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Evaluation of the Virulence Gene *Irp2* in Iraqi Patients of Urinary Tract Infections and Other Community-Acquired Illnesses

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ABSTRACT

A total of sixty-two isolates were tested to assess the presence of the *irp*2 gene in different isolates of *Klebsiella pneumoniae*. The isolates discussed in this study were obtained from patients who had acquired illnesses either within the hospital premises or in the surrounding vicinity. These isolates were sourced from three hospitals located in Baghdad, namely Al-Imam Ali, Al-Zaafaranya, and Ibin-Albady hospitals. One interesting thing about *Klebsiella pneumoniae* is that it makes siderophores, especially yersiniabactin. This is because of a gene that controls this trait. The application of DNA sequencing methodologies has facilitated the identification of the *irp*2 gene in 44% of *Klebsiella pneumoniae*. According to amino acid sequences and differentiation of nucleotide, the current work reports findings on the identification of the *K. pneumoniae irp*2 gene isolates collected from patients in Iraq. This event represents the initial recorded occurrence of such detection. The presence of this gene is considered an unconventional human pathogen. The aim is to explore the correlation between genetic analysis and the diagnosis of genetic variation by examining isolates documented in the global GenBank database (LC791754.1, LC791755.1, LC791756.1, LC791757.1, LC791758.1, LC791759.1, LC791760.1). Additionally, it seeks to provide insights into the magnitude of genetic variation observed within these isolates.

1. Introduction

Klebsiella pneumoniae was first recognized as a gram-negative, immotile, encapsulated bacterium by Carl Friedlander in 1882. The term *Klebsiella* was not officially adopted until 1886 , but prior to that, it was known as Friedlander's bacillus. It is a common colonizer of the human gastrointestinal tract (GIT) and oropharynx (Bach *et al.* 2000). *Klebsiella pneumoniae* has been implicated with a range of severe medical conditions, such as meningitis, wound infections, infections of the surgical site, bacteremia, and pneumonia (Burrus *et al.* 2002).

Gram-negative bacteria, including *Klebsiella pneumoniae* and *Enterobacter* spp., are frequently encountered in nosocomial conditions such as pneumonia, septicemia, and urinary tract infections. Whole genome sequencing revealed the presence of metallo-lactamase (blaNDM-5, blaCTX-M-15, and blaSHV-11/TEM-1) in the *K. pneumoniae* strain, the augmentation of mutations and genetic alterations within the genome has been observed to enhance the potency of virulence factors and bolster their resistance to contemporary antibiotics (Schmetterer *et al.* 2022).

Klebsiella pneumoniae and *Enterobacter* species are harmful for several reasons, including their fimbrial adhesins, polysaccharide capsules, and siderophores. Researchers want to look into the origins of carbapenem resistance in highly effective multidrug-resistant lineages like *E. coli* ST131 and *Klebsiella pneumoniae* ST307 (Shropshire *et al.* 2022). Since iron is essential for the survival of infected bacteria, and there is currently not enough in the world, most bacteria secrete siderophores, which are small molecules that bind to ferric iron (Lawlor *et al.* 2007). In addition, the discovery of hypervirulent

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Klebsiella pneumoniae in the Asia-Pacific region has led to its spread throughout the rest of the world.

The pathogen in question possesses hyper-mucoviscosity and hypervirulence, which can lead to the development of community-acquired disorders such as pyogenic liver abscess, pneumonia, meningitis, and endophthalm (Zhu *et al.* 2021; Namikawa *et al.* 2022). The results of the iron absorption test and the quantitative measurement of iron carrier production showed that iron absorption and the ability to make the *irp*2 gene could control iron carriers. Hence, these results are significant for studying the role of the *irp*2 gene in the species of the Enterobacteriaceae family (Zhang *et al.* 2021). Numerous conjugative and self-transmissible elements possessing the ability to integrate into the bacterial chromosome have been identified. (Burrus *et al.* 2004). This investigation aimed to examine the frequency of the *irp*2 gene in *Klebsiella pneumoniae* strains isolated from Iraqi patients with urinary tract infections (UTIs) and community-acquired diseases.

2. Materials and Methods

2.1. Samples Collection

The specimens were collected over the period from December 2021 to April 2022. The hospitals located in AlResafa district of Baghdad were Al- Zaafaranya, Al-Imam Ali, and Ibin- Albalady hospitals. There were a total of 250 clinical urine samples collected, 100 from women and 150 from men who were suffering from urinary tract infections after being diagnosed by doctor with urinary infection according to general urine lysis by microscope.

