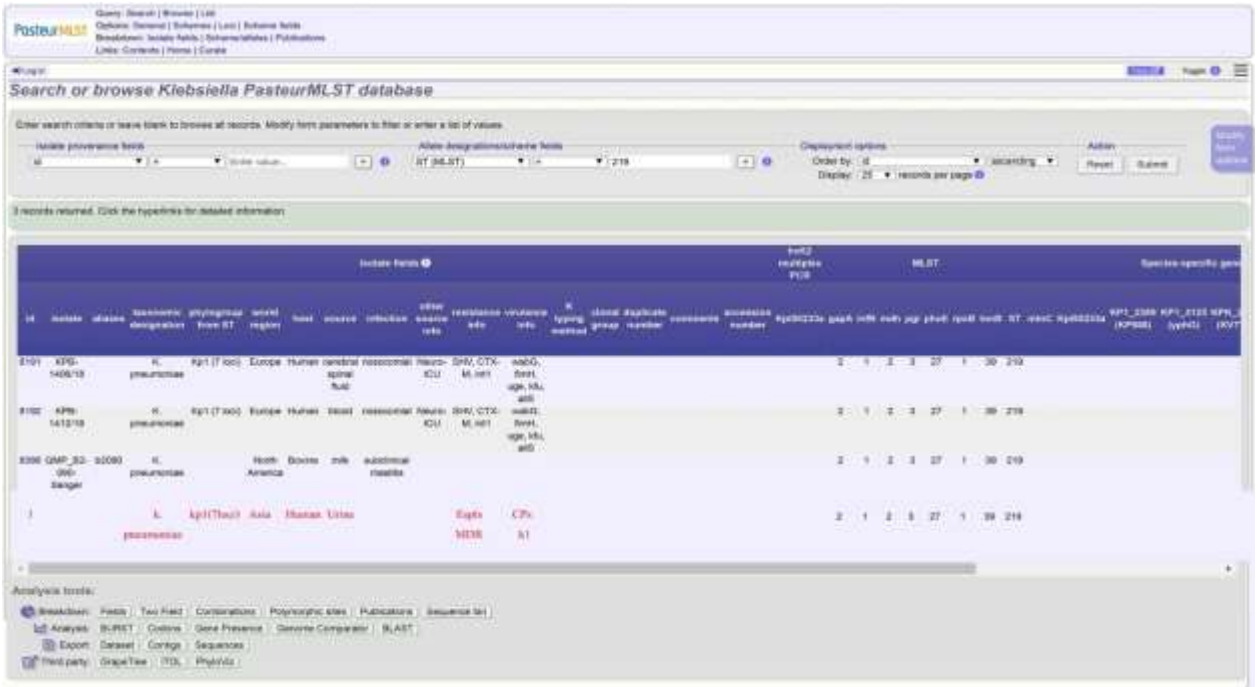

Fig. 4: Database of *K. pneumoniae* in MLST Nearest Match with (1) Strains

For (2 , 7 , 10) isolates belonged to ST15 it possibility matching with the strains in database from the website of MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?) .

For number (3) isolate belonged to ST37 it showed matching with the strains recorded in database of MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

For number (6) isolate belonged to ST307 it showed similarity with the isolates in database of MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

For number (8) isolate belonged to ST3154 it showed matching with the strains in database MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

For number (9) that belonged to ST14 it showed similsrity with the isoiates in database MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

For number (12) that belonged to ST324 it showed matching with the strains in database MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

For number (14) belonged to ST873 it showed matching with the strains in database MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

For number (17) isolate belonged to ST2286 it showed similarity with the isolates in database of MLST (https://bigs dh.phasteur.fr/cgi-bin/bigsdh.pl?).

GenBank Accession Number

New isolate and seven genes have been published in the National center for Biotechnology Information (NCBI).

This isolate got recorded name (MSAAR41934) and the seven genes were encoded and got the following serial numbers: Lc498474, Lc 498475, Lc498476, Lc 498477, Lc 498478, Lc 498479, Lc 498480.

REFERENCES

[1] Gorrie, C.L; Mirceta; M.; Wick, R.R.; Edwards, D.J.; Thomson, N.R. and Strugnell, R.A. (2017). Gastrointestinal carriage is a major reservoir of *Klebsiella pneumoniae* infection in intensive care patients Clin. infect. Dis. 65, 208 – 215.

[2] Levinson, W. (2016). Review of Medical Microbiology and Immunology. 14th ed. Mc Graw – Hill Higher Education. Newyork. 821 - 828.

[3] Dsouza, R;pinto, N.A.; Hwag, I.; Cho, Y.; Yong, D; Choi, J.; etal (2017). panel strain of *Klebsiella pneumoniae* for beta – lactam antibiotic evaluation: their phenotypic and genotypic characteization. PeeJ 5: e2896. doi: 10. 7717/ peerj. 2896.

