Molecular Detection of Some Virulence Factors genes of *Klebsiella pneumoniae* Isolated from Food Samples

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Abstract

Klebsiella pneumoniae is not only one of the main pathogens acquired from the hospital but it is as well a significance foodborne pathogen that causing liver abscesses, septicaemia ,and diarrhea.The study aimed to isolate *Klebsiella pneumoniae* from different food samples in Al-Diwaniyah city/Iraq ,determine some virulence factors genes of these isolates, and testing the resistance to antibiotics. The study extended fromAugust to November 2020.A total of 25 isolates identified as *Klebsiella pneumonia*using traditional biochemical tests ,Vitek system and 16SrRNA gene.The existence of 8virulence factors geneswasdetectedby PCR.The more commonness virulence factors genes were *Khe* (100%), *fimH*(80.0 %), *Uge* (64.0 %), *ureA* (36.0 %), *YbtA* (16.0 %) ,*alls* (12.0 %),*iucB* (8.0 %) and *ironB*(0.0%).The bacterial isolates explained risinglevel of resistanceto Amoxicillin-clavulanate,piperacillin ,gentamicin,ceftazidime , cefepime and cefotaxime(25/25,100%) , ampicillin (22/25, 88.0 %), tetracycline (20/25 ,80.0 %), amikacin (15/25,60.0 %) ,aztreonam(9/25, 36.05 %),(11/25, 44.0 %) intermediate and (5/25, 20.0 %) sensitivity rate..The sensitivity of isolates recorded to Levofloxacin ,ciprofloxacin, Ampicillin-sulbactam (SAM) and imipenem (25/25 ,100%), Chloramphenicol (20/25, 80.0 %), Moxifloxacin (19/25,76.0 %) and meropenem(9 /25,36.0 %) .The existence of virulence genes and resistance of antibiotics in *Klebsiella pneumoniae* isolated from foodsrefers to a possibility healthrisk for people.The results showa significanceformonitoring *Klebsiella pneumoniae* in food.

Keywords:*Klebsiella pneumoniae*, Food, Virulence factors, Antibiotic resistance

Introduction

Klebsiella pneumonia is a significant opportunistic bacteria which can cause a various infections in humans, involving septicemia ,liver abscesses , diarrhea ,and pneumonia (Guo *et al*.,2017).It is one of the famous pathogens acquired in hospital and is related to increasing the patients morbidity and mortality (Cabral *et al*.,2012).

Aside fromthe clinical isolates of *K.pneumoniae* is often present in diet, inclusive vegetables, dried children's powder ,fish, meat, and street foods ,and has been believed a food bornepathogen (Davis and Price,2016).

Bacterial 16SrRNA genes include nine "hypervariable regions" (V1-V9) that illustratessignificant sequence diversity among various bacteria. Species-specific sequences in the offered hypervariable region comprisebeneficial targets for

diagnostic tests and other scientific investigations" (Chakravorty *etal*.,2007).Molecular testing permits for extremelyspecific and sensitive diagnosis of a huge number of pathogenic agents in the clinical isolates .The capacity of molecular methods to detect pathogens immediately from food and clinical samples led to the fast diagnosis without   s ma from food and clinical samples makes the fast identification without tend to culture possibility (Barbut *et al*.,2011) .

Klebsiella pneumoniae canproduce a differentvirulence factors inclusive;endotoxin ,capsule ,iron scavenging system ,siderophores ,as well as adhesions ,which are shown to do significant roles in their etiology . Virulence factors likethe*allS*(the allantoin regulon activator coding, correlated with the allantoin metabolism); genes associatedwith internal toxins, *uge*, genes related to the ironacquiring system *iucB,ironB,ybtA and fimH* ; It is also believed that the *ureA* gene (sub-unit α-urease, related to invaders) is included in virulence modes (Calhau *et al* .,2013).

The appearance of antibiotics resistant within*Klebsiella* spp.is a major problem in themedicine (Hu *et al*.,2013). Drug-resistance by *K.pneumoniae*isolates from various food samples (Yaici *et al*.,2017).

Dietary intake is one of the main ways to introduce antibiotic-resistance pathogensin the human digestive system .Consumingthe certain food groups may affect the genetic diversity of antibiotic resistance .So, the aim of this study is to isolate *Klebsiella pneumoniae* from different food samples ,detect the susceptibility of the isolates to some antibiotics and molecular detection of some virulence factors genes(*khe*, *iucB*, *alls*,*ureA*, *ironB*, *fimH*, *ybtA*,*uge*).

