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Molecular detection of the K2 serotype of hyper virulent *Klebsiella pneumoniae* in local soft cheese

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Abstract

Background: *Klebsiella pneumoniae (K. pneumoniae*) is an increasingly important threat to public health since the emergence of hyper virulent strains. This study aimed to evaluate the presence of hyper virulent *Klebsiella pneumoniae* (hvkp) in the milk and milk products. **Methodology:** One hundred cow's milk and fifty local soft cheese samples from different rural areas and local markets in Baghdad city were screened. HiCrome™ Klebsiella selective agar base and indole test were used initially for bacterial identification. The isolates were also validated by Vitek®2 Compact system with the amplification of the 16S rRNA gene followed by nucleotide sequencing. **Results:** *K. pneumoniae* was totally diagnosed in thirty-three (22%) samples. PCR indicated that one (3.03%) isolate from the local soft cheese contained the K2 serotype of hvkp, together with the regulator of mucoid phenotype A (*rmpA*) gene and exhibited a high degree of multidrug resistance (MDR). The K2 serotype of the hvkp isolate and the *rmpA* gene were registered at National Center for Biotechnology Information (NCBI) under the accession numbers PX055694.1 and PX055698.1, respectively. **Conclusions:** The local soft cheese represents a potential source of hvkp strain; thus, there is a need to improve control and prevention strategies, particularly in the field of milk production.

Keywords: hvKp, K2 serotype, local soft cheese

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Introduction

Dairy products made from raw unpasteurized milk provide a favorable ground for a number of enteric pathogens. They are highly marketable commodities and represent the most common means for transmission of many pathogens from farmer dairy animals to the consumer (Khudhir, 2021). *K. pneumoniae* is an emerging human pathogen. The increasing prevalence of this pathogen is attributed to complex interactions between hosts, bacteria, and environmental factors, making the emergence of associated infections unpredictable (Wareth & Neubauer, 2021). Recent studies have shown that food is a potential source of *K. pneumoniae* transmission, it is documented in an increasing number of food products, indicating growing importance as an emerging pathogen in the food industry. Furthermore, several reports indicate that *K. pneumoniae* can contaminate dairy products, leading to food spoilage and emergence of multidrug-resistant strains (Bonardi *et al*., 2023; Jin *et al*., 2025).

K. pneumoniae is a Gram-negative bacterium, classified as an opportunistic pathogen, capable of causing a broad spectrum of infections in humans, notably immunocompromised or hospitalized individuals (Abbas *et al*., 2024). Moreover, classical and hypervirulent *K. pneumoniae* (hvKp) strains exhibit significant differences in their pathogenesis, particularly with regard to capsule structure and virulence-related genes (Choby *et al*., 2020). In *K. pneumoniae*, capsular polysaccharides (CPS) are among the most prominent protective antigens and key virulence factors. Due to the wide structural diversity of these polysaccharides, 79 different serotypes have been identified to date (Wang *et al*., 2025). CPS types "K1, K2, K20, K54, and K57" are the most serotypes associated with hvkp, and the capsule types K1 and K2 are the most virulent, representing 70% of the hvkp isolates (Al-Busaidi *et al*., 2024). Moreover, most of the isolates associated with invasive infections exhibit a characteristic hypermucoviscous phenotype, which is an important indicator of virulence. Afterwards, these strains have been shown to possess several virulence factors, notably, the regulator of the mucoid phenotype (the *rmpA* gene) (Pinpimai *et al*., 2022). In recent years, the presence of the hvkp is not limited to hospitals and patients, but there is documentation about its presence in various food sources. This increases the concern, in addition to distribution through food, and its contribution to the overall health burden (Zhang *et al*., 2018). The K2 serotype has been reported in cow milk samples, including fresh milk products in China, mastitis cows in Pakistan (Saddam *et al*., 2023), and cow milk from dairy farms in China. In contrast, limited studies have presented the virulence factors of *Klebsiella* spp. in cheese products (Gelbíčová *et al*., 2020).

Iraq is in a growing significant demand for high-quality natural foods, especially those free of artificial preservatives. In this context, evidence indicates that traditional handling practices in milk and its products may lead to its contamination and increased microorganism growth, and it became a clear indicator to the increased risk of foodborne illnesses (Dhay & Maytham, 2023). In Iraq, the phenotypic and genotypic characteristics of hvkp in foods, including dairy products, have not been investigated up to our knowledge. Therefore, this study aimed to investigate the existence of K1 and K2 serotypes of hvkp in cow milk and local soft cheese, determine the presence of the gene regulating the mucoid phenotype (*rmpA*), and the antibiotic resistance of the obtained isolates.

