Genotypic detection of Some Virulence Factors of Hypervirulent Klebsiella Pneumonia that Associated with Pathogenicity

Emad Kareem Hanoon

Department of Biology, Faculty of Science, University of Kufa, Iraq

E-Mail: imadalnajfy@gmail.com

Mahdi Alammar

Department of Biology, Faculty of Science, University of Kufa, Iraq

E-Mail: Mahdi.alammar @uokofa. edu.iq

Abstract

The study aimd to isolation and identification of Hypervirulent K. pneumonia (hvKp) from clinical samples and investigation the virulence factors genes of bacteria. The present study included 100 specimens collected from patients suffering from (, Diabetic Foot ,Wound swab, Liver abbesses , Blood , CSF, Semen, Swab) of patients to four hospitals in Najaf City from both sex Male (44) and Female (57) of age ranged between (10-50) years in the period between September to December from 2022. The bacteria were identified by Morphological, Microscopic ,biochemical and confirmed identified by VITEK-2 system were gave (80) isolates due to K.pneumoniae. In this study the results of virulence factors genes, PCR Results showed several points include : **BssS** gene gave positive result (100%) of K. pneumoniae , while **iucA** gene appear in (36%), **mrkD** gene that gave positive (100%), Molecular detection of **rmpA** gene results showed that presence in (27%), While **TraT** gene (90%) and **magA** gene were (5%).

Introduction

Klebsiella pneumoniae is a Gram-negative, non-motile, encapsulated, facultative anaerobic bacterium, which belongs to the family Enterobacteriaceae. K. pneumoniae is found in the normal flora of the mouth, skin, and intestines. this bacterium may act as an opportunistic pathogen, it is an opportunistic microorganism that causes severe diseases such as (UTI, pneumonia, septicemia, meningitis, bacteremia, wound infections and purulent abscesses at different sites) (Kwon et al.,2016). Bacterial resistance to antibiotic drugs is an increasingly serious threat to global public health besides the persister bacteria and produce a number of virulence factors that are required in the colonization, adherence, invasion, and development of the infection, including: the existence of lipopolysaccharides, capsules, adherence factors, and activity of siderophores such as hemolysins. (Mirzai et al.,2020). Klebsiella pneumoniae is classified into two types: classical K. pneumoniae (cKP) primarily occurs in hospitals and long-term care facilities, while hyper-virulent K. pneumoniae (hv KP) causes serious life threatening disease and organ failure in young healthy individuals from the community. These strains were much more resistant than classic K. pneumoniae to in vitro killing by serum or to phagocytosis by neutrophils and macrophages and caused liver abscess and meningitis in mice, they have been referred to as hypervirulent K. pneumoniae (hvKp) strains (Vading et al., 2018). The hypervirulent strains show a striking capacity to cause serious infections in young healthy individuals and immunocompetent hosts in addition to infected patients liking pyogenic liver abscess (Bulger et al., 2017; Martin and Bachman, 2018). In addition, K. pneumoniae has the potential to cause community associated infection, such as liver abscess, endophthalmitis, and meningitis, in healthy individuals (Russo and Marr, 2019). The K. pneumoniae caused liver abscess along with endophthalmitis was reported for the first time in Taiwan in the 1980s, and the causative organism was designated as hypervirulent K. pneumoniae (hvKP), Since then, hvKP has been recognized as another circulating pathotype in addition to classical K. pneumoniae (cKP), associated with high pathogenicity and mortality due to hypervirulence (Lan et al., 2020). Factors contributing to the hypervirulence mainly include capsule, siderophores, lipopolysaccharide (LPS) and fimbriae (Parrott et al., 2020). Invasive disease such as liver abscesses and endogenous endophthalmitis are associated with hypervirulent K. pneumonia (Harada and Doi,2018) .