2.1.1. Ethical Approval

The consent to collect samples from affected women was obtained. Additionally, permission was acquired from the Ministry of Health to collect hospital samples and get animals from the National Center for Pharmaceutical Control and Research (Reference number: 6999/Date 20/12/2021) Culturing of bacteria.

2.1.2. Identification of Bacteria

One microliter of urine was cultured on Blood agar and McConkey agar by using sterile loop, and incubated for 24 hours at 37 C. After that the colonies on Macconkey agar were cultured on nutrient broth to detect for molecular genetic analysis. A hypermucoviscosity test was conducted on the colony that forms a mucoviscous colony with a thread longer than (5 mm), to confirm this colony is *K. pneumoniae* (Zheng *et al.* 2018). It was applied Vitek 2 Compact for the detection bacterial isolates (ID card: GN ID card, Reference number 21341).

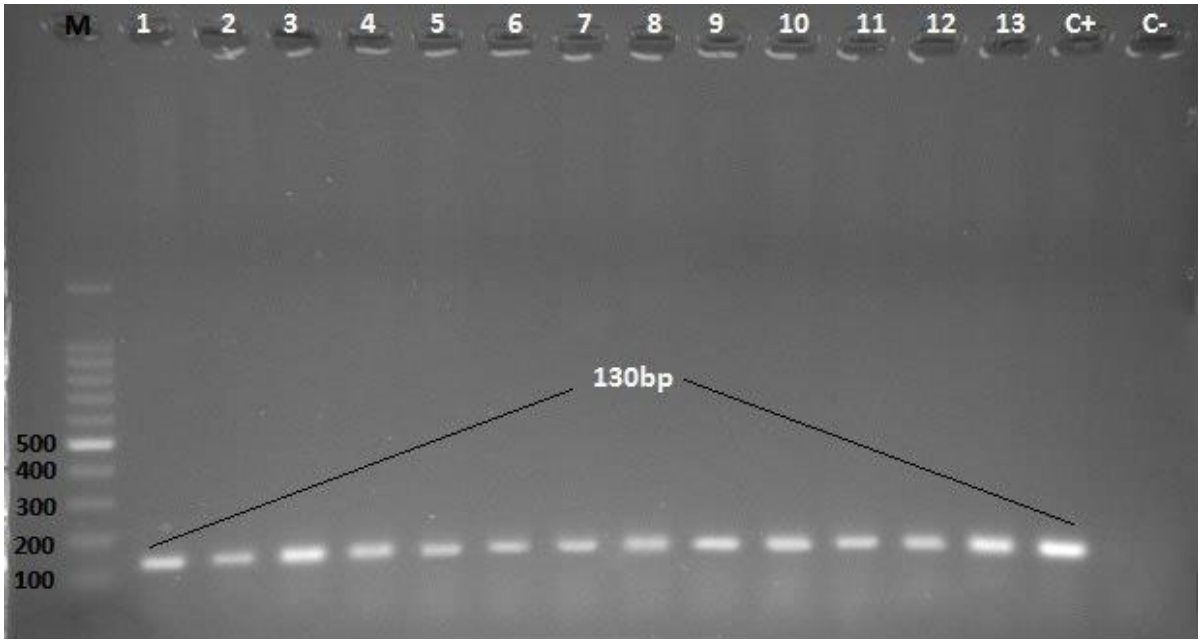

Figure 1. The PCR result of the 16SrRNA gene (130 bp) for *K. pneumoniae* was separated on a 1.5% agarose gel that was charged with 5 Volt/cm for two hours., C-: negative control, C+: positive control LC314486.1., M: DNA marker (100 bp). Lanes (1-13) are samples

2.2. Extraction of DNA and PCR Analysis

Using traditional PCR and pre-selected primer pairs for the 16SrRNA gene forward (F) 5`-ATTTGAAGAGGTTGCAAACGAT-3` and reverse (R)5`-TTCACTCTGAAGTTTTCTTGTGT TC-3` (PCR product size 130 bp) (Figure 1) (Mahmudunnabi *et al.* 2018). *K. pneumoniae* isolates were utilized to extract genomic DNA. With isolated genomic DNA and universal primers, (type1) *irp*2 (F) 5`-AAGGATTCGCTGTTACCGGAC-3` and reverse (R) 5`-TCGTCGGGCAGCGTTTCTT CT-3` (PCR product size 287 bp) and (type2) *irp*2 (F)5`-ATTTCTGGCGCACCATCT-3` and reverse (R)5`-GCGCCGGGTATTACGGACTTC-3` (PCR product size 952 bp) genes were amplified (Lopes *et al.* 2016).

