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Molecular characterization of diarrheal disease *Klebsiella pneumoniae* in children under five years of age in Tikrit, Iraq

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Abstract

Bacteria, which are classified after enteroviruses, are the second most common cause of diarrhea, as they include species and genera of the enteric family, the most widespread of which are (*Escherichia coli, Shigella, Plesiomonas,* and *Salmonella, and Klebsiella pneumoniae*) was recently added as a type of bacteria that causes diarrhea It is essential to diagnose it based on the phenotypic form and biochemical tests to determine the appropriate treatment to eliminate it, inhibit it, or reduce its spread The main objective of this study is to demonstrate that *K. pneumoniae* is a causative species of diarrhea in children under five years of age and that it may possess virulence genes that may lead to life-threatening children who may develop severe diarrhea due to this bacteria. Biochemical and biological tests were performed for the bacterial samples. The DNA extracted from the isolates was subjected to rRNA amplification by PCR. *K. pneumoniae* isolates were evaluated with specific primer genes (*KPompa*, *Khe,* and *Kpfima*) with antibiotic identification using specific primers. Moreover, the concentration of IL-6 was determined using the ELISA technique. Some disease strains have been identified by genetic trees as well. The proportions of *K. pneumoniae* bacteria were the second highest rates of bacteria diagnosed in the urine of children under five with diarrhea, so their percentage was higher than other types that cause diarrhea. It was found that *K. pneumonia* contains one or more virulence genes in the same strains. The most virulent gene identified by the primers was (*KP16S*), which scored 100% virulence, followed by the primer gene (*Shi'a gene*), which was estimated at 99%, whose effect was moderate and which was identified by the primers (*KPompa, Khe,* and *Kpfima.*) with a virulence rate of 68.5% for the three genes equally. High levels of the natural antibody interleukin (IL-6) indicate more elevated levels than normal. In this study, *K. pneumoniae* is one of the types of bacteria that cause diarrhea in children under the age of five, and its high number in people with diarrhea results from a group of its virulence genes formed in the digestive system of infected people. It may be a type of bacteria that is resistant to antibiotics. A high level of interleukin IL-6 in blood serum is a clear indication of infection with *K. pneumoniae.*

Introduction:

Accurate and timely detection of the etiology of these diseases is very important to avoid deaths in children. However, with traditional methods (apart from being laborious and time consuming), accurate and timely detection of pathogens of these diseases is essential to avoid fatalities. in children. However, traditional methods (apart from being laborious and time-consuming) often fail to identify the true etiology in an average of up to 40% of cases of diarrhea [1]. Despite significant progress in managing the causes of diarrhea in childhood and providing insight into the etiology and associated diseases of diarrhea, the mortality rate remains high in children under five. Advanced molecular diagnostic methods have resulted in significant progress in identifying pathogens, confirming that diarrheal diseases remain a major cause of malnutrition and stunting in developing and third-world countries, as 50% of diarrhea cases generally only affect children between the first and fifth years of age. These countries [2]. Bacteria, which are classified after enter viruses, are the second most common cause of diarrhea in developing countries, as they include species and genera of the enteric family, consuming) often fail to identify the true etiology in an average of up to 40% of cases of diarrhea [1]. Despite significant progress in managing the causes of diarrhea in childhood and providing insight into the etiology and associated diseases of diarrhea, the mortality rate remains high in children, particularly children under the age of five. Advanced molecular diagnostic methods have resulted in significant progress in identifying pathogens, which confirm that diarrheal diseases remain a major cause of malnutrition and stunting in developing countries and third world countries, as 50% of diarrhea cases in general only affect children between the first and fifth years of age. These countries [2]. Bacteria, which are classified after enteroviruses, are the second most common cause of diarrhea in developing countries, as they include species and genera of the enteric family, the most widespread of which are (*Escherichia coli, Shigella, Plesiomonas, Salmonella,* and *Klebsiella pneumonia* was recently added as a type of bacteria that causes diarrhea) It is essential to diagnose it based on the phenotypic form and biochemical tests to determine the appropriate treatment to eliminate it, inhibit it, or reduce its spread [3].

The use of biomarkers such as cytokines and acute phase proteins, including Interleukin (IL-6), is a natural protein produced by various cells in the body, and it helps regulate the immune response, thus; It is an important indication of the presence of high activity in the immune system, as well as an indicator of acute infection, especially in children). In recent years, a number of levels of biomarkers have been identified in serum that can help diagnose local and systemic infections, and differentiate between bacterial and fungal infections from viral infections or non-infectious conditions, which contributes to the correct and appropriate management of antibiotic therapy [4].

The main objective of this study is to prove that *K. pneumoniae* bacteria are among the types that cause diarrhea in children under the age of five and that they can have virulence genes that lead to a significant increase in their numbers in the digestive system, which may lead to danger to the lives of children who may develop severe diarrhea due to This bacte ria.