Methodology

Samples collection

The study included 100 specimens of K.pneumoniae collected from patients suffering from (Diabetic Foot ,Wound swab , , Liver abbesses , Blood , CSF, Semen, Swab) who attending to General Hospital in ALNajaf / Iraq , during the period extended from September 2022 to February 2023 for both sexes with an age ranged between (10 - 60) years. The specimens were transported by sterile transport swabs and inoculated using direct method of inoculation on culture media .

Isolation and identification of K.pneumoniae

K.pneumoniae were identification by Morphological, Microscopic, biochemical and confirmed identified by VITEK-2 (Macfadin 2011).

Molecular detection of Virulence genes

Extraction of DNA from the K. pneumoniae isolates

DNA is extracted by the boiling method. 20 ml bacterial cultures are grown for 24 hrs, take 500 Mn in abendrove small with solution D.W and then centrifuged (3,500 rpm, 5 min)., boiled for 20 min in water bath. The supernatant containing genomic DNA is collected and stored at −20℃ (Araujo et al ., 2004)**.**

Preparation of master mix for PCR reaction

Master mix was prepared in a total volume of 25 microliters, composed of Green master mix, primer solution, deionized water, and template DNA . Adding 2pmol/µl+12.5 master mix+8.5 DNA of k.pneumonia for become **25**pmol/µl , The sequence of oligonucleotide forward and reverse primers which were used to detect (BssS, iucA, mrkD, rmpA, TraT ,iutA and magA).

The Primers preparation

The primers used in this study are supplied by Macrogen Company_ Korea ,It dissolved primer (**F**,**R**) in 320 µn (D.water) , begin with give a final concentration of 100pmol/µl as a stock solution. working solution of these primers was prepared by adding 10µl of primer stock solution(stored at freezer -20 oC) to 90µl of nuclease free water to obtain a working primer solution of 10pmol/µl. as in the table (1) . DNA amplification was carried out with the following thermal cycling: an initial denaturation of DNA at 95 °C for 15 min. was followed by 30 cycles of amplification (94 °C for 30 sec., 60 °C for 90 sec and 72 °C for 90 sec), ending with a final extension at 72 °C for 10 min. and soak at 4°C for 5 min.

Table 1: Characteristics of the primers used in PCRs

Primer Target	Oligo Sequence 5′→3′	Product Size (bp)	References
BssS	**F-** 5-GATTCAATTTTGGCGATTCCTGC-3 **R**-5TAATGAAGTCATTCAGACTCATCC-3	225	Fabrice et al.,2014
iucA	**F**-5-CGAAATCGAAATAGATCACC-3 **R**-5-CTGACGCGATTTGCCGC-3	1125	Compain et al., 2014
mrkD	**F-** 5- AAGCTATCGCTGTACTTCCGGCA- **R**-5-GGCGTTGGCGCTCAGATAGG-3	340	Fabrice et al.,2014
rmpA	**F-** 5- CATAAGAGTATTGGTTGACAG-3 **R**-5- CTTGCATGAGCCATCTTTCA-3	461	Compain et al., 2014
TraT	**F-** 5-GGTGTGGTGCGATGAGCACAG-3 **R**-5 -CACGGTTCAGCCATCCCTGAG-3	461	Chuang et al., 2006
magA	**F-** 5-GGTGCTCTTTACATCATTGC-3 **R**-5- GCAATGGCCATTTGCGTTAG-3	1283	Turton et al., 2008

Statically analysis:

A statistical package (Statistical Package for Social Sciences, version 20, IBM, and Armonk, New York) was used to collect and analyze the data. The numbers and percentages were used to summarize

qualitative data, using Graph pad-prism computer software version 10.

Results And Discussion

Bacterial investigation

A total of 100 clinical specimens of K. pneumonia were collected from a number of hospitals in Najaf city , The results showed that all positive culture then streaked on general and selective media. A 100 isolates of K. pneumonia from total isolates were obtained of both sexes male(43) and female (57) ,also diagnostic by VITEK-2 .