The reactions for the (Type1) *irp*2 and (Type2) *irp*2 genes were carried out in a volume of 25 µl, which contained 2 µl (100 ng) of template DNA solution, 12.5 µl of master mix (GoTaq®Green Master Mix), 7.5 µl of DDH_2O, 1.5 µl (10 pmol) of forward primer, and 1.5 µl (10 Pmol) of reverse primer for each isolate. The free nuclease water served as the negative control. Using a thermos cycler (Eppendorf) set up for 35 cycles of 1 min at 95°C, 2 min at 65°C, and 3 min at 72°C, followed by a final extension step for 7 min at 72°C, the PCR program was run for both genes. Gel electrophoresis was performed on each 10 µl of PCR product at a voltage of 90 volts for 1.5 hours in an agarose gel containing 1.5% (w/v) agarose and a marker of 100 bp. Before being photographed using the Gel picture documentation method, Gel red 5µl (0.05%) was used to dye the gel before being shot utilizing Gel photo documentation equipment under Ultraviolet light illumination.

2.3. Sequencing of (type2) *irp*2 Gene

The researchers requested Macrogen Inc., a US-based company, to perform nucleotide sequencing of PCR products derived from *Klebsiella pneumoniae*. The primary emphasis of their study was the forward orientation of the *irp*2 gene, specifically of the type 2 primer. The nucleotide sequence was aligned and analyzed using BioEdit 7.09 by the researchers. The objective of this work was to identify differences in sequence sites and types. The comparison of the data was conducted by aligning it with the reference sequence strains provided by the National Centre for Biotechnology Information.

3. Results

3.1. *Klebsiella pneumoniae* Detection

Table 1 showed a total of 62 samples from 30 males and 32 females of varied ages were found to contain *Klebsiella pneumoniae*. Vitek2 results showed a probability 99%, *Klebsiella pneumoniae*. Also, the percentage prevalence of *Klebsiella pneumoniae* in males 20%, and females was 32%.

3.2. PCR Analysis for the *irp*2 Gene

Figure 1 shows the 16SrRNA gene (130 bp) for all *K. pneumoniae* isolates. Also, (Figures 2 and 3) show two fragments of the *irp*2 gene, at 287 and 952 base pairs, were copied from hospital infection isolates using two distinct sets of primers. According to a published worldwide investigation, the (type1) *irp*2 and (type2) *irp*2 genes are present in bacteria that cause urinary tract infections.

3.3. Analysis of the *irp*2 Gene's Sequence

Nucleotide sequencing of the *irp*2 gene has been performed for 7 randomly selected strains *K. pneumoniae* database (LC791754.1, LC791755.1, LC791756.1, LC791757.1, LC791758.1, LC791759.1, LC791760.1). Cluster analysis showed isolates had been sequenced diversity in the distribution of isolates due to differences in their sequence. They were organized into four groups (Figure 4). The isolation number 58 was categorized into the fourth group, exclusively comprising Asian isolates. This grouping suggests a tight relationship among these isolates, indicating a potential common origin from ancestral sequences that were horizontally transmitted to different bacterial species. Regarding isolates 9 and 50, they were arranged separately from one another and from the remaining isolates that had been documented.

Table 1. The proportion of *Klebsiella pneumoniae* strains isolated from urinary tract infection patients and other community-acquired illnesses

Sex	Number of sample	No. of *Klebsiella pneumoniae* isolates	Percentage of isolation
Male	150	30	20%
Female	100	32	32%
Total	250	62	24.8%