Materials and Method

Isolation and diagnosis

In this study, 130 samples were collected from patients attending Tikrit Hospital, 100 infected patients, and 30 control samples without diarrhea, who were hospitalized and reviewed in the city of Tikrit from November 2020 to April 2021. The patient's information was recorded in a particular form that included his general clinical symptoms such as abdominal pain, fever, vomiting and the number of times of diarrhea. Biochemical and biological tests were carried out for bacterial samples. Tests were carried out according to (Leber, 2016) [5], where colonies of bacterial isolates were purified by re-growing them on blood agar medium, MacConkey agar medium, and nutrient agar medium using the planning method and incubating them for 24 hours at a temperature of 37°C. Bacterial colonies were initially diagnosed. Depending on their morphological and culture characteristics on the culture media, including the size, colour, borders, and height of the colonies, their ability to ferment the sugar lactose, and their ability to produce mucus and hemolysin, the characteristics of the cells were observed under the microscope after staining them with a Gram stain, and then biochemical tests were performed. Several biochemical tests were conducted to diagnose bacteria developing on the nutrient media after 24 hours of growth.

DNA extraction and PCR Amplification

The PCR process includes several steps. First, during primary denaturation, the double-stranded DNA template is heated to a high temperature, and then the temperature is lowered, allowing the primers to bind to complementary sequences of the DNA. DNA polymerase synthesizes a new DNA strand using the DNA template and free nucleotides. This process is repeated several times, with each cycle amplifying the target DNA.

PCR reactions were carried out under sterile conditions and included the following steps: The primers used in the PCR assay are *khene*,*kp16s*,*kpompA*,*kpfima* and *shia*gene.

The molecular diagnosis of toxin genes is by extracting the bacterial DNA(Geneaid kit) and then subjecting the DNA to the polymerase chain reaction (PCR) test. The process of extracting DNA from the sample: part of the sample was taken and placed in the tube of the device, then we added the lysates, closed the tube and mixed it well with the vortex device to ensure the destruction of the bacterial wall. The suspension is transferred to the spin column. We perform a precise centrifugation of 3-5 seconds for the suspension centrifuge, and then the suspension is transferred to another second tube with the addition of lysate buffer. Then, we run a second centrifuge to remove any protein material attached to DNA or RNA. Then, it is transferred to a third tube, and we add the nucleus buffer, then a third centrifugation process in which the pure nucleic acid is deposited.

Amplification of DNA from the eye

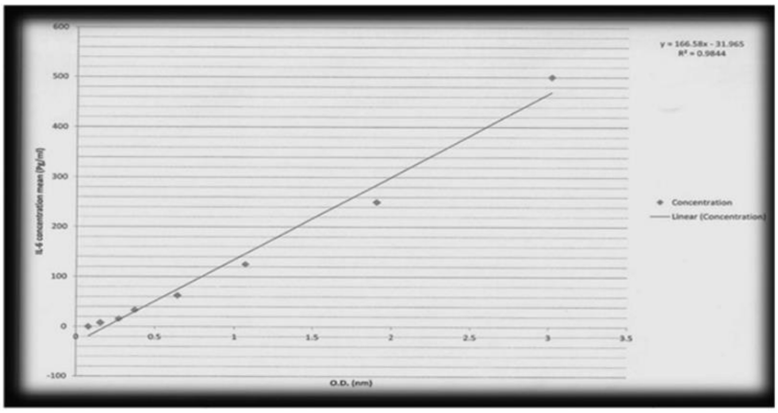
This process uses different temperatures to separate DNA strands at a temperature of 95 C^o then changing the temperature to obtain pads. According to the manufacturer's instructions, the tubes were carefully inserted into the thermopolymer device for each primer to perform the PCR reaction using the appropriate replication program for each primer based on the research recommendation used for primers. Then, the results were recorded.

Table 1. The primers used in this study.

Primers	**Sequence (5′ → 3′)**		**Annealing temperature**	**PCR product**	**References**
KP16S	F	CGGACGGGTGAGTAATGTCT	57	510bp	This study
	R	CGGGGATTTCACATCTGACT			
KpompA	F	TCCAAAGGCAACTACCGCTTC	58	339bp	This study
	R	ACCTTCCGGTTTCAGGGTAG			
KpfimA	F	CAATCCGGTTCGTTATTTCG	57	434bp	This study
	R	CCGGTATTGTCGAGGATCTG			
Khegene	F	TGATTGCATTCGCCACTGG	59	428bp	[6]
	R	GGTCAACCCAACGATCCTGG			
Shiagene	F	TCACCTTACTGGTATGAACTC	56	451	[7]
	R	TCCAGGGCCAGACATATTCA			

IL-6 serum Analysis

The assay kit determined the concentration of IL-6 from (the Chinese company Sun Lung). This test depends on the binding of IL-6 in the serum with specific antibodies installed at the bottom of the fine plate after adding peroxidase-tagged anti-IL-6 antibodies to the microplate. The immune complex is formed during the appropriate incubation period, and all materials not associated with washing are removed. Then the base substance, TMB, is added to create a blue colour that is directly proportional to the concentration of interleukin in the sample, and when the solution to stop the reaction is added, the yellow dye that is measured at λmax = 450 nm and the concentration of interleukin in the sample is determined by using the standard curve as shown in the Fig. 1.