Materials and Methods

Isolation and Identification of Bacterial isolates

A total of(190)food samples collected from super market ,house eat ,Different restaurantsas well s from farm in different region in Al-Diwaniyah city during a period from August to November 2020.The sample including   meat(fresh, freezing,mincedand cooked), aquatic products (fish), Fruits(Orange, Grapes) vegetables (Tomato, Cucumber and lettuce),grainsand appetizers. The food samples put in discrete sterilized bags and thereafter directly transferred in icebox to the laboratory (under 4˚C) and treated during 4-6 h.

Nearly, 2.5grams of each sample was enriched in 10 ml of nutrient broth for 24h at 37˚C.Then, inoculate from nutrient broth was cultured in MacConkey agar ,incubated overnight at 37˚C . From the growing colonies with pink color and mucoid texture ,three colonies were selectedand subcultured in the nutrient agar and incubated overnight at 37˚C , after that the identification of isolates performed by using Vitek system (Biomerieux,France) .Insured isolates were preserved in Brain-heart infusion broth with 20% glycerol and stocked at -20˚C stored at -20˚C for furthermore study.

Molecular detection of*Klebsiella pneumoniae* by 16S rRNA gene:

DNA extraction

A DNA extraction kit (Geneaid, Taiwan) was used to perform the process of extracting the DNA from fresh growth of *Klebsiella pneumoniae*. The procedure was generated depending on the protocol of the manufacturer .The measurement of DNA concentration was done for quality and   quantity by a NanoDrop(Nabi-Korea) .

Amplification of 16S rRNA gene by PCR

The primers (Forward primer ; AGCGTCAGTCTTTGTCCAGG   and Reverse primer; GATGACCAGCCACACTGGAA) used to amplify regions in the 16S rRNA gene have been designed by using program of Primer 3 Plus. The reaction of the PCR employed by using Mastermix (Bioneer, Korea) with at total volume (20 μl) of reaction mixture; including 5μl DNA template, 2μl (10pmol) for each primer, 11 μl of nuclease free water . The conditions of the thermocycler were 95˚ C for 2 minute of the first denaturation, 35 cycles including the denaturing with 95˚C for 30 sec., the annealing was 58˚C for 30 sec. and the extension was 72˚C for 1 minute) and the ultimate extension was at 72 ˚C for 5 minute.Using 1.5 %-agarose gel including 0.5μg/ml Red Safe dye   , DNA bands were separated via electrophoresis and visualized via a Gel documentation system (Wisd-Korea).

Antimicrobial susceptibilityTesting

The strains were examined for antibiotic sensitivity for 17antibiotics using the method of disk diffusion in Muller Hinton agar (Mast, UK) following the guidelines of (CLSI,2020). These tested antibiotics: ampicillin(10μg),amoxicillin-clavulanic acid amoxicillin-clavulanic acid (30μg), cefotaxime (30μg), ceftazidime (30μg) , piperacillin(100μg), imipenem(10μg), gentamicin(10μg), amikacin (30μg), Cefepime(30μg), Aztreonam(30μg), Meropenem(10μg), Levofloxacin (5 μg), Ampicillin-Sulbactam (20μg), Ciprofloxacin(5μg), Chloramphenicol(30μg), Moxifloxacin (5μg), and teteracycline (30μg). The selected antibiotics represent the major classes of antibiotics which are substantial to treat bacterial diseases . The isolates recorded   as a sensitive , moderate and resistance depending on the guidelines of CLSI.

Detection of virulence genes in *K.pneumoniae* isolates

The assured isolates of *Klebsiella pneumoniae*were cultured in thenutrient broth for 24 h. at 37˚C.The extraction of DNA was done by using specific kit for extraction (Geneaid- Taiwan) depending on the instructions of the manufacturer. Eight singular PCRs carried out to determine the existence of virulence factors genes (*allS*, *ybtA*, *iucB*, *ironB*, *fimH*, *ureA*, *uge*, *Khe*) in the isolates of *Klebsiella pneumoniae*as recorded in the former studies (Brisse *et al*.,2009 and Turton,2010). The sequences of primers ,and the size of amplicons are clarified in Table 1.

The product of PCR analyzed in 1.5% agarose gel that contain Safe Red (0.005% v/v) in TAE buffer,and imaged by using Gel documentation system (Wisd-Korea) .

