JURNAL BIOMEDIKA DAN KESEHATAN
(JOURNAL OF BIOMEDIKA AND HEALTH)

Vol. 6 No. 2 (2023) pp. 196-215 e-ISSN: 2621-5470

ORIGINAL ARTICLE

Molecular Epidemiology genes detection of *Klebsiella pneumoniae* Clinical Isolates from the Adult Patients with Comorbidities in Baghdad hospitals

Deteksi gen secara Epidemiologi Molekuler pada Isolat Klinik *Klebsiella pneumoniae* dari Pasien Dewasa dengan komorbid di Rumah Sakit Baghdad

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https://doi.org/10.56186/jbk.196-215

ABSTRACT

Background

Klebsiella species is one of the most worldwide opportunistic pathogens in the world that cause infections at multiple sites like lung, urinary tract, bloodstream, wound or surgical site, and brain. *Klebsiella pneumoniae* has many types of virulence factors that give the bacteria the ability to invade the host and cause infections.

Methods

A total of (105) human clinical samples were collected from different patients with different cases. *Klebsiella pneumonia* were isolated from 30 samples. The bacteria were identified by biochemical tests and certified by using VITEK 2 system and genetically by amplify using PCR for 16 rRNA gene. One of the Klebsiella pneumoniae virulence factors is the capsule that is responsible for the macrophage resistance and its also responsible for serotype. So to confirm the strains isolated, PCR Gene detection of, MagA, K2A, RmpA, and Kfu, where (magA, k2A, rmpA, kfu) gens are responsible for capsule synthesis of *Klebsiella pneumonia* have been done.

Results

There were 30 strains of *Klebsiella pneumoniae* were identified. Epidemiological studies show that infections are preceded by gastrointestinal colonization, with the gastrointestinal tract being the most important reservoir for transmission. The primary bacterial isolates were identified based on their cultural, microscopic, and biochemical traits. The API system (API 20E) and VITEK 2 systems were used to confirm the identity of the bacterial isolates. Colony morphology revealed that *Klebsiella pneumonia* was Gram-negative, non-motile, microscopic straight rods grouped singly or in pairs. Biochemical tests showed that neutral end products prevailed over acidic end products, with *Klebsiella* producing negative results for indole. The Kligler Iron Agar (KIA) test showed that

Klebsiella isolates changed the color of the slant and butt, producing an acidic slant and acid butt, along with gas production. The urease test for *Klebsiella* distinguishes between Enterobacter and Klebsiella isolates, as Klebsiella can manufacture the urease enzyme. The indole test distinguishes K. pneumonia from *K. pneumonia* from *K. mobiliz*. The API 20E system was used for biochemical testing, and the VITEK 2 system was used for identification. The VITEK 2 system was used to test for biochemical and antibiotic susceptibility, and the results supported the findings from morphological and biochemical analyses. PCR amplification was used for molecular detection, and all nine isolates tested positive for the magA and k2A genes. The ferric iron uptake system gene (kfu) and the extracapsular polysaccharide synthesis regulator gene (rmpA) were amplified using specific primer pairs. The VITEK 2 system demonstrated promising outcomes for K. pneumoniae identification. However, standard diagnostic methods are costly and time-consuming.

Conclusions

Navigating the complex landscape of infectious diseases, collaborative research projects have become indispensable in unraveling the mysteries surrounding pathogens. The project in Baghdad, having stood as a shining example of such collaboration, provided valuable insights into the identification, characterization, and molecular aspects of Klebsiella pneumoniae strains. The findings, have not only contributed to the scientific community's understanding of bacterial infections but also paved the way for enhanced strategies in diagnosis and treatment.

Keywords: Capsule, *Klebsiella pneumonia*, 16 rRNA, *MagA, K2A, RmpA*, *Kfu*,

ABSTRAK

Latar Belakang

Spesies *Klebsiella* adalah salah satu mikroorganisme yang paling patogen di dunia yang menyebabkan infeksi di paru-paru, saluran kemih, aliran darah, luka atau situs bedah, dan otak. *Klebsiella pneumoniae* memiliki beberapa faktor virulensi yang mampu menyerang inang dan menyebabkan infeksi.

Metode

Seratus lima (105) sampel klinis diperoleh dari pasien dengan kasus yang berbeda. *Klebsiella pneumoniae* yang berhasil diisolasi adalah 30 buah. Bakteri diidentifikasi dengan uji biokimia dan disertifikasi dengan menggunakan sistem VITEK 2. Identifikasi secara genetik dengan menggunakan PCR pada gen 16 rRNA. Salah satu faktor virulensi *Klebsiella pneumoniae* adalah pada kapsulnya, yang memiliki kemampuan resistensi terhadap makrofag dan juga bertanggung jawab untuk serotipe. Sehingga untuk mengidentifikasi strain yang diisolasi dilakukan deteksi gen PCR, MagA, K2A, RmpA, dan Kfu terhadap gen (magA, k2A, rmpA, kfu) yang bertanggung jawab untuk sintesis kapsul Klebsiella pneumonia.

Hasil

Ada 30 strain *Klebsiella pneumoniae* yang diidentifikasi. Studi epidemiologi menunjukkan bahwa infeksi didahului oleh kolonisasi gastrointestinal, dengan saluran pencernaan menjadi reservoir paling potensial dalam penularan. Isolat bakteri primer diidentifikasi berdasarkan sifat kultur, hasil pemeriksaan mikroskopis dan biokimia. Sistem API (API 20E) dan sistem VITEK 2 digunakan untuk identifikasi isolat bakteri. *Klebsiella pneumoniae* adalah koloni yang memiliki morfologi bakteri batang lurus, Gram-negatif, non-motil, dan secara mikroskopis bakteri ini dapat tunggal atau berpasangan. Pemeriksaan secara biokimia menunjukkan bahwa hasilnya lebih bersifat netral dibandingkan asam, dan hasil negatif untuk uji *indole*. Pemeriksaan *Kligler Iron Agar* (KIA) menunjukkan bahwa isolat *Klebsiella* mengubah warna pada *slant* dan *butt*, dan menghasilkan *slant* dan *butt* yang asam disertai dengan gas. Pemeriksaan urease dilakukan untuk membedakan antara isolat Enterobacter dan *Klebsiella*, dengan hasil pemeriksaan pada *Klebsiella* akan memproduksi enzim urease. Uji indol dilakukan untuk membedakan *Klebsiella pneumoniae* dan *Klebsiella mobiliz*. Pemeriksaan biokimia menggunakan sistem API 20E, sedangkan untuk identifikasi menggunakan sistem VITEK 2. Sistem VITEK 2 adalah pemeriksaan penunjang yang digunakan untuk pemeriksaan biokimia dan kerentanan antibiotik, yang hasilnya akan menunjang hasil uji analisis morfologi dan biokimianya. Pemeriksaan PCR dilakukan untuk mendeteksi secara molekuler, dan kesembilan isolat juga menunjukkan hasil yang positif pada pemeriksaan gen magA dan k2A. Gen ferric iron uptake system (kfu) dan gen regulator extracapsular polysaccharide synthesis regulator (rmpA) diamplifikasi menggunakan pasangan primer spesifik. Sistem VITEK 2 menunjukkan hasil yang menjanjikan untuk identifikasi K. pneumoniae. Namun, metode diagnostik standar ini memerlukan biayan yang mahal dan waktu yang lama.