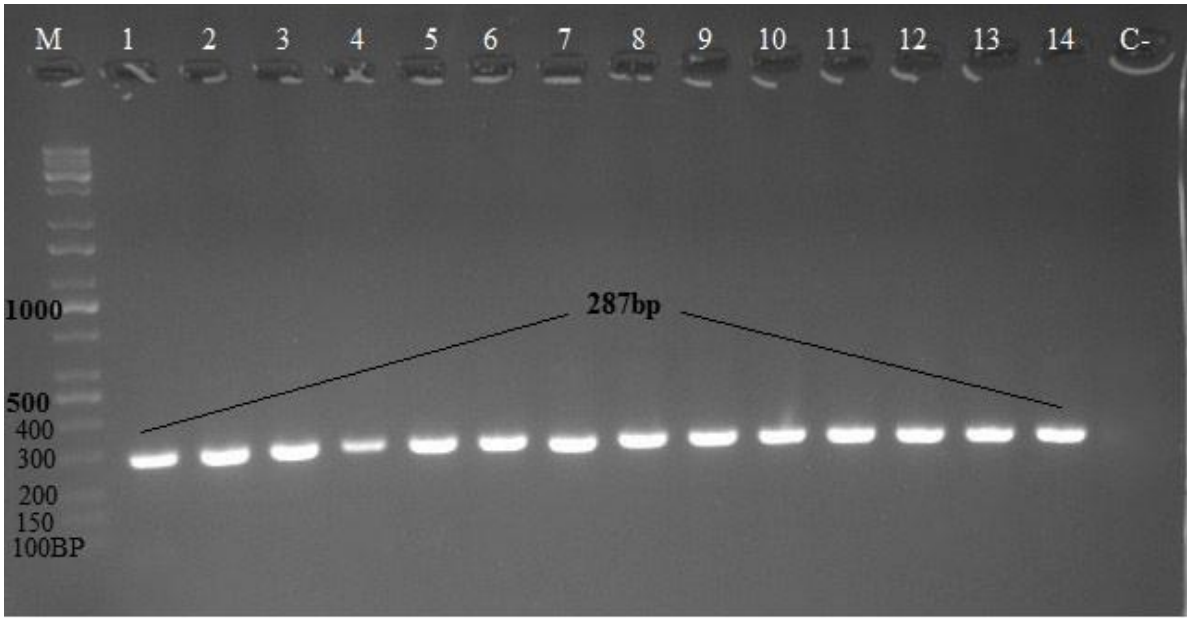

Figure 2. The (type1) *irp*2 gene PCR product (287 bp) from *K. pneumoniae* isolates was run on a 1.5% agarose gel at 5 Volt/cm for 2 hours. Positive PCR results for each of the 14 lanes. C- Means DNA-free control .DNA marker, 100 base pairs; abbreviated "M"

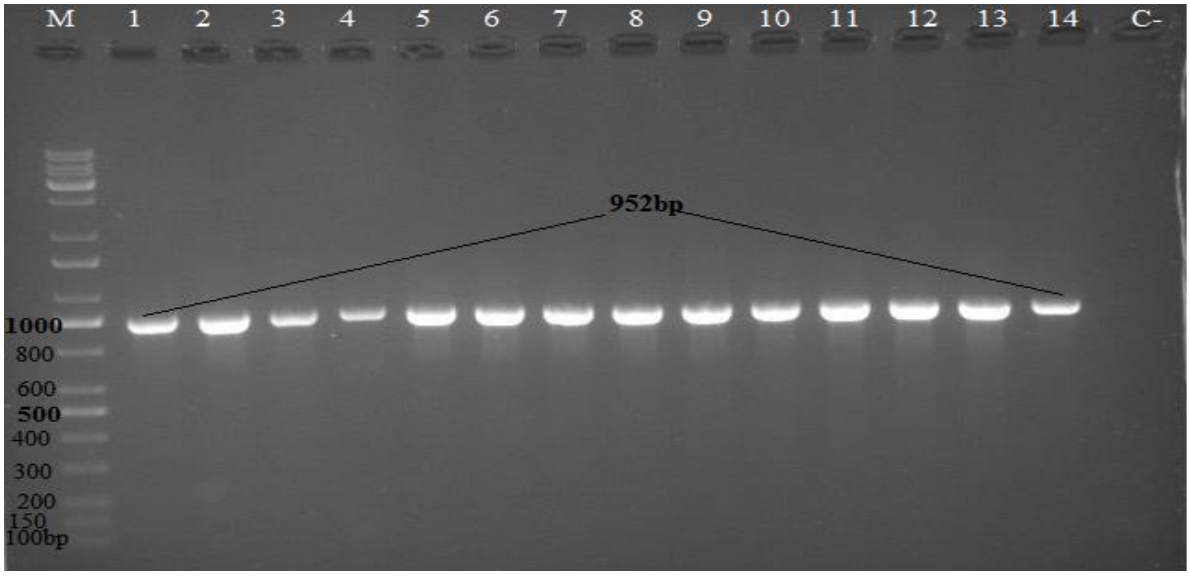

Figure 3. The PCR product for the (type2) *irp*2 gene from *K. pneumoniae* isolates was 952 base pairs, and it was processed at 5 volts/cm for two hours on a 1.5% agarose gel. Positive PCR results for each of the 14 lanes. C- Means DNA-free control. DNA marker, 100 base pairs; abbreviated "M"

The findings of the sequencing analysis of the *K. pneumoniae* (type2) *irp*2 gene were summarized in (Table 2) as shown in (Figure 5 and 6). The results in (Table 2) represented the number and position of substitutions in the gene for 13 isolates in seven samples.