Table(1) PCR primers used in this study

Target virulence genes	Primer Sequence(5′-3′)	Size (bp)	References
Khe	F: TGATTGCATTCGCCACTGG R: GGTCAACCCAACGATCCTG	428	Neuberger *et al.*, 2008
Alls	F: CCGTTAGGCAATCCAGAC R: TCTGATTTA(A/T)CCCACATT	1090	Brisse *et al.*, 2009
YbtA	F: ATGACGGAGTCACCGCAAAC R: TTACATCACGCGTTTAAAGG	960	He, 2012
iucB	F: ATGTCTAAGGCAAACATCGT R: TTACAGACCGACCTCCGTGA	948	He, 2012
UreA	F: GCTGACTTAAGAGAACGTTATG R: GATCATGGCGCTACCT(C/T)A	337	Brisse *et al.*, 2009
fimH	F: GCTCTGGCCGATAC(C/T)AC(C/G)ACGG R:GC(G/A)(A/T)A(G/A)TAACG(T/C)GCCTGGAACGG	423	Brisse *et al.*, 2009
Uge	F: GATCATCCGGTCTCCCTGTA R: TCTTCACGCCTTCCTTCACT	534	Brisse *et al.*, 2009
ironB	F: GGCTACTGATACTTGACTATTC R: CAGGATACAATAGCCCATAG	992	He, 2012

Results

Identification of *K. pneumoniae* by biochemical and molecular assays

The overall of 25isolateswere diagnosed as *K.pneumoniae*from (190) food samples that collected from different sources in Al-Diwaniyah city/Iraq, by traditional biochemical tests and Vitek system;distributed by 4 isolates from Pomegranate , 3 from Cucumber, 4 from Orange , 2 from Graps , one isolate from fish , 2 isolates from shredded colored Walnuts, 4 from Hummues , 3 from Minced meat, one isolate from fresh meat and one fromrestaurant Appetizers).Also ,we confirmed the identification of isolates by molecular detection of 16SrRNA gene as clarified in figure.1.
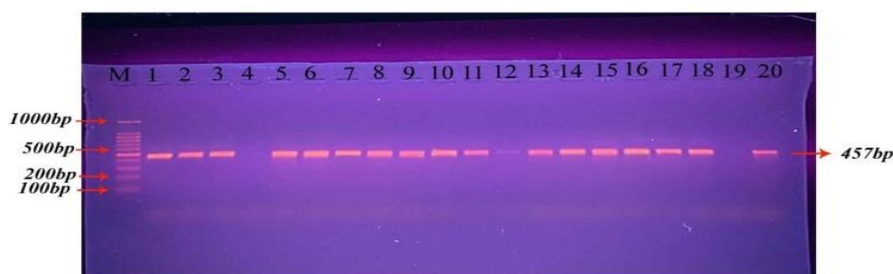
Figure1: Image of Agarose gel electrophoresis showed the PCR product withsize457 bp for identification gene(16SrRNA)of *Klebsiella pneumoniae* isolates. Where Marker ladder (100-1000bp), lane (1-20): Isolates numbers.

Antibiotic susceptibility of *K. pneumoniae* isolates:

The Rates of sensitivity and resistance to antibiotics by *Klebsiellapneumoniae* samples are shown in table 1. Amoxicillin-clavulanate (AMC), piperacillin (PRL), Gentamicin (GEN), Ceftazidime (CAZ),Cefepime (CPM) and Cefotaxime (CTX) showed (25/25 ,100%) resistance rate, Ampicillin (AM) showed 22 (88.0 %) resistance rate and 3 (12.0 %) sensitivity rate,Tetracycline (TE) showed 20 (80.0 %) resistance rate and 5 (20.0 %) intermediate rate, Amikacin (AK) showed (15/25,60.0 %) resistance rate and (10/25, 40.0 %) intermediate rate.Aztreonam (ATM) showed (9/25,36.0 %) resistance, (11/25, 44.0 %) intermediate and (5/25, 20.0 %) sensitivity rate. Meropenem (MEM) showed 5 (20.0 %) resistance rate, 11 (44.0 %) intermediate rate and 9 (36.0 %) sensitivity rate.

Levofloxacin (LEV) ,Ciprofloxacin (CIP) andImipenem (IPM) ,Ampicillin-sulbactam (SAM) showed 25 (100%) sensitivity rate. Chloramphenicol (C10) showed 20 (80.0 %) sensitivity rate and 5 (20.0 %) resistance rate. Moxifloxacin (MFX) showed 19 (76.0 %) sensitivity rate and 6 (24.0 %) resistance rate.Therefore in terms of sensitivity, the best antibiotics are Levofloxacin, Ciprofloxacin, imipenem and ampicillin-sulbactam followed by Chloramphenicol and then by Moxifloxacin.