Kesimpulan

Pada navigasi secara lanskap pada penyakit menular yang komplek, memerlukan penelitian secara kolaboratif untuk memecahkan masalah patogenitas. Penelitian di Baghdad adalah sebuah contoh kolaborasi yang baik dan dapat memberikan informasi yang baik mengenai identifikasi, karakterisasi, dan dari aspek molekuler pada strain *Klebsiella pneumoniae*. Temuan ini, tidak hanya berkontribusi pada pemahaman komunitas ilmiah tentang infeksi bakteri tetapi juga membuka wawasan bagi peningkatan strategi dalam menegakkan diagnosis dan tatalaksana.

Kata Kunci: Kapsul, *Klebsiella pneumonia*, 16 rRNA, *MagA, K2A, RmpA, Kfu*

INTRODUCTION

Klebsiella pneumonia is a rod-shaped, Gram-negative, lactose-fermenting bacillus with a prominent capsule. Typical *K. pneumoniae* is an opportunistic pathogen that is widely found in the mouth, skin, and intestines (Agarwal, 2018). *Klebsiella pneumonia* creates an acidic capsule polysaccharide.[1]

Klebsiella colonizes different places in the human body like the nasal and digestive tract but the colonization does not cause any symptomatic disease. However, the colonization can turn into an infection when the host immunity fails to control the pathogen growth, and when the immune system is affected by diseases or other medications like diabetes patients, or patients on glucocorticoid therapy, and those who have received organ transplantation. Studies showed that the prevalence of Klebsiella colonization ranges from 18.8 to 87.7% in Asia and 5 to 35% in Western countries.[2]

K. pneumonia has historically been divided into 79 different capsule types based on serotyping of the K antigen.[3] Due to increased production of capsule polysaccharide (CPS), which is known as the most important virulence factor of *K. pneumonia* and is defined by the appearance of hyper mucoviscous colonies grown on agar plates, a few serotypes, primarily K1 and K2, have a distinct hyper mucoviscous (hyper virulent) phenotype.[4]

Seven major bacterial components make up *K. pneumonia's* virulence factors: the capsule (to inhibit phagocytosis), the lipopolysaccharide (for inhibiting the factors of host serum complement), the fimbriae (for adhesion)[5], the siderophores (for iron acquisition), the bacteriocin[6], the serum resistance[7], and the extended-spectrum β-lactamases ESBLs (for protection from extended-spectrum cephalosporins).[8] *Klebsiella pneumoniae* has a chromosomal system for antibiotic resistance like *K. pneumoniae* strain HS11286 chromosome has putative type II TA.[9] In addition, The chromosomal encoded kacAT bicistronic operon of *K. pneumoniae* strain HS11286 has a functional locus with kacA encoding the antitoxin to the toxic product of kacT Activation of this gen toxin plays an important role in bacterial multidrug tolerance.[10]

To study the epidemiology of *K. pneumoniae* we investigate the presence of *K. pneumoniae* in (105) human clinical samples. The samples were collected from different patients with different cases. The positive samples with *K. pneumoniae* was subjected to PCR in order to determine the frequency of the rmpA, magA, k2A, and kfu genes that are responsible for capsule synthesis, all the target genes are chromosomal gens except *rmpA* gene.[11] A collection of genes known as the capsular polysaccharide genes are responsible for producing different serotypes of CPS.[12] Several of these genes, including *magA*, k2A, *rmpA*, and *kfu*, have been identified as virulence markers.[13]

METHODS

Samples collection

Using sterile containers and travel swabs dampened with regular saline, 105 samples were taken from various patients who visited hospitals in Baghdad such as Ibn Al-Baladi, Central Children, Al-Kindy, Al-Wasity, and Medical City/Educational Lab. Samples were gathered between January 6 and January 10, 2021, from individuals of various ages and genders. The table provides a list of the types and quantities of clinical samples (Table 1).

Table 1. Clinical specimens from different types and numbers

No.	Sample source	Number of sample
1	Urine	50
2	Wound	15
3	Sputum	15
4	Ear swab	10
5	Blood	10
6	Burn	5
Total		105

***Klebsiella pneumonia* isolation**

All isolates were derived from clinical samples and cultivated on MacConkey and blood agar plates. Before looking for bacterial growth, these plates underwent an overnight aerobic incubation at 37 Co. The colonies with pink color and mucous texture were sub-cultured onto MacConkey agar to separate lactose-fermenting bacteria (pink) from non-lactose fermenting bacteria (colorless), whereas onto Blood agar they appeared pale and resulted in gamma-hemolysis.

***K. pneumonia* identification:**

In a hospital library, probable isolates were identified using colony morphology, staining reactions, and biochemical assays.[14-16]

Colony morphology:

Following overnight incubation at 37 C°. Using MacConky agar and Blood agar, all isolates were categorized mostly based on the colony's general cultural features (color, shape, texture, and size). The fermentation of lactose and blood lysis were two additional characteristics that were seen.

Staining reaction:

Gram stain:

After being stained with Gram stain[14], all bacterial isolates were inspected to check the microscopic appearance of cell shape, aggregation, and Gram reactivity.

Capsule stain:

The following steps were carry out for the capsule stain test to identify capsule production: Setting up a typical colony slide from fresh grown colony that have been incubated on MacConky agar for 24 hours at 37 C. The slide was not heated and lefted to dry by air. Then methylene blue was spilled onto the slide for three to five minutes. Finally, Nigrosine was used to rinse the slide for one minute. Microscopic examination under oil immersing 100X objectives.