Standard curve of interleukin-6 for determination the concentration of IL-6 in humans' sera

Fig. 1 Standard curve IL-6

Statistical analysis

The data, which was carried out using the IBM SPSS Statistics 21 software, to calculate means and standard deviations and to make correlations between the various parameters, a correlation is considered significant if the p-value is less than 0.05.

Results and Discussion

It is clear from Fig . 2, that the percentages of *K. pneumoniae* bacteria were the second highest percentages of bacteria diagnosed in the urine of children under the age of five with diarrhea, so their rate was higher than other types that cause diarrhea in a significant way, such as *salmonella* and *Shigella*, and this may be clear evidence that *K. pneumoniae* is one of the causes of diarrhea. This result is similar to the study Guerin *et al.*, [8]. This confirmed the infection of a group of samples with regular human diarrhea, and the cause was the bacteria *K. pneumoniae* vision. After conducting tests, we found that these samples were negative to test other types of Bacteria, such as salmonella, *Shigella*, and *Escherichia coli*, in addition to testing for amoebic dysentery.

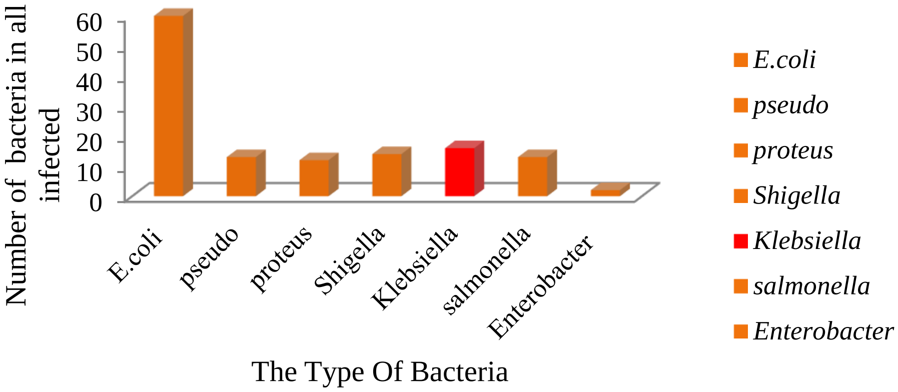


Fig. 2 Percentages of numbers of bacteria in all samples infected with diarrhea

It was also found that bacteria contain one or more virulence genes in the same strains. The most virulent gene determined by the primer was (*KP16S)* which recorded a virulence rate of 100%, followed by the primer gene(*Shia gene*), which estimated 99%, with the effect of the genes moderate determined by the primers (*Kpompa, Khe* and *Kpfima*) genes virulent rate of 68.5% for the three genes equally It is also noticeable, according to Tables (2), (3) and Fig.2, which are specific to the interleukin (IL-6) test, that the levels of this natural antibiotic secreted by the immune system rise when a person is exposed to a bacterial infection, with rates ranging from 40% to 85%. % than the standard limit, according to the type of sample and the severity of the infection, and this is in line with some studies such as the study (Agata, 2022) [9], which showed a high percentage of anti-inflammatory agents when the body of *K. pneumoniae* was exposed in any part of the body.

Table 2. One sample test shows a higher level of interleukin IL-6 compared to the control group

One-Sample Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Control	30	7.6076	2.28398	.41700
K. pneumoniae	10	63.7718	18.09234	5.72130

Table 3. Showing the highest and lowest levels of interleukin IL-6 for a bacteria test

One-Sample Test						
	Test Value = 0					
	t	Df	Sig. (2-tailed)	Mean Difference	95% Confidence Interval of the Difference	
					Lower	Upper
Control	18.244	29	.000	7.60758	6.7547	8.4604
K. pneumoniae	11.146	9	.000	63.77185	50.8294	76.7143

We conclude in this study and according to Fig. 8, that the presence of interleukin (IL-6) in high levels in the immune system of children, which recorded the highest percentage compared to other types of bacteria, results from infection with *K. pneumoniae*, which may be the second cause of diarrhea in children. , and not any different kind of bacteria that causes diarrhea specifically, and this is similar to the study [10, 11], which studied the resistance of bacteria that cause diarrhea and the role of antibiotics and their impact on types of bacteria and supporting the immune system by secreting types of interleukins and cytokines.

By looking at Table (4) resulting from the PCR test, the virulence genes or the virulence genes resulting from the pram rat test led to the appearance of symptoms of toxins formed from *K. pneumoniae* bacteria between severe, medium and simple in varying degrees according to the type of virulence gene, so the most harmful gene was the effect on children with diarrhea It is diagnosed by primer (*Kp16s)*. The least aggressive gene is the gene that primer (*Khe* gene) diagnosed. Still, in all cases, these virulence genes led to complications in children with diarrhea under the age of five, including fever, vomiting, an increase in the number of diarrhea times, and an apparent loss of fluids from the body. This is consistent with the study Dong, *et al.*[12], which showed the effect of *K. pneumoniae* toxins on infected people, whether children or older adults.