**Table 1**: Rates of sensitivity and resistance to antibiotics by *Klebsiella pneumoniae*eisolates

Antibiotic	R	I	S
AMC	25 (100.0 %)	0 (0.0 %)	0 (0.0 %)
ATM	9 (36.0 %)	11 (44.0 %)	5 (20.0 %)
PRL	25 (100.0 %)	0 (0.0 %)	0 (0.0 %)
GEN	25 (100.0 %)	0 (0.0 %)	0 (0.0 %)
AK	15 (60.0 %)	10 (40.0 %)	0 (0.0 %)
MEM	5 (20.0 %)	11 (44.0 %)	9 (36.0 %)
AM	22 (88.0 %)	0 (0.0 %)	3 (12.0 %)
CAZ	25 (100.0 %)	0 (0.0 %)	0 (0.0 %)
LEV	0 (0.0 %)	0 (0.0 %)	25 (100.0 %)
CIP	0 (0.0 %)	0 (0.0 %)	25 (100.0 %)
C10	5 (20.0 %)	0 (0.0 %)	20 (80.0 %)
IPM	0 (0.0 %)	0 (0.0 %)	25 (100.0 %)
TE	20 (80.0 %)	5 (20.0 %)	0 (0.0 %)
CPM	25 (100.0 %)	0 (0.0 %)	0 (0.0 %)
MFX	6 (24.0 %)	0 (0.0 %)	19 (76.0 %)
SAM	0 (0.0 %)	0 (0.0 %)	25 (100.0 %)

CTX	25 (100.0 %)	0 (0.0 %)	0 (0.0 %)

AMC: Amoxicillin-clavulanate; **ATM**: aztreonam; **PRL**: piperacillin; **GEN**: Gentamicin; **AK**: Amikacin; **MEM**: meropenem; **AM**: Ampicillin; **CAZ**: ceftazidime; **LEV**: Levofloxacin; **CIP**: Ciprofloxacin; **C10**: Chloramphenicol; **IPM**: imipenem; **TE**: tetracycline; **CPM**: cefepime; **MFX**: Moxifloxacin; **SAM**: ampicillin-sulbactam; **CTX**: Cefotaxime; **R**: resistant; **I**: intermediate; **S**: sensitive

Detection of Virulence factors genes

The rate in terms of cases numbers of virulence factors genes in *Klebsiella pneumoniae* isolated from various food samples is shown in figure 2. The virulence factor gene ,*fimH* was seen in 20 (80.0 %) cases, *alls* in 3 (12.0 %). *Uge* in 16 (64.0 %), *iucB* in 2 (8.0 %), *ureA* in 9 (36.0 %), *YbtA* in 4 (16.0 %) and *Khe* in 25 (100.0 %) .Whereas, *ironB* was not seen in the enrolled isolates.The identification of these virulence factors was done using conventional PCR techniques and the results of gel electrophoresis are shown in figures 3 through 9.
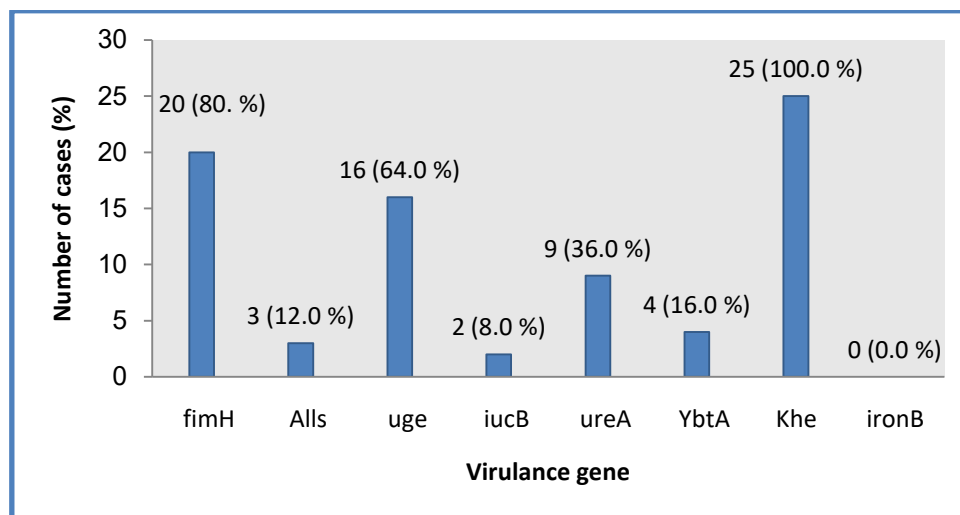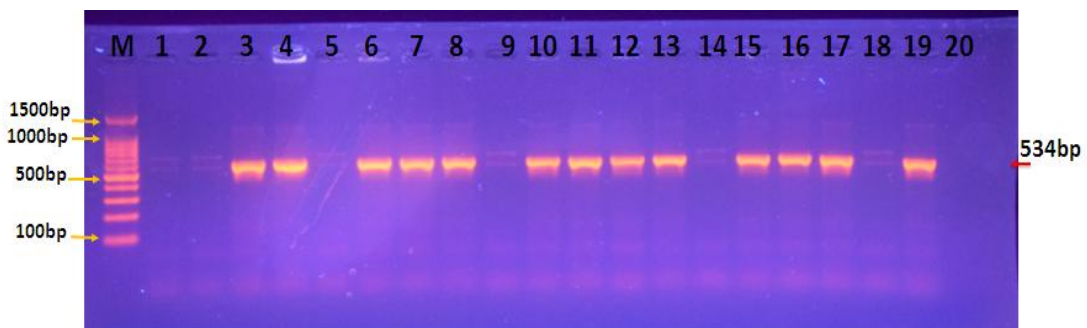