Biochemically showed that all isolates of K. pneumoniae gave positive for catalase test indicated by bubbles formation of O2 generated by the enzyme catalase, citrate utilization , urease ,while most isolates gave negative indol test due to incapability isolates for the secreted of enzyme tryptophanase, and gave negative result for methyl red test (Forbes et al., 2014). Lactose and Glucose were fermented with the formation of acid (A/A) and gas (CO2), H_2S was not formed by kligler iron agar, showed that K. pneumoniae isolates converted the color of both the butt and slant, which produced acid butt (yellow) and acidic slant (yellow) accompanied by gas creation (bubbles formation), without black precipitate formation, which indicated that glucose and lactose fermentation had occurred and no H_2S was produced(Brooks et al., 2007;Forbes et al., 2014). The **Vitek-2** system was used to confirm the identification. Showed results that 80 isolates belonged to K. pneumoniae, while 20 isolates were not related to K. pneumoniae (Ozgen and Eyupoglu , 2020).

Genotypic detection of virulence genes in H.V.K. pneumoniae:

Molecular detection of BssS gene in K. pneumoniae:

The results showed that present **BssS** gene was detected in all isolates with percentage (**100**%) and Which is the most widespread of the genes study, the bands appeared for all favorable isolates within the expected size of the gene (225bp) in gel electrophoresis as in figure (1).

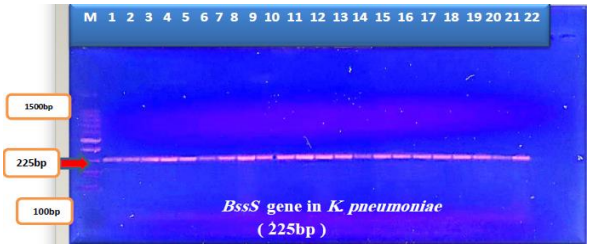

Figure 1: Virulence genes amplification assay for K. pneumoniae,image of Agarose Gel : electrophoresis for BssS in K. pneumonia isolates with product size 225bp , M: Marker ladder (100-1500bp), : primer sequences for BssS gene.

The sequences of the biofilm gene (bssS) of K. pneumoniae isolates (that showed an increase in their gene expression by RT-PCR) were submitted to GenBank , Data analysis of these sequences using the gene showed 98% identity of these nucleotide sequences with K. pneumoniae biofilm gene (bssS) (Grela et al .,2015). In other study Presence BssS gene of virulence factors in K. pneumoniae , the biofilm gene (bssS) in 60% of the tested isolates which indicates on found this the in K. pneumoniae isolated (Abdelaziz et al., 2019).

Molecular detection of iucA gene in K. pneumoniae:

The findings this gene appearance present in (29) isolates from all isolates (80) that give positive result for iucA gene to K. pneumoniae with percentage (36%). The gave positive of **iucA** gene in**(Diabetic foot**, **burns**, **Sputum**, **Wound**) only and Shows there this gene in hypervirulence K.pneumoniae (hvkp) strains compared to classical K. pneumoniae,In these study the present iucA gene was estimated among **36**% of K. pneumoniae isolates. It was found that 42 (**35.5**%) isolates exhibited serum resistance, The hypervirulent strains show a striking capacity to cause serious infections in young healthy individuals and immunocompetent hosts in addition to infected patients (Bulger et al.,2017).

Figure 2: Virulence genes amplification assay for K. pneumoniae,image of Agarose Gel electrop: horesis :for iucA in K. pneumonia isolates with product size 1125bp , M: Marker ladder (100-1500bp): Isolates numbers and that primer sequences for iucA gene.