Biochemical assessment:

The following assessments were conducted to investigate *K. pneumoniae*:

IMVC test:

Indole production assessment:

Fresh bacteria were introduced to peptone broth, which was then cultured at 37°C for 24 hours. 10 drops of Kovac's reagent were then added to each test tube following that. A successful

outcome is indicated by the emergence of a red ring at the top of the soup within 10 minutes. This examination looks for the presence of the tryptophane enzyme, which hydrolyzes tryptophane into indole, pyruvic acid, and ammonia in bacteria.[15]

Methyl red assessment:

The bacterial culture was added to methyl red-Voges Proskauer broth, which was then incubated for 24 hours at 37 C. Each tube received five drops of methyl red solution, and the results were read right away. A successful outcome is shown by the color red. This test is used to identify bacteria that can ferment glucose and end up producing acid. [15]

Voges-Proskauer test

After adding 0.6 ml of VP1 and 0.2 ml of VP2, the MR-VP medium was infected with the bacterial culture and incubated at 37 Co for 24 hours. A positive result was indicated by the reagent turning red after 15 minutes; a negative result was confirmed if the reagent's color remained unchanged. This examination looks for the presence of bacteria that can ferment glucose and create acetone.[15]

Citrate utilization test:

Fresh bacterial isolates were stabbed into Simmon citrate agar slants, which were then incubated at 37 Co for 24-48 hours. A successful outcome is shown by the color changing from green to blue. This test was performed to determine whether bacteria could use sodium citrate as a source of carbon.[15]

Hyper microvasculitis assessment:

The bacterial culture was seeded onto MacConky agar and cultivated for 24 hours at 37C°. A string-like development in the colony that was contacted by a loop and then pulled vertically from the surface of an agar plate was identified as the mucous phenotype. Except for K. pneumonia colonies, which showed a length of 5 cm and displayed the high mucoviscosity phenotype. Test of gas generation and sugar fermentation. After being seeded onto MacConky agar, the bacterial culture was cultured at 37C for 24 hours. The mucous phenotype was characterized as being present when a string-like growth was noticed in the colony that was contacted by a loop and then pulled vertically from the surface of an agar plate. Except for K. pneumonia colonies, which showed a length of 5 cm and displayed the high microviscosity phenotype. Test of gas generation and sugar fermentation. (Kligler iron agar test (MCH)): [15,16]

After being seeded onto MacConky agar, the bacterial culture was cultured at 37C for 24 hours. A string-like development in the colony that was contacted by a loop and then pulled vertically from the surface of an agar plate was identified as the mucous phenotype. Except for K. pneumonia colonies, which showed a length of 5 cm and displayed a high micro-viscosity as the phenotype. Test of gas generation and sugar fermentation.

Table 2. Results of the MCH agar test

Slant/Butt.	Color or Bubble	Reaction
acid / Alkaline	Yellow / Red (Butt. / top)	Glucose only fermented
Acid / acid	Yellow / Yellow (Butt. / top)	Glucose (anaerobic conditions) and Lactose (aerobic conditions) are fermented
Alkaline/alkaline	Red / Red (Butt. / top)	Neither lactose nor glucose are fermented
H_2S production (In the Butt.)	Black precipitation	The reaction between H_2S and ferrous sulphate in the medium
Gas production (In the Butt.)	Bubbles formation	Causing the agar to float to the top shows CO2 production.

The fermentation of sugar caused the color of the phenol red indicator to change from red to yellow, yielding a good result, whereas the absence of bubbles and the formation of black residue yielded a negative result. The initial identification of the Enterobacteriaceae genus was done using this medium.

Urease test:

By inoculating the surface of urea agar slants with bacterial growth and incubation at 37 C for 24 hours, urease activity in K. pneumonia was discovered. While preserving the medium's current yellow-orange tint implies a poor outcome, changing its color to purple-pink indicates a favorable outcome. This examination looks for bacteria that can manufacture the urease enzymes needed to break down urea into ammonia and carbon dioxide.[14]

Motility test:

A single vertical stab was used to introduce the suspected colony into the semi-solid nutritional medium, which was then incubated at 37 Co for 24 hours. Mobile organisms can be identified by movements away from the stab line or by a hazy appearance through the media.[16]

Oxidase test:

Filter paper was used for this test, and a few drops of a freshly made solution of oxidase reagent were used to wet it (tetramethyl-p-phenylenediamine dihydrochloride). Using a sterile toothpick, a clump of cells cultivated on brain heart infusion agar was aseptically removed from the growth and spread on the filter paper. A successful outcome is indicated by the emergence of a violet or purple tint within 2–10 seconds.[14]

Catalase test:

With the use of a sterile toothpick, one colony was transferred onto a clear glass microscope slide. Next, the colony was given a drop of hydrogen peroxide (3 percent).The presence of catalase, which hydrolyzes hydrogen peroxide to water and oxygen, is shown by the generation of gaseous bubbles.[14]

Mannitol fermentation:

At 37°C for 24 hours, mannitol semisolid agar medium was inoculated and incubated. Positive mannitol fermentation outcomes are indicated by the medium's hue changing to yellow.[17]

API 20E identification of *Klebsiella pneumonia*:

This kit was used to investigate Klebsiella spp. isolates and the reagents and indicators IND, TDA, and VP1-VP2 had been prepared in accordance with the manufacturer's instructions from (BioMrrieux).

Preparation of the strips:

The 20 microtubes in the API 20E strip are filled with dehydrated substrates. The incubation tray's wells were filled with five milliliters of tap water to produce a humid environment before an API test strip was removed from its sealed container and placed within.

Preparation of the inoculums:

MaCconky agar was used to choose a single colony, which was then suspended into sterile 5 ml of NaCl (0.85 percent) and thoroughly mixed. The infected solution must be utilized right away after preparation.

Inoculation of the strip:

- A sterile pasture pipette was used to transfer the bacterial suspension to the slanted API test strip. The pipette tip was pressed against the side of the cupules to fill the microtube.
- The CIT, VP, and GEL microtubes had their cupule and tube portions filled.
- To create anaerobic conditions, the cupule parts of the ADH, LDH, ODC, H_2S, and URE microtubes were entirely filled with sterilized mineral oil.
- The Incubation box (Tray and lid) was incubated at 37°C for 24 hours.
- The reagents were applied to TDA, IND, and VP microtubes after incubation.

Reading the strip:

Adding the reagents was done as follows:

- One drop of Kavoc's reagent (Indol reagent) was added to the IND tube, and the results were read right away.
- To the TDA tube, one drop of the TDA reagent (3.4 percent ferric chloride) was introduced.
- One drop of reagents VP1 (40 percent potassium hydroxide) and VP2 to the VP tube (6 percent alpha naphthol) was introduced, and the results were read after 10 minutes.