**Figure 2:** The rate (%) of virulence factors genes in *Klebsiella pneumonia* isolates from various food sources.
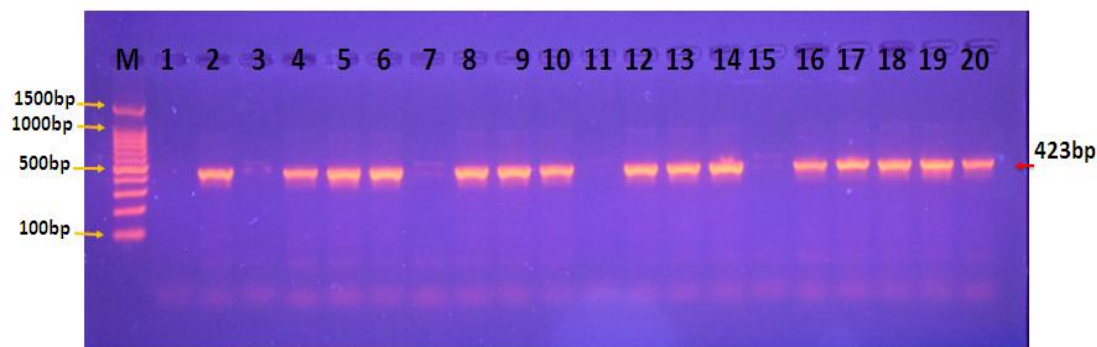
Figure 3: Image of agarose gel electrophoresis showing the product of PCR of virulence factor gene,*fimH* of *Klebsiellapneumoniae* isolates with product size 423bp .Where Marker ladder (100-1500bp), lane (1-20): Isolates numbers.
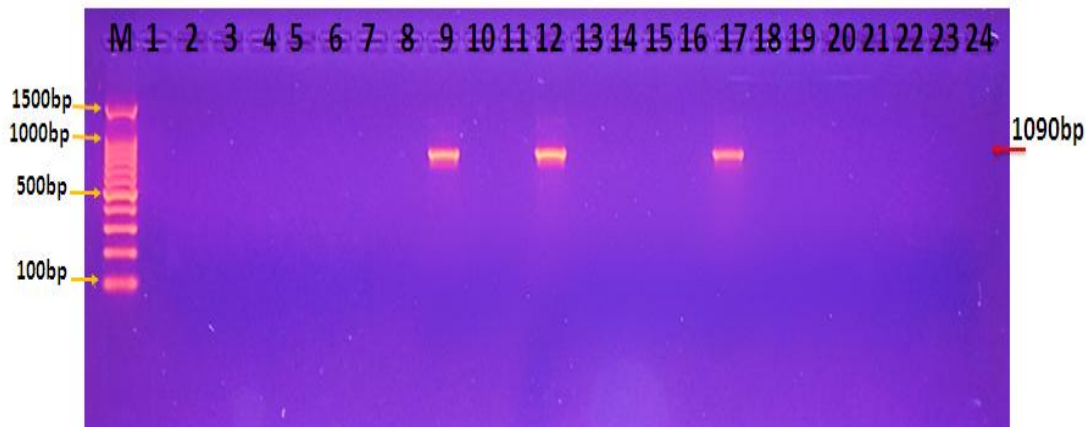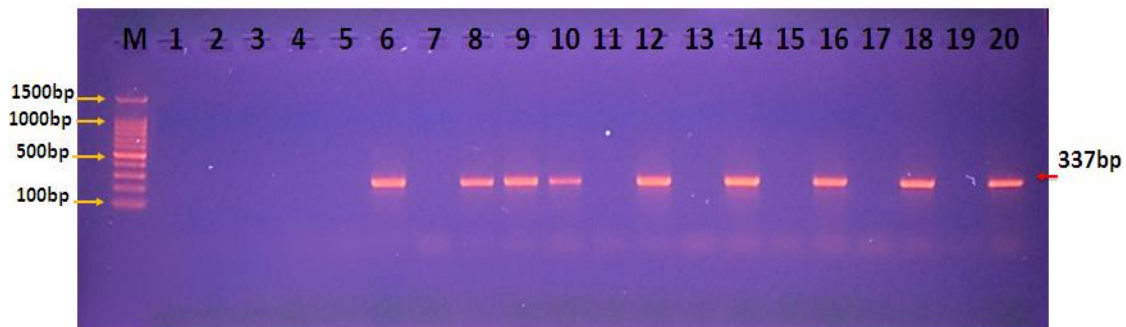
Figure 4: Image of agarose gel electrophoresis showing the product of PCR ofvirulence factor gene,*allS* of *Klebsiella pneumonia*isolates with product size1090bp. Where Marker ladder (100-1500bp), lane (1-24):Isolates numbers.
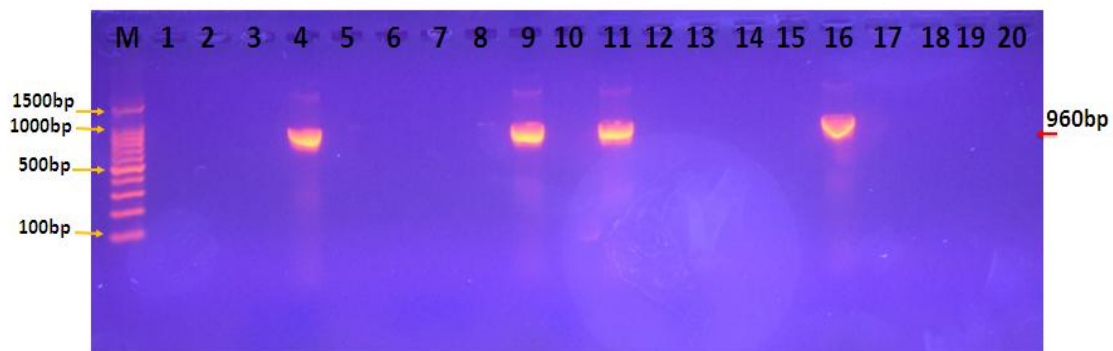