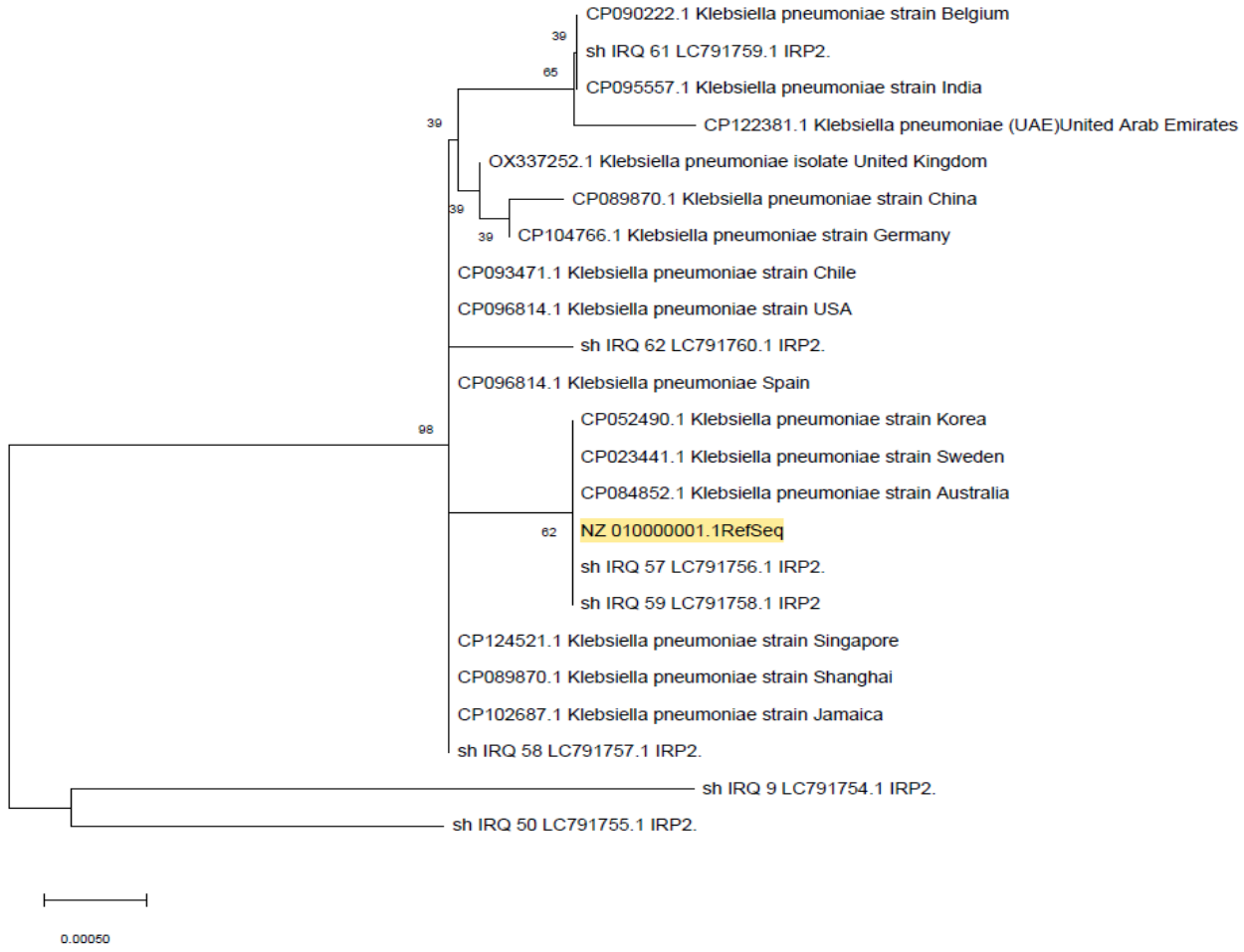

Figure 4. Neighbor-joining phylogenetic study using seven *irp*2 gene sequences from *K. pneumoniae* strains found in Iraq and eighteen strain sequences from strains reported worldwide (found in the public database GenBank)

Table 2. Substitution in the *K. pneumoniae* bacteria's (type2) *irp*2 gene sequence