When the strip is divided into 7 groups, each group has 3 tests with the numbers 1-2-4, using the numerical profile, identification is obtained. The unique digit number that corresponds to each positive test's sequence in the strip was provided to it; The special digit number 0 was given to each negative test. Therefore, if it is, the three-digit number for each group was added up.

VITEK 2 system for identification of *Klebsiella pneumonia* isolates

The following Klebsiella spp. were identified using the VITEK 2 system in this analysis:

To identify the company's turbidity supplier, a single pure colony of the bacterial isolate was suspended in 3 ml of physiological normal saline in a sterile production tube. The bacterial suspension was then compared to a standard turbid static solution (Turbidity measurement instrument). The following Klebsiella spp. were identified using the VITEK 2 system in this analysis: To identify the company's turbidity supplier, a single pure colony of the bacterial isolate was suspended in 3 ml of physiological normal saline in a sterile production tube. The bacterial

suspension was then compared to a standard turbid static solution (Turbidity measurement instrument).

The tubes were placed in their respective racks after being introduced to the VITEK 2 Cassette for each tube evaluation based on the diagnostic gram stain, which has a specific strip for gram-negative bacteria.

- Transferring the cassette and tube rack to the system, inserting the first filler (which automatically fills the cassette with bacterial suspension) and waiting for the procedure to
- The first sliced the cassettes and provided order (burden), and the second field reader (reader) was transmitted to it ever since. The rack holds tubes that are moved from the device to the computer connected to the VITEK system to provide the data for each sample.

Molecular study

DNA extraction kit

Automation machine (Mag purix/Zinextes life science corp/ Taiwan) have been used for DNA Extraction, for that Bacterial DNA extraction kit *200 tests, (***Company**: MagPurix, **Origin**: Taiwan) automated kit used. The process was carried out for the automated genomic DNA extraction after the *Klebsiella pneumoniae* Isolates were cultured in nutrient broth for the night at 37 °C. The purified nucleic acid-containing elute tubes were taken out after the process finished. The extracted DNA was kept at a temperature of -20 °C until it was needed,

Preparation of primers

In this investigation, particular areas were amplified using primers. To create a stock solution with a final concentration of 100 pmol/l, the primers were dissolved in a nuclease-free solution. The final concentration of the primer solution, which was employed in the PCR procedure, was 10 pmol/l after adding 10 mM of this solution to 90 mM of free nuclease water.

Amplification reaction

2 litres of DNA template (100 ng/litre) were amplified in a 25 litre PCR reaction using 12.5 litres of Go Taq® green master mix (Promega, USA), 1 litre of each primer (10 pmol/liter) for each individual gene, and 8.5 litres of nuclease-free water. The PCR premix, primers, and extracted DNA were defrosted at 4°C, vortexed, and then briefly centrifuged to force the contents to the tubes' bottoms. After multiple tests, the polymerase chain reaction was optimized. All components of the negative control were present; however, the DNA was substituted with D.W. The Thermal cycler was set to run PCR procedures (Applied BioSystem, Singapore).

Measuring DNA concentration and purity

By evaluating the nucleic acid content of samples in quantities between 1 L and 1 L, the concentration and purity of extracted DNA were determined using a Nanodrop spectrophotometer. DNA absorbs ultraviolet light, with an absorption maximum of 260 nm wavelength. The absorption at 260 nm and 280 nm are contrasted in order to evaluate the DNA with regard to protein contamination. Protein contamination can be detected by measuring the 260:280 ratio; a value of 1.8 or below is regarded as "relatively pure." DNA, a secondary indicator of nucleic acid purity, also detects impurities that absorb light at 230 nm. Expected 260/230 readings typically fall between 2.0 and 2.2.

Agarose Gel Electrophoresis

Agarose gel electrophoresis is used to verify the presence and integrity of the extracted DNA after genomic DNA has been extracted.[18]

Agarose Gel Preparation (1%): 100 ml (1X) of TBE was added to the beaker after diluting (TBE buffer 10X) to 1X by adding 900 ml of distilled water to 100ml of TBE buffer 10X. The buffer received 1 gm of agarose powder. The mixture was microwaved until it reached a boiling temperature, dissolving all of the gel particles. Ethidium bromide in an amount of 30 ul with a concentration of 10 mg/ml was added to the agarose solution, and it was carefully stirred to prevent bubbles, After the mixture cool to 50 degrees Celsius.

The casting of the agarose gel for horizontal electrophoresis.

The gel tray's edges are sealed with adhesive tape before the agarose solution is added, and the comb is attached 1 cm from one tray edge. The gel tray is placed in the gel tank and submerged entirely in 1x TBE buffer after the gel tray has solidified at room temperature (18 to 22 o C) for 30 minutes. The comb is then carefully removed.[18]

Electrophoresis and DNA loading

Three liters of the 6X loading dye were combined with seven liters of DNA. Samples were carefully placed into each well of the gel, and after 30 minutes of electrical power being applied at 70 volts, the DNA began to flow from the cathode (-) to the anode (+) poles. The gel's ethidium bromide-stained bands were seen with a UV transilluminator and captured on camera.

RESULTS

Klebsiella pneumoniae isolation

Between January 6 and January 10, 2021, 105 clinical samples were gathered from several hospitals in Baghdad and Iraq, including Al-kindy, Ibn Al-baladi, Al-Wasity, Central Children, and the educational lab at Medical City. 30 strains of Klebsiella pneumonia were identified. The transport swabs were wet and sterile containers were used to collect the strains. Total salt units (Table 3), the overall variety of samples utilized to isolate germs. About 90 (85.7%) of the samples were clinically positive, whereas the remaining 15 (14.2%) were negative.

Table 3. Total amount of various samples utilized for the bacteria isolation.

Different Clinical samples	Positive (growth)	Negative (no growth)
105	90	15
Percentage	85.7%	14.2%

On the other hands not all the samples that show growth showed *K . pneumonia* isolates. Table 4 and figure 1 show the number of the positive samples for *K . pneumonia* growth and the source of the samples.