According to the foregoing, we conclude in this study that *K. pneumoniae* is one of the types of bacteria that cause diarrhea in children under the age of five, and that its high number in people with diarrhea results from a group of virulence genes that it forms in the digestive system of infected people. It may be a type of bacteria that is resistant to antibiotics. Therefore, new methods must be found to inhibit the growth of these bacteria because they have become a clear threat to the health of children under the age of five.

Table 4. Shows the virulence genes and the activity of each gene, as measured by the degree of diarrhea specific to bacteria *K. pneumonia*

Active virulence genes *K. pneumoniae*	Percentage of severe diarrhea for samples under study		
	Achronic	Medium	Simple
kpompa	60%	100%	90%
Kp16s	100%	100%	100%
kpfima	60%	90%	100%
Khe gene	70%	10%	100%
Shiagene	90%	100%	100%

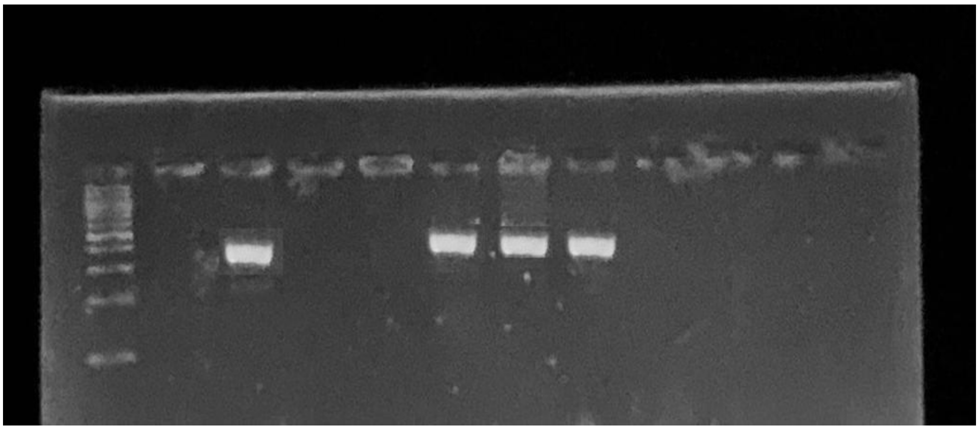


Fig. 3 Agarose (At a concentration of 0.5 μg/L, 1-5V/cm) gel electrophoresis for virulence gene lanes positive for KpfimA

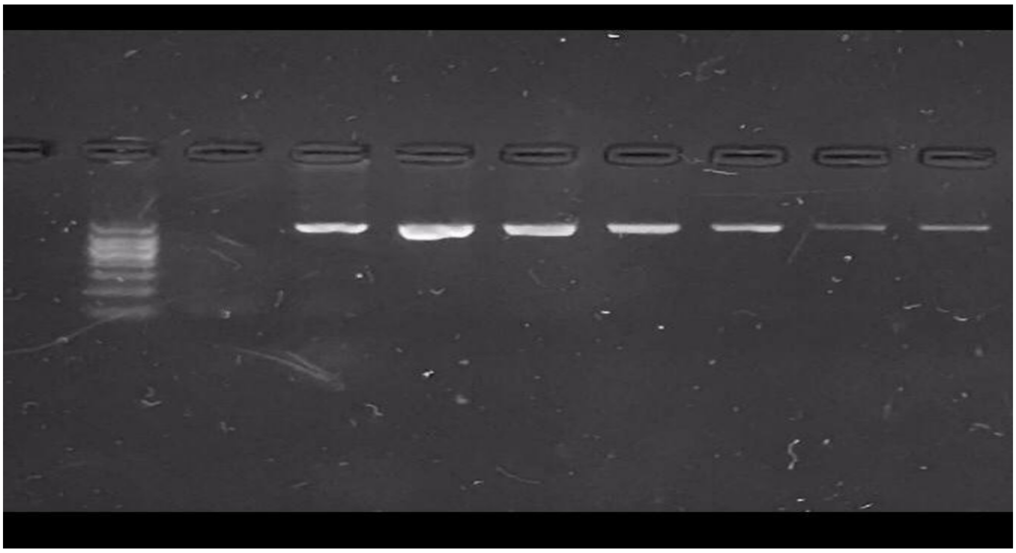


Fig 4 Agarose(At a concentration of 0.5 μg/L, 1-5V/cm) gel electrophoresis for virulence gene lanes positive for shia gene