Materials and Methods

Ethics approval

All samples were collected from different rural areas and local markets in Baghdad city after getting approval from the committee of care and use of the animal in research at the College of Veterinary Medicine, University of Baghdad, with Ethics No. 1205/P.G. on 19/5/2025.

Collection of samples

During the study period, 100 cow milk and 50 local cheese samples were collected from different rural areas and local markets in Baghdad city without heat treatment or salting. 250-500 ml and/or grams of milk and/or cheese were placed in a sterile container inside a refrigerated box and immediately transported to the Public Health Laboratory, College of Veterinary Medicine, University of Baghdad.

Bacterial isolation and identification

According to Gelbíčová *et al.* (2020) and Azwai *et al*. (2024), *K. pneumoniae* was detected from cows' milk and local soft cheese. In brief, 25 g or 25 ml from each sample was transferred into a sterile polyethylene stomacher bag and enriched in 225 ml of buffered peptone water (Oxoid, UK) at 37°C overnight. Then, roughly 10 µl (loopful) were inoculated onto HiCrome Klebsiella Selective Agar and MacConkey's agar (Himedia, India) and incubated at 37°C overnight. The next day, magenta-colored colonies and large mucoid pink colonies were looked for.

Identification of *K. pneumoniae* by the indole test

Pure single colony of the test organism was mixed with tryptone broth and inoculated at 35°C (+/- 2°C) for 24 to 48 h, after that five drops of Kovac's reagent were added. The expected results were either positive (formation of pink ring) or negative (no color change).

String test

K. pneumoniae isolates were inoculated overnight into MacConkey's agar plates at 37°C. The next day, an inoculation loop was used to stretch the top colony on an agar plate. The positive result deemed mucus filaments longer than 5 mm (AlKhafaji *et al*., 2024).

Extraction of DNA

Genomic DNA was extracted from the bacterial isolates according to the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (FAVORGEN Biotech Corporation, Taiwan).

Molecular identification of the *16S rRNA* and virulence genes

The primer pair targeting the 16S rRNA gene was designed using NCBI-GenBank, based on its partial sequence (MT634697.1). While the primers targeting the K1 and K2 serotypes as well as the *rmpA* gene were obtained from Pinpimai *et al*. (2022). These primers were provided by Macrogen, Korea, and are listed in Table 1. The primers were used to detect K1 and K2 serotypes and the *rmpA* gene using the polymerase chain reaction (PCR). Each 25 µl PCR reaction mixture composed of 12.5 µl of GoTaq® Green PCR master (Promega, USA), 5 µl DNA template, 1.25 µl of each forward and reverse primers and 5 µl of PCR distilled water to complete the final volume. Thermo cycler (Promega, USA) was used in this study, and its program was as follows: The operation was done by setting up the initial denaturation for 1 cycle and for 5 min at 94°C, while for 16S rRNA it was done for 5 min at 95°C; denaturation was conducted for 35 cycles for 30 sec at 94°C, while for 16S rRNA it was done for 30 sec at 95°C; annealing involved 35 cycles for 30 sec at 64°C, while for *16S rRNA* it was for 30 sec at 58°C; extension included 35 cycles for 45 sec at 72°C, while that of *16S rRNA* was done for 5 min at 72°C; and final extension involved 1 cycle for 8 min at 72°C, while for *16S rRNA* it was conducted for 5 min at 72°C (Pinpimai *et al*., 2022; Optimase Protocol Writer™ online application). The final PCR products were loaded onto 1% agarose gel, stained with 3 µl of ethidium bromide, and later visualized under UV transilluminator.

Table 1: Primers used to detect hvkp isolates and their sizes

Primer	Gene	Primers' Sequences (5'- 3')	Amplicon Size (bp)
Capsular type K1	*K1magA*	F: GGTGCTCTTTACATCATTGC R: GCAATGGCCATTTGCGTTAG	١٢٨٢
Capsular type K2	*K2wzy*	F: GACCCGATATTCATACTTGACAGAG R: CCTGAAGTAAAATCGTAAATAGATGGC	٦٤١
RmpA	*rmpA*	F: ACTGGGCTACCTCTGCTTCA R: CTTGCATGAGCCATCTTTCA	٥١٦
16S rRNA	*16S rRNA*	F:CCTGGACAAAGACTGACGCT F: CCTGGACAAAGACTGACGCT R: AGTTGCAGACTCCAATCCGG	٥٨٤

DNA sequencing

The PCR positive products, one isolate for the K2 serotype and the *rmpA* gene from the same isolate were sent to the Macrogen Company (South Korea) for sequencing. Later, the sequences were submitted to the GenBank/NCBI website to be registered under a specific accession number.