Table 4. Klebsiella pneumoniae isolate from different Samples

No.	Samples source	No. of sample	Positive samples with K. pneumoniae	Percentage of positive samples with K. pneumoniae
1	Urine	50	17	34
2	Wound	15	4	26.67
3	Sputum	15	6	40
4	Ear swab	10	0	0
5	Blood	10	3	30
6	Burn	5	0	0

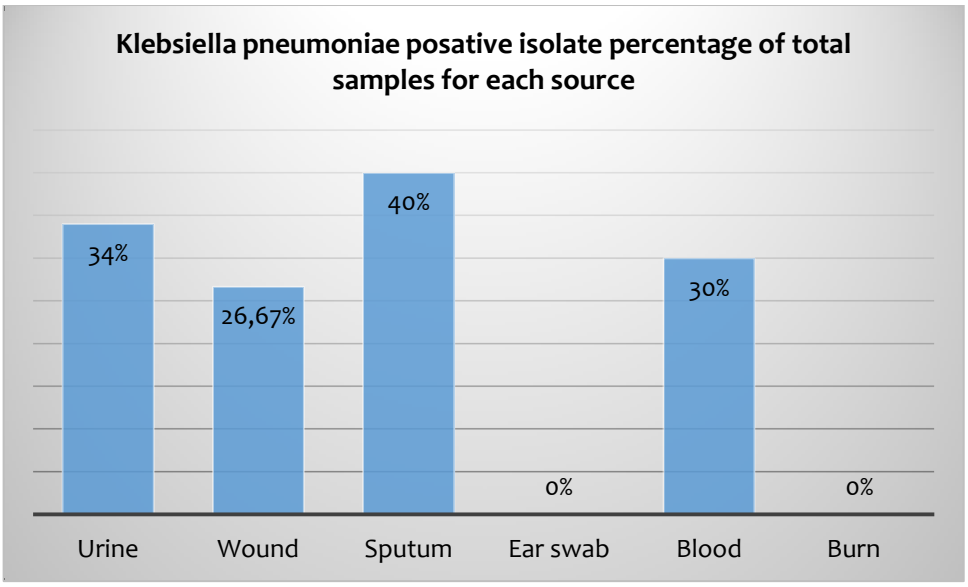

Figure 1. Klebsiella pneumoniae posative isolate percentage of total samples for each source

We can read from the table and the figure that the samples from the burns and ear swabs did not show *Klebsiella* in the growth compare to wounds and urine samples and sputum and blood samples. Epidemiological studies have shown that K. pneumoniae infections are preceded by gastrointestinal colonization, and the gastrointestinal tract is believed to be the most important reservoir for transmission of the bacteria. the carrier rate of Klebsiella in fecal samples ranges from 5 to 38%.[19] On the other hand, K. pneumoniae is a frequent cause human UTI and, in epidemiological studies of Klebsiella bacteraemia, the urinary tract is reported to be the most frequent infection.[20]

Identification of *Klebsiella pneumoniae*

To identify bacterial isolates, a number of morphological and biochemical tests were conducted. Results showed that *Klebsiella pneumonia* constitutes 30 isolates. Bacterial isolates were identified based on their cultural, microscopic, and biochemical traits.[14,17]
Additionally, the API system (API 20E) and VITEK 2 systems were used to confirm the identity of the bacterial isolates.

Colony Morphology:

The primary bacterial isolates were identified after they were grown aerobically on MacConkey agar and blood agar at 37°C for 24–48 hours. On MacConkey agar, Klebsiella colonies generated big, pink, spherical, mucoid-textured colonies with straight edges that were lactose-fermenting colonies K. oxytoca was 3–4 mm in diameter. On the other hand, K. terrigena was 1.5–2.5 mm in diameter and was slightly mucoid. Samples are grown on MacConkey agar using a selective enrichment technique to identify Klebsiella Figure (2A). While preventing the development of Gram-positive bacteria and some fastidious Gram-negative bacteria, bile salts and crystal violet in this medium favor the growth of Enterobacteriaceae and related enteric Gram-negative rods. In this medium, lactose is the sole carbon source that distinguishes lactose-fermenting bacteria from non-lactose-fermenting bacteria. The first can be identified by the formation of pink colonies brought on by neutral red indicator dye conversion at pH values lower than 6.8. The Non-lactose bacterial growth, on the other hand, appears transparent or colorless.[21] Regarding enrichment Large, mucoid, white to grey, and non-hemolytic colonies were the characteristics that set Klebsiella apart from other species of the bacteria that grew similarly on MacConkey agar but produced hemolytic blood, such Serratiaspp.[22]

The string test revealed that all K. pneumonia isolates were positive, indicating the presence of the hyper mucoviscosity phenotype as demonstrated by the production of a string that was around 7 cm long (figure 2 B). According to Cardenas *et al.*[23], this characteristic was not present in the noninvasive K. pneumonia strains recovered from patients with noninvasive infections.

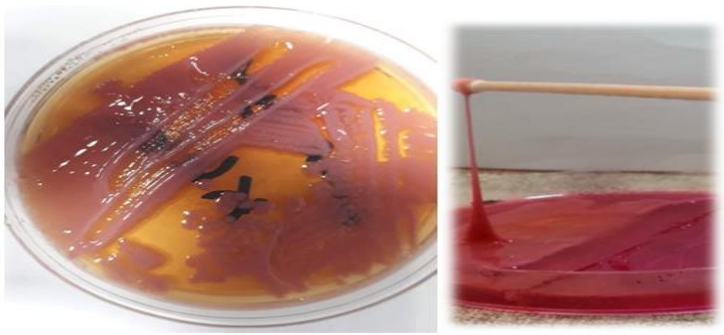

Figure 2. Mucoid colonies of *K.pneumoniae* of MacConkey agar at 37C for 24hrs (A) single colony (B) the Hyper-mucoviscosity phenotype

Staining reaction:

Under a compound light microscope, the most likely Klebsiella pneumonia was discovered to be Gram-negative, non-motile, microscopic straight rods grouped singly or in pairs (figure 3 A), according to Brenner et al.[16]

Under a compound light microscope, the most likely Klebsiella pneumonia was discovered to be Gram-negative, non-motile, microscopic straight rods grouped singly or in pairs (figure 3 A), according to Brenner et al.[16]

One of the several virulence traits that the bacteria K. pneumoniae is known to express is a thick polysaccharide capsule. The capsule shields the bacteria from polymorph nuclear leukocyte phagocytosis and may prevent the bacteria from being killed by bactericidal serum components.[16]