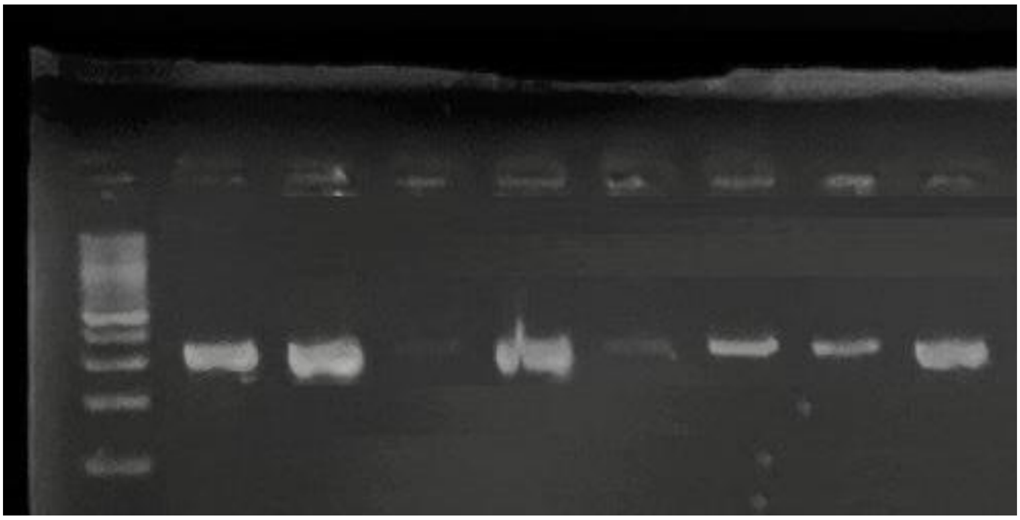


Fig 5 Agarose gel(At a concentration of 0.5 μg/L, 1-5V/cm) electrophoresis for virulence gene lanes positive for KpompA

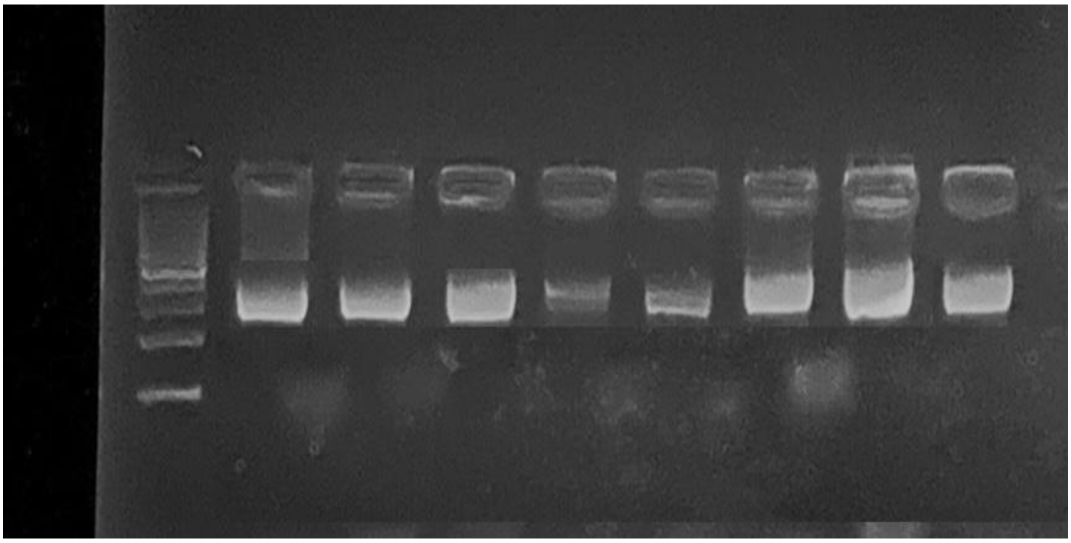


Fig. 6 Agarose(At a concentration of 0.5 µg/L, 1-5V/cm) gel electrophoresis for virulence gene lanes positive for Khegene

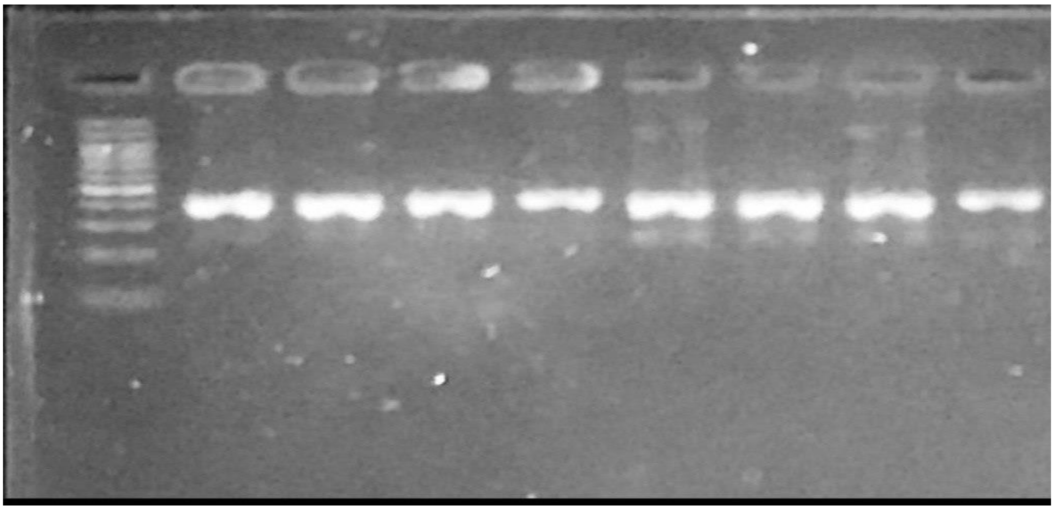


Fig. 7 Agarose(At a concentration of 0.5 µg/L, 1-5V/cm) gel electrophoresis for virulence gene lanes positive for Kp16S.