Figure 5: Image of agarose gel electrophoresis showing the product of PCR of virulence factor gene,*uge*of*Klebsiella pneumoniae*isolates with product size534bp. Where Marker ladder (100-1500bp), lane (1-20):Isolate numbers. .
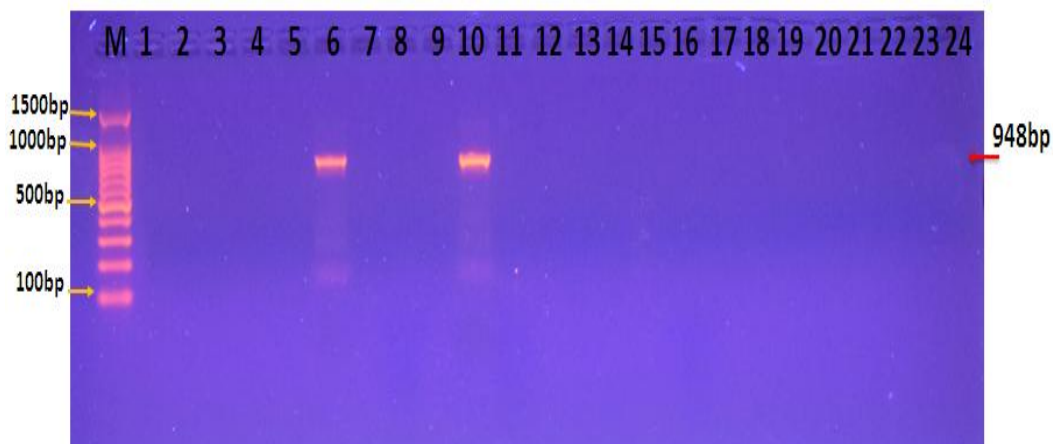
Figure 6: Image of Agarose gel electrophoresis showed PCR product of virulence factor gene,*iucB*of *Klebsiella pneumoniae* isolateswith product size948bp. Where Marker ladder (100-1500bp), lane (1-20): Isolates numbers.