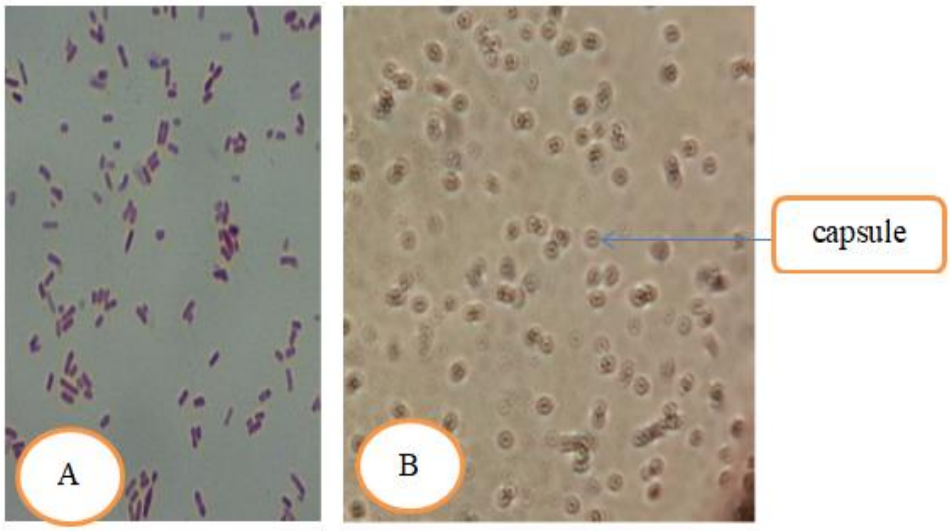

Figure 3. Klebsiella pneumonia (A) Gram stain (B) Capsule stain, magnification power=100x

Biochemical tests:

For a more thorough identification of bacterial isolates, biochemical assays were performed. All isolates of Klebsiella were positive or negative for methyl red and positive for voges-proskauer (VP), indicating that neutral end products prevail over acidic end products. Acetoin and 2,3-butanediol were formed from partial fermentation of glucose.[24]

They are distinct from the lactose fermenter genera E. coli, Citrobacter, and Serratia based on the results of IMVC. For indole, Klebsiella produced negative results (except K. oxytoca). Tryptophanase, an enzyme that breaks down the amino acid tryptophan into indole, pyruvic acid, and water, is a feature of some enteric bacteria that can hydrolyze tryptophan to indole in the indole test. Since Klebsiella and other indole-negative bacteria did not make tryptophanase, a red ring would not form when Kovac's reagent was introduced to a broth without indole.[17]

One of the many essential physiological tests used to identify members of the Enterobacteriaceae family is the use of citrate. The citrate in the simmon citrate medium is essential to determining whether the bacteria isolates can grow on it as a unique source of carbon and energy because Klebsiella showed good reactions to it. In addition, bromothymol blue, a pH indicator, is included in Simmon's medium. The CO_2 that Klebsiella produces combines with the medium's components to create an alkaline molecule, which changes the pH indicator's (bromthymol blue's) color from green to blue and indicates a positive citrate test.[15]

DISCUSSION

Based on their patterns of carbohydrate fermentation and H_2S generation, the Enterobacteriaceae genera are distinguished from one another in the Kligler Iron Agar (KIA) test. KIA slants have a 1% lactose and 1% glucose content. In the presence of acids, the pH indicator (phenol red) caused the medium's hue to shift from orange red to yellow. Additionally, KIA contains ferrous sulfate, which forms a black precipitate to distinguish H_2S-producing bacteria from other types, and sodium thiosulfate, a substrate for H_2S generation.

The findings revealed that Klebsiella isolates changed the color of the slant and butt, producing an acidic slant (yellow) and acid butt (yellow), along with gas production (bubble formation), but

without the formation of a black precipitate, indicating that lactose and glucose had been fermented and no H2S had been produced. These findings corroborated those made by Brenner et al.[16]

As the urease test was positive for Klebsiella but negative for Enterobacter, it distinguishes between Enterobacter and Klebsiella isolates. The urease enzyme catalyzes the breakdown of urea, and the bacteria that can make it are able to detoxify the waste products and use it to generate metabolic energy, changing the medium's color from yellow to purple-pink and showing a positive urease test. Since Klebsiella can manufacture the urease enzyme, the test for urease is positive.[14]

The Klebsiella isolates failed the motility test. The bacteria are motile if they move away from the stab line or seem hazy as they move through the semisolid media. However, the linear growth implies a bad outcome, a characteristic of Klebsiella.

The isolates of Klebsiella were either catalase positive or negative and oxidase negative (Hajjar, et. al., 20 20). The findings of the indole test distinguish K. pneumonia from K. oxytocait was positive for that species and negative for others.[14]

The fact that K. *pneumonia* could not survive at 10 C separates them from K. *oxytoca* and K. *terrigena*, which can survive at this temperature.[25] All K. *pneumonia* isolates were discovered to be non-motile, separating them from the motile species K. *mobilis*.[24] Since K. *ozaenae*, K. *rhinoscleromati*, K. *oxytoca*, and K. *terrigena* are unable to create gas from lactose at 44.5 Co, all isolates of K. *pneumonia* can. This distinguishes them from K. *ozaenae*, K. *rhinoscleromati*, K. *oxytoca*, and K. *terrigena*.

Identification of Klebsiella pneumoniae using the API 20E system

Using the API 20E system, the results of biochemical testing for *Klebsiella pneumoniae* (30 isolates) were verified (3). Results from every bacterial isolate agreed with those mentioned above.

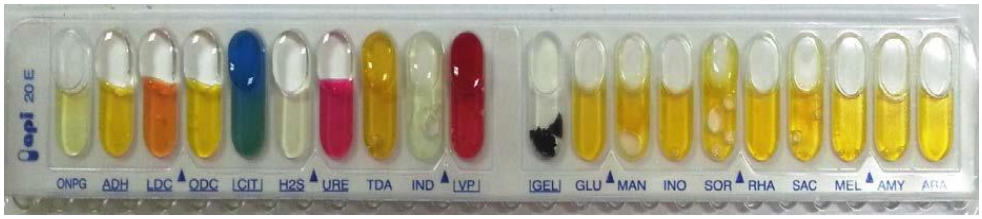

Figure 4. Characterization of K. pneumoniae using the API 20E system

The diagnostic API 20E system could be able to distinguish Klebsiella pneumoniae and produce excellent results. Additionally, this system's diagnostic is quite accurate.[11] However, standard means of diagnosis, such as the API 20E system and biochemical tests, are costly and time-consuming.

VITEK 2 System for Identification of *Klebsiella pneumoniae*

This approach was utilized to support the final K. pneumoniae diagnosis. This technology discovered germs more quickly, effectively, and far from contaminants that may limit pathogen identification.[26]

The outcomes of the tests utilized in this approach supported the findings from morphological and biochemical analyses. Therefore, it is proven for all thirty isolates that were previously recognized as Klebsiella pneumoniae.
The VITEK 2 system offers to test for both biochemical and antibiotic susceptibility. demonstrated promising K. pneumonia outcomes.

PCR amplification for K. pneumonia molecular detection:
DNA from Klebsiella pneumonia was extracted.