The immune response of the species to the cytokines under study

IL-6 immune response to diarrhea severity

The high level of cytokines, including interleukins, in the immune system is evidence of infection caused by pathological bacteria, which leads children to fever, vomiting and frequent diarrhea. This clearly indicates the strength and ferocity of these bacterial strains that cause diarrhea in children. It may be the result of two things. The results were as mentioned previously, and it is noted according to Table (5) and Fig .8, that the severity of diarrhea was on three levels (severe, medium and simple) and according to the readings on the sample group, there was a significant difference in the severity of diarrhea compared to Std. Deviation in the control group, where the rate of severe diarrhea was 63.27, while it was 18.39 for the control group. This is consistent with many studies such as the study [11].

Table 5. The level of interleukin-6 in the blood serum of a group of patients according to the severity of diarrhea.

Severity	Severe	Moderate	Mild
Number of values	31	15	14
Minimum	25.87	30.55	30.66
Maximum	89.15	71.39	65.01
Range	63.27	40.83	34.35
Mean	57.64	48.60	46.87
Std. Deviation	18.39	15.02	10.01

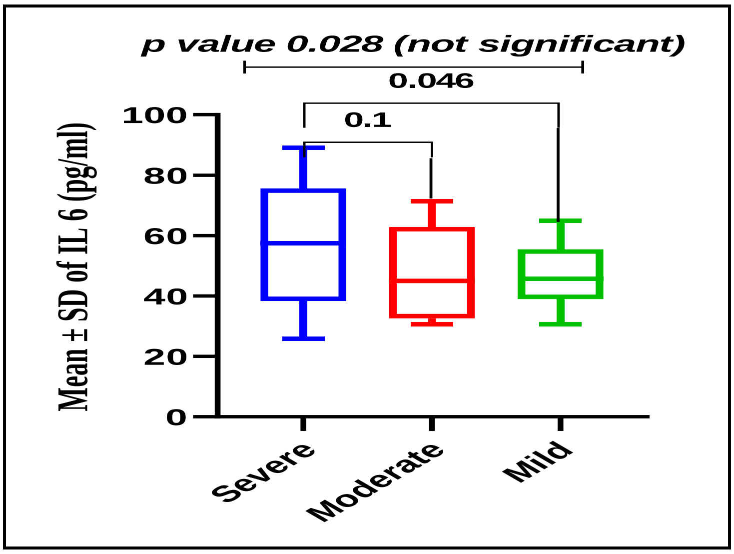


Fig. 8 *Serum IL 6 level among the group of patients according to the severity of diarrhea*.

IL-6 immune response to fever severity

The immune response to fever in children with diarrhea was evaluated at two levels (high and low) based on the immune response to the formation of interleukin IL-6.

The highest intensity of fever was 81.09, and the lowest severity was 30.55, with a rate of 50.53, compared to the control group, which recorded the average of the highest severity of 14.50. There is a significant difference between the two cases. While there was no significant difference between the levels of fever (high and low) for fever, as it was 50.53 and 63.27, respectively, Table (6) and Fig .9, and this also confirms the association of interleukin IL-6 with the appearance of fever as one of the essential symptoms. It was associated with diarrhea and interleukin secretion in children. The results in this study may agree with (Tajbakhsh *et al*, 2016) [13].

Table 6. Serum IL-6 level among the group of patients according to the level of fever

	Moderate fever	High fever
Number of values	21	39
Minimum	30.55	25.87
Maximum	81.09	89.15
Range	50.53	63.27
Mean	53.54	52.53
Std. Deviation	14.50	17.66

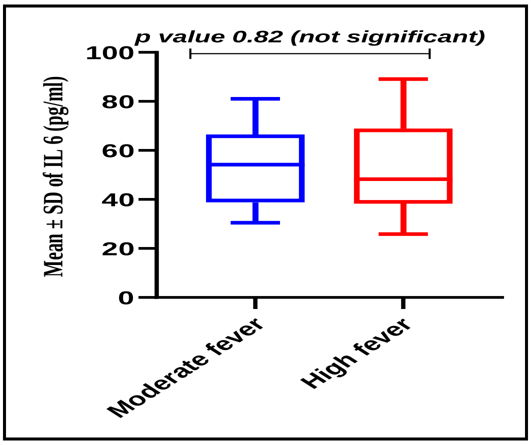


Fig. 9 Serum IL-6 level among the group of patients according to the level of fever.

Level IL -6 in blood serum among the patients group by type of bacteria

The level of interleukin IL. 6 was high in samples diagnosed and infected with *K. pneumonia* isolates, so it was 89.15, as it recorded the highest level ever for all types of bacteria under study, including *E. coli*, which recorded 76.34. There was a real and apparent significant difference in the rate of IL formation. 6 between *K. pneumonia* and *E. coli*. It was 51.79 and 37.37, respectively. Table (7) and this is an inconclusive indication that *K. pneumonia* is one of the types of bacteria that cause diarrhea, as it was, according to this study, the second most common bacterial type that causes diarrhea in children.