The sample number	(Acc. NO.)	The location of substitution
Sh-IRQ-9-IRP2	LC791754.1	5175142 C/T
		5175154 T/C
		5175370 T/G
Sh-IRQ-61-IRP2	LC791759.1	5175492 C/A
Sh-IRQ-62-IRP2	LC791760.1	5175557 C/T
Sh-IRQ-9-IRP2,Sh-IRQ-50-IRP2	LC791754.1, LC791755.1	5175616 T/C
Sh-IRQ-58-IRP2,Sh-IRQ-61-IRP2 ,Sh-IRQ-62-IRP2	LC791757.1, LC791759.1 LC791760.1	
Sh-IRQ-9-IRP2, Sh-IRQ-50-IRP2	LC791754.1, LC791755.1	5175877 G/T
Sh-IRQ-9-IRP2	LC791754.1	5175890 C/G
Sh-IRQ-50-IRP2	LC791755.1	5175899 G/C
Sh-IRQ-9-IRP2, Sh-IRQ-50-IRP2	LC791754.1, LC791755.1	5175904 G/T
Sh-IRQ-50-IRP2	LC791755.1	5175917 G/T
Sh-IRQ-9-IRP2, Sh-IRQ-50-IRP2	LC791754.1, LC791755.1	5175947 C/G
Sh-IRQ-50-IRP2	LC791755.1	5175951 A/G
Sh-IRQ-50-IRP2, Sh-IRQ-9-IRP2	LC791755.1, LC791754.1	5175954 A/T
Sh-IRQ-9-IRP2	LC791754.1	5176005 G/T

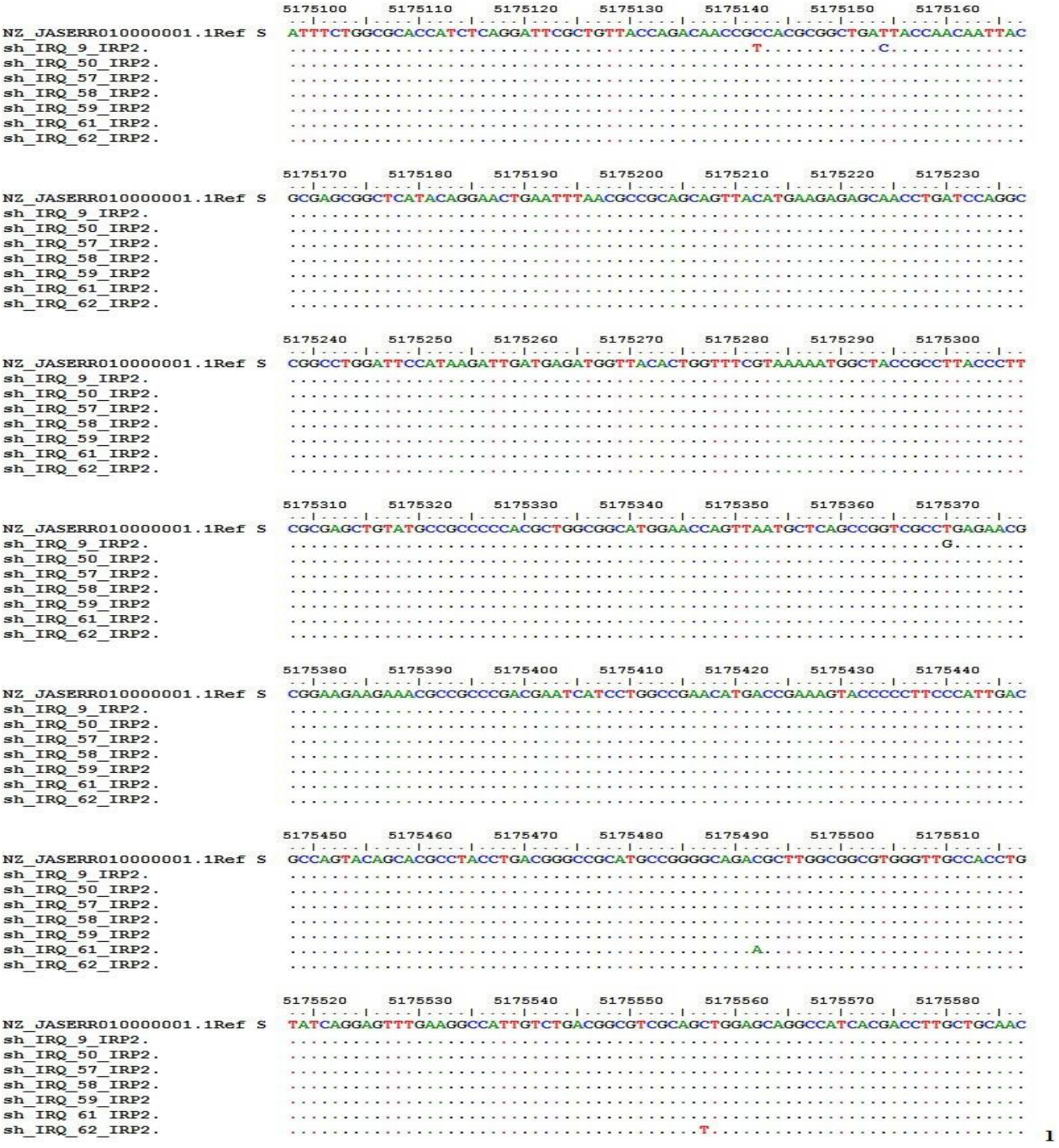

Figure 5. Alignment (type2) *irp*2 sequences from seven different *K. pneumoniae* isolates (5175100-515587) with the sequence of a reference strain (NZ-JASERR010000001)