Molecular detection of mrkD gene in K. pneumoniae:

The findings obtained in this study showed that the presence of **mrkD** gene in K. pneumoniae positive with percentage (100%) according sequences isolated that use in PCR , with different sources that indicate in these study mrkD gene is weak in classical(UTI) and strong in hyper isolates K. pneumoniae as **figure(3).** Compain et al. (2014) showed that all isolates **100**% while the findings obtained in this study showed that the presence of mrkA, **mrkD**, fimA and fimH genes in K. pneumoniae was **100**% respectively (Doneli et al., 2014) El Fertas-Aissani et al. (2013) reported that the most common virulence genes were , **mrkD** (**96.3**%), with rmpA was (3.7%) and magA not detected in 54 isolates.hvKP; that possess **mrkD** (Nahavandinejad et al.,2017)**.** Kuş et al. (2017)

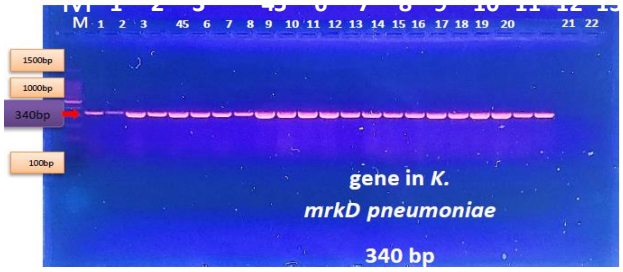

Figure 3: Virulence genes amplification assay for K. pneumoniae,image of Agarose Gel electrophoresis for mrkD in K. pneumonia isolates , size 340 bp , M: Marker ladder (100-1500bp): Isolates numbers and that primer sequences for mrkD gene.

Molecular detection of rmpA gene in K. pneumoniae

The result of this study showed that the presence of **rmpA** gene in K. pneumoniae with percentage (27%) according **Figure (4).** Virulence and capsule serotype genes were identified by using PCR technique **that rmpA** in (**38.7** %), (Alizade et al., 2018). The virulence factor **rmpA** gene was in 5.7 % in the study of Shakib .,(2018) and in 62 % in the study of (Hamam et al. ,2019).

Recent studies have revealed that the rmpA gene is responsible of producing of capsule which protect bacteria from phagocytosis. In another study reported by Zhan et al. (2017) from China found the virulence-associated genes among 21 isolates included entB 95.2%(20), ybtS 95.2%(20), and iutA 90.5%(19). **rmpA** and ybtS were found in **57.1**%(12) isolates.

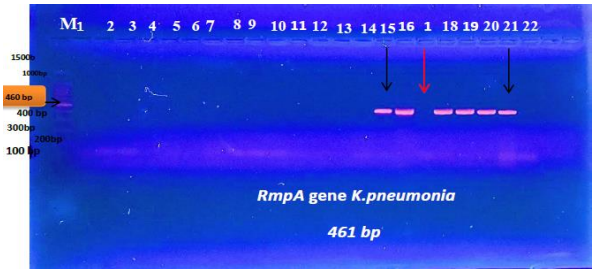

Figure 4: Virulence genes amplification assay for K. pneumoniae,image of Agarose Gel electrophoresis for rmpA in K. pneumonia isolates , size 461 bp , M: Marker ladder (100-1500bp): Isolates numbers and that primer sequences for rmpA gene.

Molecular detection of TraT gene in K. pneumoniae:

The result that obtained in this study showed that the presence of **TraT** in K. pneumoniae with percentage (**90%)** according sequences isolated that use in PCR , Other isolates gave positive result for TraT gene of K. pneumoniae . as **Figure (5)**. In a previous study of Atmani et al., (2015), **traT** gene was present at low rate (31%) in municipal wastewater-treatment plant isolates and was absent in hospital.

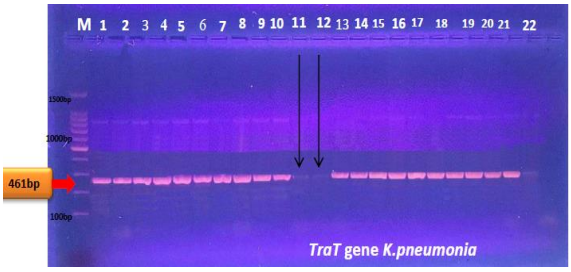

Figure 5: Virulence genes amplification assay for K. pneumoniae,image of Agarose Gel electrophoresis for TraT gene in K. pneumonia isolates , size 461 bp , M: Marker ladder (100-1500bp), lane : Isolates numbers and that primer sequences for TraT gene.