Figure 7:Image of Agarose gel electrophoresis showed PCR product of virulence factor gene,*ureA*of *Klebsiella pneumoniae* isolateswith product size 337bp.   Where Marker ladder (100-1500bp), lane (1-20): Isolates numbers.
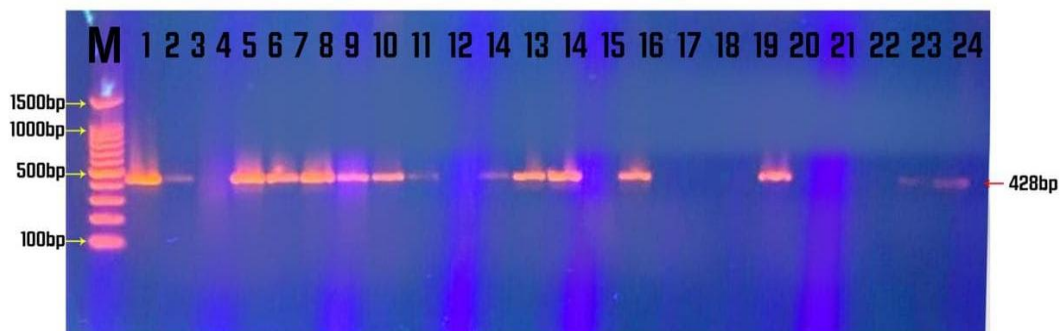

Figure 8: Image of Agarose gel electrophoresis showed PCR product of virulence factor gene,*YbtA*of *Klebsiella pneumoniae* isolateswith product size960bp.   Where Marker ladder(100-1500 bp) ,lane (1-20):Isolates numbers.

Figure 9: Image of Agarose gel electrophoresis showed PCR product of   virulence factor gene,*khe*of *Klebsiella pneumoniae* isolates with product size 428 bp.   Where Marker ladder (100-1500bp), lane (1-20): Isolates numbers .

Discussion

In addition, to being a principal hospital acquired bacterial pathogen, *Klebsiella pneumonia*e is a food born agent that can cause a variety of clinical features such as diarrhea, liver abscess and septicemia (Zhang *et al*., 2018). The genotypic characteristics of food born *K.pneumoniae* strains have not been fully investigated in our country, thus, the aim of the current study was to evaluate the genetic characteristics in terms of virulence factors expression as well as the phenotypic characteristics in terms of antibiotic sensitivity and resistance.

In the current study, *Klebsiella pneumonia* isolated from 25 food samples according to laboratory investigations by traditional biochemical tests and molecular diagnosis using 16SrRNAgene, these isolates were tested for antibiotic resistance. The resistance to Amoxicillin-clavulanate was seen in all cases. In a previous Chinese study done by Zhang *et al*. (2018) the resistance to this antibiotic was not reported in any of the their samples, in the contrary to our results; however, in another study done by *Hartantyoet al*.(2020) in Singapore, resistance to Amoxicillin-clavulanate was reported in 6.1 %. Therefore, the strain affecting the food item in our study is most probably different form that food isolates in China and Singapore because it was 100 % resistant to Amoxicillin-clavulanate.

With respect to aztreonam, the resistance was seen in 36 %. Neither the Chinese study nor the Singapore has assessed the resistance to this antibiotic.

Regarding piperacillin, we reported 100 % resistance rate. The Chinese study reported only 3.2 % resistance rate while the Singapore did not assess the resistance to this antibiotic. This result also supports our belief that the isolated Iraqi *Klebsiella pneumoniae* strains are different from that isolated in China.

The resistance to Gentamicin, was seen in all cases, while the Chinese study has reported 6.5 % resistance rate and the Singapore study has reported no resistance at all. For amikacin, we reported 60.0 % resistance rate, whereas, the Chinese study has reported no resistance and the Singapore study has reported 1.5 %.

The antibiotic, meropenem, showed 20.0 %resistance rate, while both Chinese and Singapore studies has mentioned nothing about this antibiotic. For ampicillin, we reported resistance in 88.0 % of cases, which is comparable to that reported by Chinese study (82.3 %) and Singapore study (97.0 %) , ceftazidime, reported resistance in 100 % of cases, while the Chinese study, reported no resistance and the Singapore study did not include this antibiotic. For Levofloxacin, we reported no resistance; the Chinese and the Singapore study did not include this antibiotic. Regarding Ciprofloxacin, we reported no resistance; the Chinese study reported 4.8 % and the Singapore study reported 12.1 % resistance rate.

Regarding Chloramphenicol, we reported resistance in 20 % of cases; the Chinese study 6.5 % of cases and the Singapore study reported resistance in 7.8 %, thus, the resistance rate in the present study is slightly risingthanthe rate recorded by both Chinese and the Singapore studies. For imipenem, no resistance were recorded for our cases and in accordance with our findings, the Chinese study reported resistance in none of the cases. With respect to tetracycline, we reported resistance in 80.0 % of cases; the Chinese study reported resistance in 11.3 % while the Singapore study reported resistance in 19.7 %. Again, these results support our belief that the isolated

Iraqi *Klebsiella pneumoniae* strains are different from that isolated in China and in Singapore. Concerning Cefotaxime, we reported resistance in 100% of cases, while the Chinese study reported resistance in only 3.0 % of cases supporting the great discrepancy between our genetic strains and their isolated genetic strains.