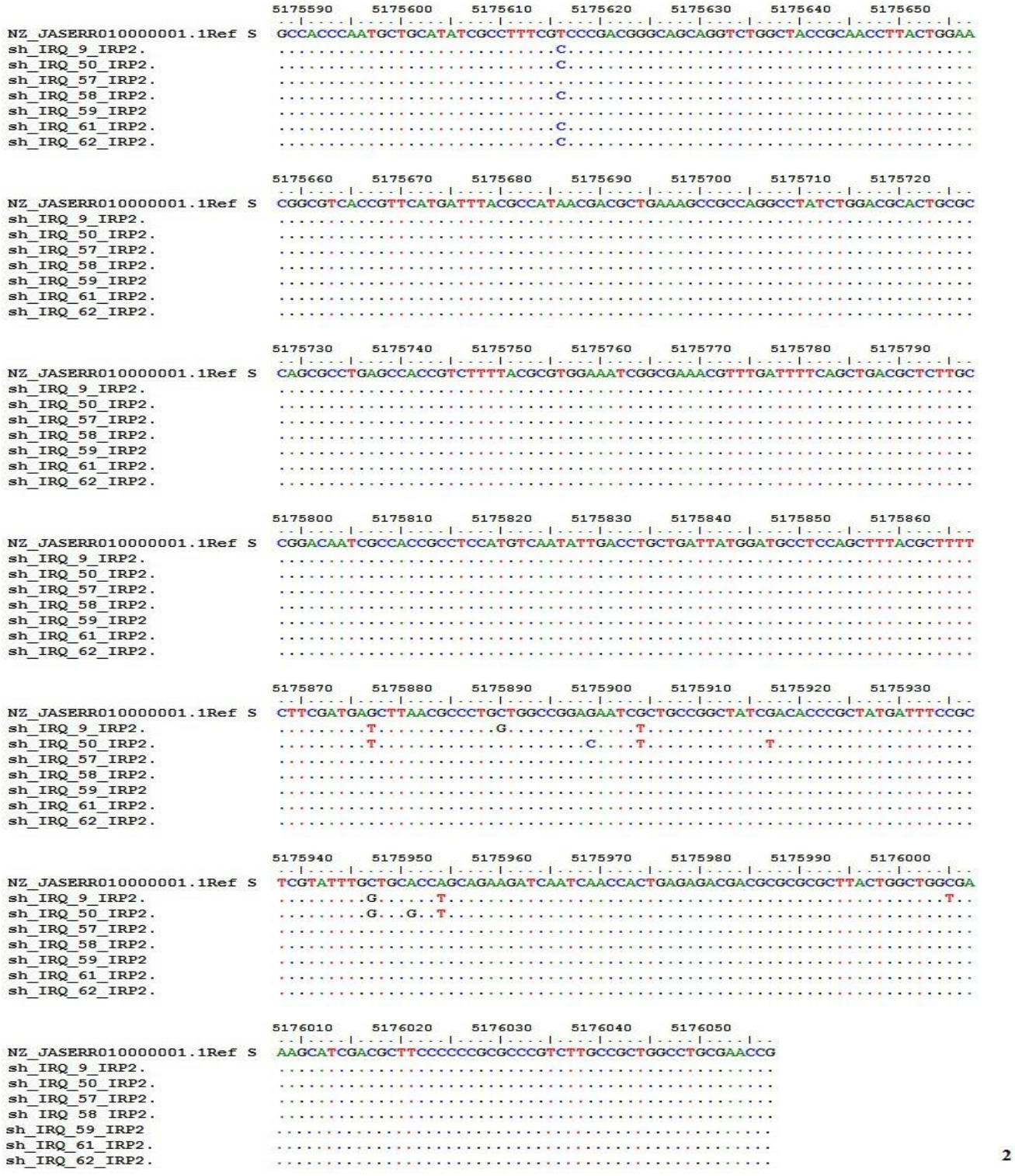

Figure 6. The GenBank database contains recorded alignments of partial (type2) *irp*2 sequences from seven isolates of *K. pneumoniae* (515588-51760557) with the standard isolate of *K. pneumoniae* (NZJASERR010000001)

4. Discussion

The high-pathogenicity phenotype in pathogenic *K. pneumoniae* is associated with the presence of an iron uptake high-pathogenicity island (HPI) and the importance of the role of plasmids in their spread. Studies were conducted to evaluate the application of adaptive sampling to enrichment in low-abundance plasmids, and using different instruments has potential cost savings for laboratories (Ulrich *et al.* 2022). Galy *et al.* have demonstrated the mutability of this gene and its susceptibility to mutagenesis by utilizing both conventional and novel methodologies. Their study was done wherein specific mutations were successfully induced in the genes encoding the IRP-1 and IRP2 regulatory proteins, employing a conventional gene trap-building method (Galy *et al.* 2004).