Table 7. The level of IL 6 in blood serum among the group of patients by type of bacteria.

IL. 6	*E. coli*	*K. pneumonia*
Number of values	7	10
Minimum	38.98	37.36
Maximum	76.34	89.15
Range	37.37	51.79
Mean	56.74	63.77
Std. Deviation	14.98	18.09
P-value	0.4124	

Some bacterial strains, the degree of relationship between them, and the nature of the gene appearing in the genetic tree of Klebsiella Pneumoniae bacteria

It is necessary to know the genetic sequence of the bacteria or pathogenic bacterial strain so that we can ultimately diagnose the pathogenic strain and find the appropriate treatment to eliminate it. Virulence genes have been identified and the ratios of pathogenic to non-pathogenic strains known.

According to Fig .10, the most virulent genes were identified by electrophoresis, and it was found that the most virulent is the *Kp16s* gene, which appeared generally in *Klebsiella pneumoniae* strains, so it may be the most virulent gene in bacterial strains that cause diarrhea in the genus *K. pneumoniae*, while The gene specified in the KR190100 primer, as well as the gene in addition to the gene, depending on the form of the genetic tree, which may belong to *K. pneumoniae* bacteria, may also be the most neutral genes of this genus of bacteria, and these results may agree with many studies on virulence factors or virulence genes that are formed by pathogenic bacteria under consideration . In addition to the group of other virulence genes, Fig. 11, the genetic tree and table (8).

Table 8. Some bacterial strains, the degree of relationship between them, and the nature of the gene appearing in the genetic tree of *K. pneumoniae* bacteria

K. pneumonia			
Unsatisfactory (symbiotic) strains	Some virulence genes By breed	satisfactory strains	some major strains pathological
E16S.ab12188	SUS2K 16S	KF991498	MZ891624
E16S.ab12956	Kpompa	ON063355	MF980918
E16S.ab12146	Kp16s	SWHIN 106	MZ203699
E16S.ab12451	UIWRF0599 16S	KR190100	GU428702
E16S.ab12298	NRC140 16S	CP055106	KM377646