Nine K. pneumoniae isolates had their genomic DNA retrieved utilizing an automated genomic DNA extraction method. To create a PCR template for amplification, DNA was extracted. The recorded DNA concentration range was 48.4-125.8 ng/l, and the DNA purity ranged from 1.6 to 2.0, according to the results. The amounts and DNA quality that were obtained were sufficient for PCR amplification. Increased DNA template concentrations enhance the possibility of producing non-specific PCR results. With less template, the amplification's accuracy declines. Based on the extinction coefficients of nucleic acids at 260 and 280 nanometers, 1.8 is the predicted value for a pure DNA preparation.[18]

Amplification of *16S rRNA* gene

The 16S-F and 16S-R primers, which are specific for the PCR amplification of K. pneumonia 16S rRNA, were used for the molecular identification of the nine isolates. According to figure 5. of the outcomes, the amplified fragments were around 130 bp in size, which is comparable to the size of those produced by Turton et al.[11] when they used the same primer. The 130 bp bands on each of the nine isolates were positive, classifying them as K pneumoniae. The PCR amplification results confirmed the earlier findings and showed that each and every isolate was K. pneumoniae.

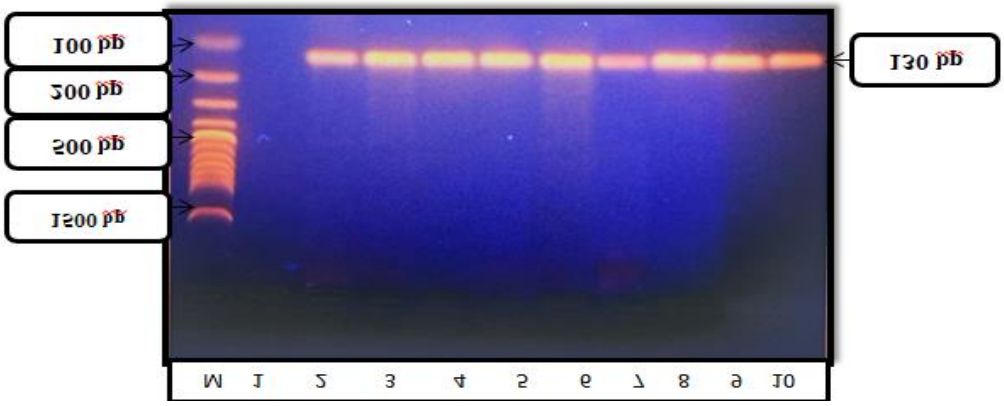

Figure 5. Gel electrophoresis for application of 16S rRNA gene of K.peumoniae. Electrophoresis wa performed on 1.5% agarose gel and run with a 70 volt/35 mAmp current for 2 hours. Lane M is a (100 bp) ladder.

Amplification of *magA*and *k2A* genes

Via a specific primer pair and amplified magA gene, K. pneumonia was identified using PCR (magA-F and magA-R). The magA gene was amplified from K. pneumonia isolates using this primer, and all 9 isolates tested positive for the gene. The PCR result was 1283 bp in size, the same size as that obtained by Turton et al.[11] when they used the same primer, as shown in Figures 6 and 7.

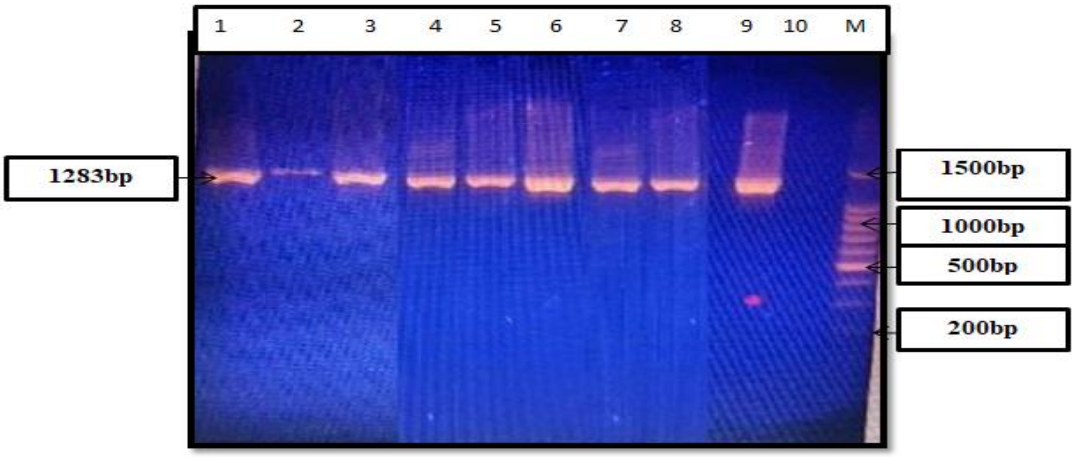

Figure 6. Gel electrophoresis for amplification of *MagA* gene usingspecific primers of K.*pneumoniae*. Elecrophoresis was performed on 1.5% agarose gel and run with a 70 volt/35 mAmp current for 2 hours. Lane M is a (100 bp) ladder.

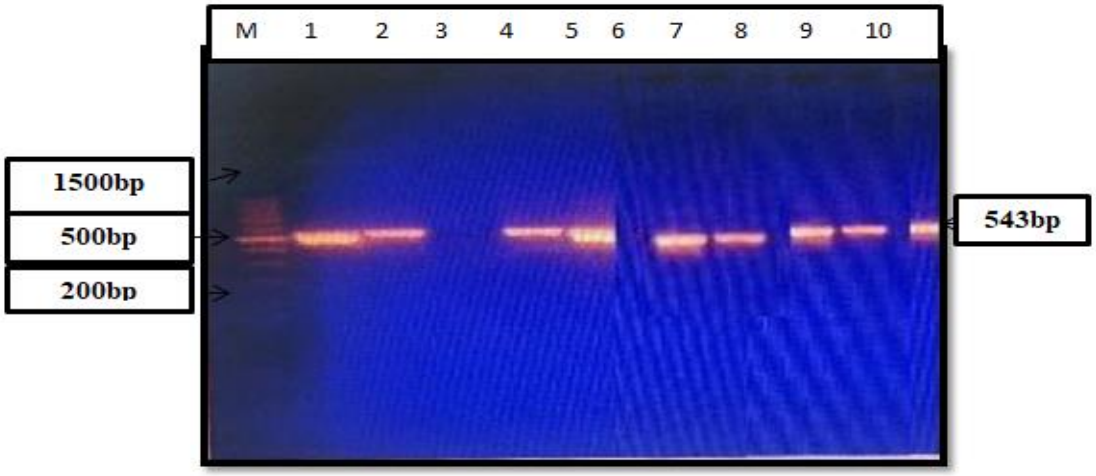

Figure 7. Gel electrophoresis for amplification of *k2A* gene using specific primers of K.*pneumoniae* serotype K2. Elecrophoresis was performed on 1.5% agarose gel and run with a 70 volt/35 mAmp current for 2 hours.