[4] Derakhshan, S.; peerayeh, S.N. and Bakhshi; B. (2016). Association between presence of virulence genes and antibiotic resistance in clinical *Klebsiella pneumoniae* isolates. Lab Med. 47, 306 – 311.

[5] Reza, R. Bahman, A. and Nematollah, J. (2014). The prevalence of Beta Lactamase Producing *Escherichia coli* strains Isolated from the Urine samples in Valiasr Hospital. Biomedical & pharmacology Journal. 7 (2) : 425 – 431.

[6] Abdullah Hasan Jabbar et al, 2020, "Green Synthesis and Characterization of Silver Nanoparticle (AgNPs) using Pandanus Atrocarpus Extract", International Journal of Advanced Science and Technology, 29 (3), 4913- 4922.

[7] Magiorakos, A.p.; Srinivasan, A.; Carey, R.B.; Carmeli, Y.; Falagas, M.E.; Giske, C.G.; etal (2012). Multidrug – resistant, extensively drug – resistant and pandrug – resistant bacteria: an international expert proposal for interim strandard definitions for acquired resistance. Ciln. Microbiol. Infect. 18; 268-281.

[8] Abbas, M.S.M. (2018). Molecular study of Multidrug Resistant *Klesbiella pneumoniae* Isolated from Different clinical samples. a thesis M.Sc. University of Bagdad, the college of Education for pure science / Ibn – Al –Haitham.

[9] CLSI (clinical & Laboratory standards institute) (2018). Performance standard foe antimicrobial susceptibility testing seventeenth in formational supple.

[10] Olivier. C.; David, G. and Erick, D. (2015). Gudie to the various phylogenetic classification schemes for *Escherichia coli* and correspondence among schemes. 161: 980-988.

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020

[11] Abdullah H. Jabbar et al., 2019. "Enhanced bioactivity of polystyrene-silver nanocomposite (PS/Ag NCs)-an antimicrobial study." *AIP Conference Proceedings*, 2151 (1), 0200024.

[12] Faria – Junior and Alex, L. pereira. (2017) Virulence Markers and Phylogenetic Analysis of *Escherichia coli* strains with Hybrid EAEC/UPEC Genotypes Recovered from Sporadic cases of Extra intestinal Infections frontiers In Microbiology. 8 (146): 1- 11.

[13] Turton, J.F.; Perry, C.; Elgohari, S. Hampton. C.V. (2010) PCR characterization and typing of *Klebsiella pneumoniae* using capsular type – specific, variable number tandem repeat and virulence gene targets. *J. Med. Microbiol*. 59: 541-547.

[14] Paczosa, M.K. and Mecsas, J. (2016) *Klebsiella pneumoniae* going on the offense with a strong defense. *Microbial. Mol. Biol. Rev.* 80: 629 – 661.

[15] Hameed, Z.R. (2019) Detection of NDM-1 in Cabapenem-Resistant *Klebsiella pneumoniae*. a thesis M.Sc. Biology University of Diyala, the college of science.

[16] Al-Obadi, T.H.Z. (2014). Molecular Identification of *Klebsiella pneumoniae* Using Capsule Genes. a thesis M.Sc. Biotechnology. University of Al – Nahrain the college of science.

[17] Moghadasi, M.; Mirazaee, M. and Mehrabi, M.R. (2016). Frequency of Quinolone Risstance and qnrB and qnrC genes in clinical isolates of *Klebsiella Pneumoniae*. *J.Med Bacteriol.* 5(5):39-45.

[18] Al-Garawyi, A.M.A. (2016). Molecular expression of blaCIT and blaFox in *Klebsiella* spp. Isolates from Al-Hussien teaching hospital in Al-Muthanna province – Iraq. *Kufa J.vet.Med. Sci.* 7(2):33-47.

[19] El-Badawy, M.F. Tawakol, W.M., El-Far, S.W.; Maghrabi, I.A.; Al-Ghamdi, S.A.; Mansy. M.S. and Shohayeb. M.M. (2017). Molecular Identification of Aminoglycosides-Modifying Enzymes and plasmid-Mediated Quinolone Resistance Gene among *Klebsiella pneumonia* clinical isolates recovered from Egyptian patients. *Int. J. Microbiol.* 1-12.

[20] Ali Jabbar Abdullah (2018). "Effect of climate change on occurrence of the vectors borne and infectious disease." *Journal of Global Pharma Technology*, 10 (08): 159-164.

[21] Lalitha,M.K.(2004).Manual on antimicrobial susceptibility testing performance standards for anti-microbial testing: *Twelfth Informational supplement*, 56238, 454-456.

DOI: 10.37200/IJPR/V24I5/PR202018
Received: 27 Feb 2020 | Revised: 22 Mar 2020 | Accepted: 02 Apr 2020