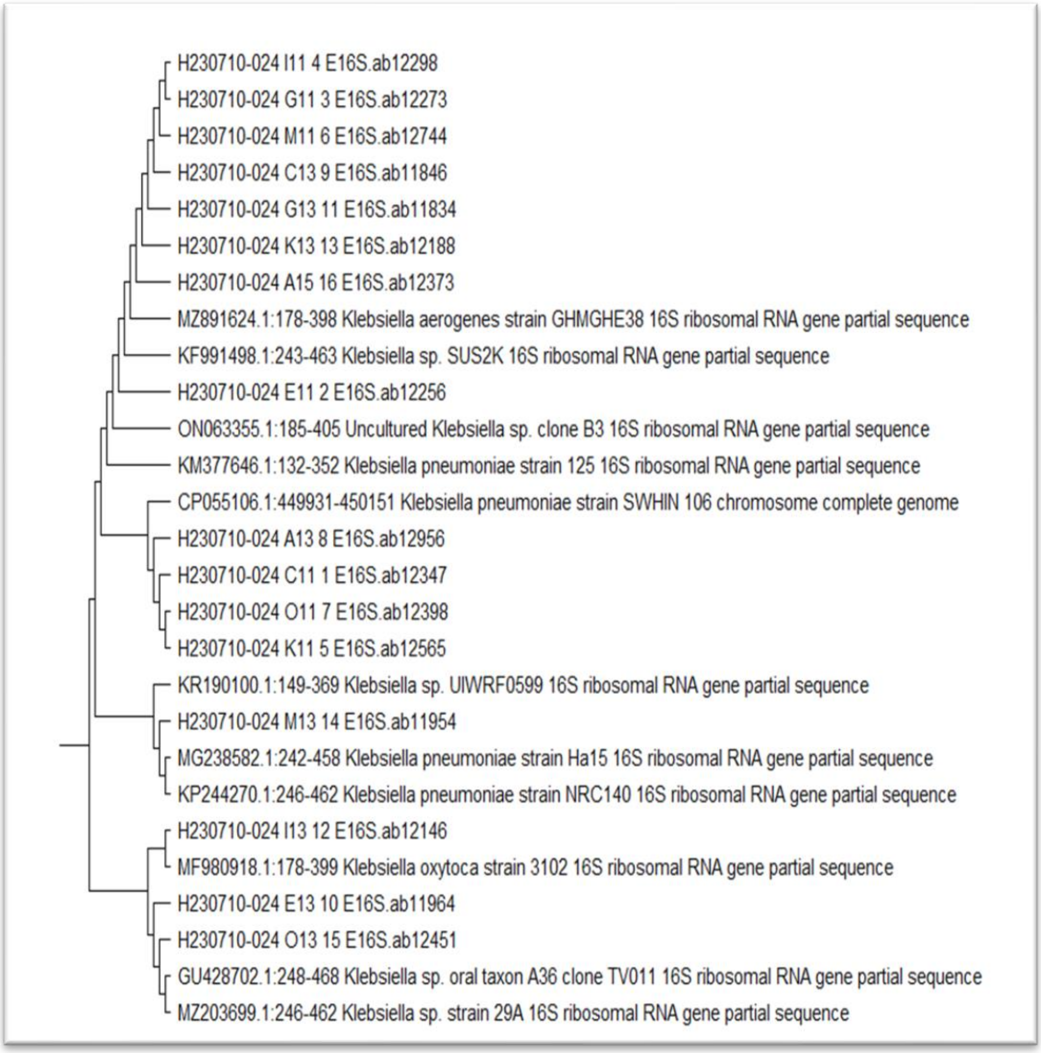


Fig.10 *K. pneumoniae genetic tree*

Conclusion

This study demonstrated that *K. pneumoniae* strains coexist with the human digestive system and cause diarrhea, especially in children. In addition to that, it may cause bloody diarrhea in infected samples. It may synergize or unite with other known types of diarrhea-causing bacteria to increase the suffering of children with diarrhea that is difficult to control, and the high number of them in people with diarrhea results from a group of virulence genes formed in the digestive system in children. Infected persons. It may be a type of bacteria that is resistant to antibiotics. An elevated level of interleukin IL-6 in serum is a clear indicator of infection with *K. pneumoniae*.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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التوصيف الجزيئي لمرض الإسهال كليبسيلا الرئوية لدى الأطفال دون سن الخامسة في تكريت، العراق

محمد أحمد صابر*، معن حسن صالح، هالة عبد الخالق عوض
قسم علوم الحياة، كلية العلوم، جامعة تكريت، تكريت، العراق

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الموبايل:

الخلاصة:

تعد البكتيريا التي تصنف بعد الفيروسات المعوية ثاني أكثر الأسباب شيوعاً للإسهال، حيث أنها تشمل أنواع وأجناس الفصيلة المعوية، وأكثرها انتشاراً هي (الإشريكية القولونية، والشيجلا، والبليسيوموناس، والسالمونيلا، والكلبسيلا الرئوية). أضيفت مؤخرا كنوع من البكتيريا المسببة للإسهال ومن الضروري تشخيصها بناء على الشكل المظهري والاختبارات البيوكيميائية لتحديد العلاج المناسب للقضاء عليها أو تثبيطها أو الحد من انتشارها. الهدف الرئيسي من هذه الدراسة هو إثبات أن كليبسيلا نيومونيا هي مسبب للإسهال لدى الأطفال دون سن الخامسة وقد يمتلك جينات الفوعة التي قد تؤدي إلى أطفال مهددين للحياة وقد يصابون بإسهال شديد بسبب هذه البكتيريا. تم إجراء الاختبارات البيوكيميائية والبيولوجية للعينات البكتيرية. تم إخضاع الحمض النووي المستخرج من العزلات لتضخيم بواسطة PCR. تم تقييم عزلات كليبسيلا نيومونيا الرئوية باستخدام جينات تمهيدية محددة (*KPompa*، *Khe*، و*Kpfima*) مع تحديد المضادات الحيوية باستخدام بادئات محددة. وعلاوة على ذلك، تم تحديد تركيز IL-6 باستخدام تقنية ELISA. وقد تم التعرف على بعض سلالات الأمراض عن طريق الأشجار الوراثية أيضًا. وكانت نسب بكتيريا كليبسيلا نيومونيا ثاني أعلى معدلات البكتيريا التي تم تشخيصها في بول الأطفال دون سن الخامسة المصابين بالإسهال، لذا كانت نسبتها أعلى من الأنواع الأخرى المسببة للإسهال. لقد وجد أن كليبسيلا نيومونيا تحتوي على واحد أو أكثر من جينات الفوعة في نفس السلالات. وكان الجين الأكثر فتكاً الذي حددته البادئات هو (*KP16S*) الذي سجل فوعة 100%، يليه الجين التمهيدي (الجين الشيعي) الذي قدر بـ 99%، والذي كان تأثيره معتدلاً والذي تم التعرف عليه بواسطة البادئات (*KPompa*، *Khe*، و*Kpfima*.) بنسبة ضراوة 68.5% للجينات الثلاثة بالتساوي. تشير المستويات العالية من الجسم المضاد الطبيعي إنترلوكين (IL-6) إلى مستويات مرتفعة أكثر من المعتاد. وفي هذه الدراسة، تعد بكتيريا كليبسيلا نيومونيا أحد أنواع البكتيريا المسببة للإسهال لدى الأطفال دون سن الخامسة، وينتج ارتفاع عددها لدى الأشخاص المصابين بالإسهال عن مجموعة من جينات الفوعة الخاصة بها والتي تتشكل في الجهاز الهضمي للأشخاص المصابين. قد يكون نوعًا من البكتيريا المقاومة للمضادات الحيوية. يعد المستوى المرتفع للإنترلوكين IL-6 في مصل الدم مؤشرًا واضحًا على الإصابة ببكتيريا K. الرئوية.