Cluster analysis showed that the isolates had varied sequences in the distribution of isolates due to differences in their sequences, so they were organized into four groups (Figure 4). It is noteworthy that isolate 61 was arranged with the first group, which also contained the isolate from the Emiratis. While isolates 57 and 59 were grouped with Korea, Sweden, Australia, and reference isolate, isolate 62 was similar to both the American isolate and the isolate from the country of Jelly. The isolation number 58 was categorized into the fourth group, comprising Asian isolates and Jamaica. This grouping suggests a tight relationship among these isolates, indicating a potential common origin from ancestral sequences that were horizontally transmitted to different bacterial species. Regarding isolates 9 and 50, they were arranged separately from one another and from the remaining isolates that had been documented.

The present study investigated the level of similarity between the isolates obtained from Iraq and the isolate containing the same gene from a neighboring country, specifically the United Arab Emirates, with a similarity rate of 98.85%. These findings suggest the extent of genetic resemblance, *Irp*2 type 2 sequence alignment of seven different strains of *Klebsiella pneumoniae* for this study, including the UAE isolate and reference strain; all isolates have a Substitution in T/C when compared to the reference strain. The substitution in the nucleotide sequence, as demonstrated in (Figure 5 and 6), has led to modifications in the amino acid composition and, subsequently, the iron proteins that HPI encodes. It has been observed that clinical isolates of *Klebsiella pneumoniae* are capable of producing enterobactin, with a limited number of strains also exhibiting the ability to make aerobactin (Vernet *et al.* 1995).

The process of sequencing and creating a phylogenetic tree plays a significant role in enhancing the identification, structure, and epidemiological prevalence of bacteria (Chakravorty *et al.* 2007). Previous studies have demonstrated the effectiveness of using 16S rRNA gene sequences for the identification of clinically harmful bacterial isolates (Patel 2001).

In the recently conducted study, it was observed that the *irp*2 virulence gene was detected in 44% of *K. pneumoniae* isolates. The stated proportion is fairly analogous to the findings of El Fertas-Aissani *et al.* who observed the presence of HPI in 46.3% of isolates of *K. pneumoniae* (El Fertas-Aissani *et al.* 2013). Koczura and Kaznowski (2003) detected the HPI in 18% of faeces and urine *K. pneumoniae* isolates, respectively. Among 67 *Enterobacteriaceae* isolates from 18 species, including *Klebsiella*, Bach *et al.* found that nine isolates (13.4% of the total) carried the island. These researchers also found that all nine isolates produced the HPI-encoded iron-repressible proteins HMWP1 and HMWP2, similar to what is seen in *Yersinia* sp. *Enterobacteriaceae* strains that cause extraintestinal illnesses in humans and animals include septicemia, meningitis, wound infections, and urinary tract infections. All have a functional HPI (Schubert *et al.* 2000; Mokracka *et al.* 2004). The substitution in the nucleotide sequence, as demonstrated in (Figure 5 and 6), has led to modifications in the amino acid composition and, subsequently, the iron proteins that HPI encodes. It has been observed that clinical isolates of *Klebsiella pneumoniae* are capable of producing enterobactin, with a limited number of strains also exhibiting the ability to make aerobactin (Vernet *et al.* 1995). The necessity of incorporating a third siderophore, such as yersiniabactin, remains unclear. Each of these siderophores potentially fulfills a distinct role in cellular metabolism. Yersiniabactin assumes greater significance under conditions of iron deficiency, whereas enterobactin operates effectively in the presence of sufficient iron (Lawlor *et al.* 2007). Enterobactin has been observed to

be associated with conventional pathogenicity in *K. pneumoniae*. On the other hand, aerobactin and yersiniabactin have been found to enhance invasiveness in susceptible populations potentially. It is worth noting that siderophore production is also correlated with bacterial virulence (El Fertas-Aissani *et al.* 2007).

In conclusion, the present study indicated the existence of the *irp*2 gene in *K. pneumoniae* isolates obtained from Iraqi patients. This is considered the first Iraqi recording of this gene detection. The existence of this gene is regarded as an atypical human pathogen.

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