Molecular detection of magA gene in K. pneumoniae

Result of molecular detection of magA gene in all isolates a many K. pneumoniae , The result of **magA** gene in figure (6) were isolates with percentage **(5%)** and isolates positive due to **Ear swab** , other isolates gave negative result for **magA**

gene as **Figure (6)**. According to the results of screening virulence genes, was the most prevalent among all the clinical isolates, followed by the mrkD (n=46, 65.7 %), ybtS (n=42, 60%), iutA (n=8, 11.4%), kfu (n=8, 11.4%), rmpA (n=4, 5.7%), **magA** (n=**1**, **1.43**%), and K2 was not detected in any of the isolates (Amraie et al., 2014)**.**

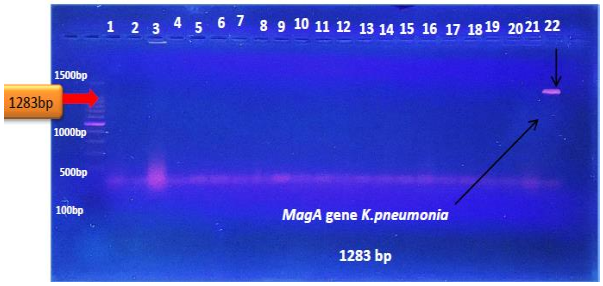

Figure 6: image of Agarose Gel electrophoresis for maqA in K. pneumonia isolates with product size 1283bp , M: Marker ladder (100-1500bp): Isolates numbers and that primer sequences for maqA gene.

Prevelence of virulence Factor encoding genes in Virulence K. pneumoniae

The result show that (BssS , MrkD and TraT) genes were high production 100% a many K. pneumoniae isolates, While the genes (iucA and RmpA) were moderate prevalence , in addition to the virulence gene magA were low level 5% of K. pneumoniae isolates. Nahavandinejad and Asadpour (2017) reported that **magA** gene was confirmed in 2 (**3.07**%) isolates and 10 (15.38%) isolates were positive for the presence of rmpA gene from 65 isolates. Fu et al. (2018) found 43 isolates carried entB, ybtS and mrkD gene, followed by allS 34(79.06%), iutA 27 (62.79%), kfu 25 (58.13%), ybtS16(37.20%). rmpA14(32.55%) and **magA** 5(**11**.62%).

Table 2: Number and percentage of virulence factors genes of Klebseilla pneumoniae in clinical samples from different of sources.

Gene / Sources	**BssS**	**iucA**	**mrkD**	**rmpA**	**TraT**	**magA**	**Total**	**Percentage %**
1-Diabetes	17	12	17	0	17	0	63	78%
2-Urine	19	0	19	0	19	0	57	71%
3-Burns	15	9	15	0	15	0	54	68%
4-Wound	10	5	10	10	10	0	45	56%
5-Sputum	5	3	5	0	5	0	18	22 %
6-Liver	3	0	3	3	3	0	12	15%
7- Semen	3	0	3	3	3	0	12	10%
8- Blood	2	0	2	2	2	0	8	10%
9- CSF	2	0	2	2	2	0	8	15%
10-Vaginal	2	0	2	2	2	0	8	10%
11 Ear swab	2	0	2	0	2	2	8	10%
Total	**80** 100%	**29** 36%	**80** 100%	**22** 27%	**72** 90%	**5** 5 %		

The result show that the K. pneumoniae isolate were carry high rates (78% ,71% , 68% and 56%) isolates from (Diabetes foot , Urine , Burns and Wound) , while isolates (Sputum and Liver) were carry the moderate , and other isolates were carry lower rates of virulence Factor encoding genes (Semen , Blood , Ear swab , Vaginal swab , CSF).

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