Amplification of *rmpA*and *kfu* genes:

Two additional virulence factors that were researched were the ferric iron uptake system gene (kfu), necessary for the metabolism of iron to maintain growth in the host, and the extra capsular polysaccharide synthesis regulator gene (rmpA), linked to the hyper mucoviscosity phenotype.[27,28]

In the current study, PCR was used to amplify rmpA and kfugenes using primer pairs that were specifically designed to do so (rmpA-F and rmpA-R), respectively (kfu-F and kfu-R). Using the rmpA and kfu primers, the amplified DNA produced a PCR product with a band that measured around 536 and 797 bp in molecular size, respectively. Nine isolates produced successful results.

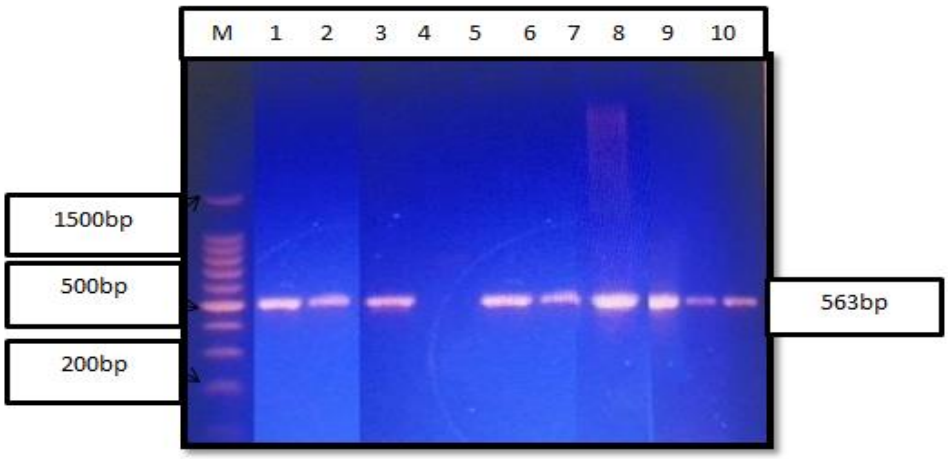

Figure 8. Gel electrophoresis for amplification of *rmpA* gene using specific primers of K.*pneumoniae*. Elecrophoresis was performed on 1.5% agarose gel and run with a 70 volt/35 mAmp current for 2 hours.

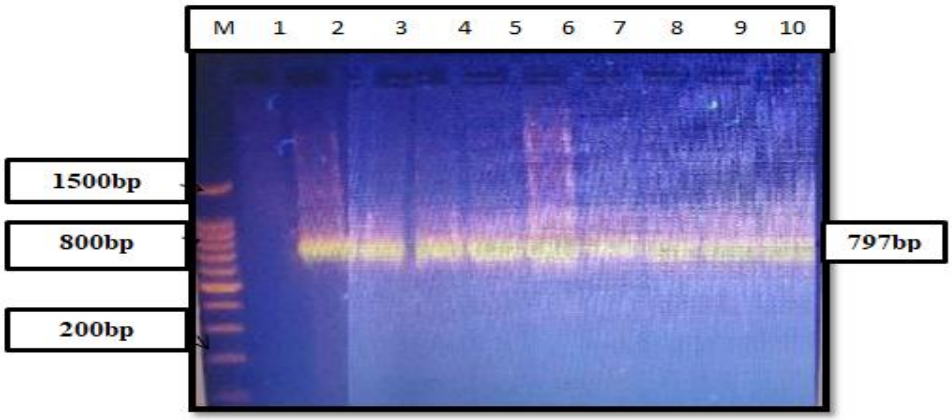

Figure 9. Gel electrophoresis for amplification of *kfu* gene using specific primers of K.*pneumoniae*. Elecrophoresis was performed on 1.5% agarose gel and run with a 70 volt/35 mAmp current for 2 hours.

CONCLUSION

Based on the clinical samples collected from various hospitals in Baghdad and Iraq between January 6 and 10, 2021, **30 strains of Klebsiella pneumoniae** were identified. Epidemiological studies suggest that gastrointestinal colonization precedes Klebsiella pneumoniae infections, with the gastrointestinal tract being the most important reservoir for transmission. The primary bacterial isolates were identified based on their cultural, microscopic, and biochemical traits. The API system (API 20E) and VITEK 2 systems were used to confirm the identity of the bacterial isolates.

The colony morphology of Klebsiella pneumoniae was Gram-negative, non-motile, microscopic straight rods grouped singly or in pairs. Biochemical tests showed that neutral end products prevailed over acidic end products, with Klebsiella producing negative results for indole. The Kligler Iron Agar (KIA) test showed that Klebsiella isolates changed the color of the slant and butt,

producing an acidic slant and acid butt, along with gas production. These findings corroborate those made by Brenner et al.

The urease test for Klebsiella distinguishes between Enterobacter and Klebsiella isolates, as Klebsiella can manufacture the urease enzyme. The indole test distinguishes K. pneumonia from K. pneumonia from K. mobiliz. The API 20E system was used for biochemical testing, and the VITEK 2 system was used for identification. The VITEK 2 system was used to test for biochemical and antibiotic susceptibility, and the results supported the findings from morphological and biochemical analyses. PCR amplification was used for molecular detection, and all nine isolates tested positive for the magA and k2A genes. The ferric iron uptake system gene (kfu) and the extra capsular polysaccharide synthesis regulator gene (rmpA) were amplified using specific primer pairs. The VITEK 2 system demonstrated promising outcomes for K. pneumoniae identification.

ACKNOWLEDGEMENT

The success of this research project is a testament to the dedication and collaboration of a diverse team of professionals. Researchers, clinicians, laboratory technicians, and support staff from various hospitals in Baghdad and Iraq worked seamlessly to collect, analyze, and interpret the clinical samples. Their collective expertise and commitment to advancing medical knowledge have contributed to the elucidation of critical information regarding Klebsiella pneumoniae.

Conflict of interest: This study does not pose any conflict of interest in its interaction with other studies.

AUTHORS CONTRIBUTION

None

FUNDING

None

CONFLICT OF INTEREST

None

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