For cefepime, the resistance rate was 100 %. Concerning Moxifloxacin, we reported resistance in 24 % of cases. Regarding ampicillin-sulbactam, we reported no resistance at all. Both Chinese study and Singapore study did not include these antibiotics.

We compared our results with previous results in China and Singapore because there are notmany studies about *Klebsiella pneumoniae* isolated from food samples.

K.pneumoniae causes a wide range of infections both in the community and health-care setting leading to increased morbidity and mortality(Brisse *et al*.,2009). Pathogenicity of *K.pneumoniae* is due to the presence of various virulence factors such as capsule, endotoxins, siderophores, iron-scavenging systems and adhesins. These factors help this bacterium to evade immune system and cause various infections(Brisse *et al*.,2009 and Alhasani,2016).

It has been detected the following virulence genes in food isolates of *K.pneumoniae*with corresponding rates: *fimH* in 80.0 %; *AllS* in 12.0 %; *Uge* in 64.0 %; *iucB* in 8.0 %; ureA in 36.0 %; *YbtA* in 16.0 %; *Khe* in 100% and *ironB* in 0.0 %.

In the Chinese study, the virulence genes were reported in the following rates: *fimH* in 85.5 %; *allS* in 6.5 %; *uge* in 65.5 %; *iucB* in 0.0 %, *ureA* in 79.0 %, *ybtA* in 1.6 % and *iroNB* in 0.0 %. Therefore, our study is approximately similar to the Chinese study in all included genes with exception of *khe* gene which has not been mentioned by the Chinese study.

The findings of the virulence factors detection proposed that the *Khe*, ,*fimH* ,*ureA* ,and *uge genes* were the most common genes founded in the food isolates , which are agree with that results recording for clinical isolates (Yu *et al*.,2008; Calhau *et al*.,2014 and Cheng *et al*.,2015).The existence of those genes in bacterial strains from food samples proposed the pathogenic possibility of the isolates and a possible hazards to health of human.

Hemolysins is cytolyic toxin that lysis erythrocytes which are represent as a significant cause of destruction to are consider as an important causes of damage to ease the diffusion of bacteria, extra-intestinal diseases as well releasing of host nutrients, and may also modify the pathways of the host by influencing on different pathways,include host cell survival,inflammatory response ,cytoskeletal dynamics (Schmidt *et al*.,1995).
Type 1 fimbriae are essential for the ability of *K. pneumonae*to cause urinary tract infection (UTI) and found on all members of *Enterobacteriacae*. They exert their adhesive properties by virtue of the *fimH* adhesion located on the tip of fimbriae that recognizes mannose-containing glycoproteins present on many mammalian host tissues and their expression is phase variation encoded by operon fim (Schembri *et al*.,2005).

Numerous species of bacteria ,involving *K.pneumoniae* have the ability to use urea as a nitrogen source by virtue the capacity of hydrolyzing the urea to ammonia and

CO2 by the urease enzyme (Mobley and Island,1995). The production of the alkaline ammonia may be the cause of tissue destruction ,and sometimes a key agent in the existence of pathogens (Burne and Chen,200). The disability to metabolize the urea may therefore effect the ability of *K.pneumoniae* colonization in the GI tract, where the urea is found.

The *uge* gene, which encodes a uridine diphosphate galacturonate 4-epimerase and is required for biosynthesis of the capsule and smooth lipopolysaccharide(Regué*et al*.,2004).

Thepresence of *allS*gene that associated with allantoin metabolism (*allS*) is used by bacteria to obtain carbon and nitrogen from the environment(Paczosa and Mecsas,2016).

Other virulence factors have also been detected, aerobactin (*iucB*).Where, production of erobactin could be correlated with virulence(Jian-li*et al*.,2017). *and YbtA* , transcriptional regulator, is presumed to be the central regulator of yersiniabactin production together with the ferric uptake regulator   (iron uptake and transport genes) .

Conclusions

Our findingdemonstrate that *K.pneumoniae* isolated from food samples appeardifferent virulence factors genes and resistance to antibiotics .The possiblehealth hazardsshowedby the isolates mustnot be underassessment. Theresultshighlighted requirementofrisingthemonitoring of *Klebsiella pneumoniae*in diet. Try as much as possible not to eat restaurant appetizers because they contain a mixture of bacteria, including *K.pneumoniae.*

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