VITEK-2 Compact: Biochemical analysis and antibiotic susceptibility testing

AVitek®2 Compact (BioMérieux, France) system was used for the biochemical analysis and antibiotic susceptibility testing (AST) of the test organism using specific Gram-negative kits (VITEK®2GN ID and VITEK®2 AST–N222). In brief, new colonies (2–3) were dropped into a sterilized physiological NaCl solution using DensiChek™, and the McFarland turbidity was adjusted to 0.50–0.63. Then, 5 ml of the suspension was placed onto each card. Finally, the cards and suspension tubes were inserted into the VITEK2 cassette. In case of GN ID, the unidentified isolates were compared with the symmetric reference strains kept in the VITEK2 Compact software to ensure identification. The VITEK®2 Gram negative susceptibility cards were used to assess the antibiotic resistance and susceptibility against positive serotype isolates according to minimal inhibitory concentrations (MICs) (CLSI, 2024).

Results and Discussion

Isolation and identification of *K. pneumoniae*

Overall, *K. pneumoniae* was identified in 33(22%) out of 150 samples (Table 2) using HiCrome™ Klebsiella Selective Agar Base and indole test (Figure 1).

Table 2: Isolation of *K. pneumoniae* from cows' milk and local cheese

Type of Samples	Total Samples	Positive	Negative
Cows' milk	١٠٠	٢٣(٪٢٣)	٧٧(٪٧٧)
Local cheese	٥٠	١٠(٪٢٠)	٤٠(٪٨٠)
Total	150	33 (22%)	117 (78%)

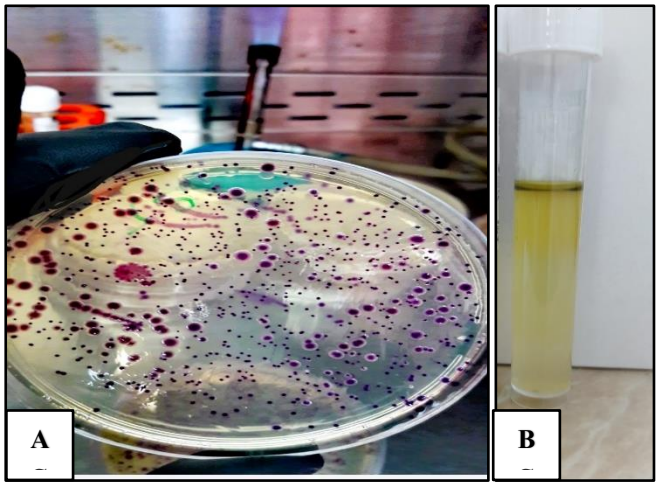


Figure 1: Identification of *K. pneumoniae*. A: Growth on HiCrome™ Klebsiella Selective Agar Base, which shows purple-magenta colored colonies. B: Negative indole

Molecular identification of *K. pneumoniae*

Bright bands of approximately of 584 bp from 23 isolates were observed on the agarose gel. These bands are suspected to be the partially amplified *16S rRNA* gene of *K. pneumoniae* (Figure 2).

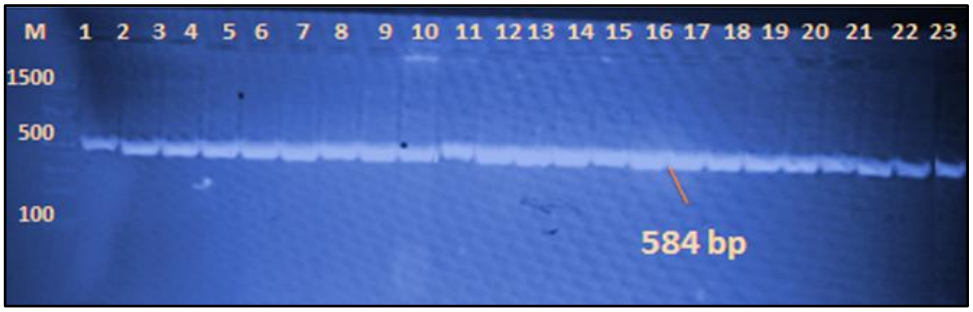


Figure 2: Agarose gel electrophoresis shows bands of approximately 584 bp suspected to be the *16S rRNA* gene of *K. pneumoniae* amplified by the PCR technique. Ladder marker at 1500 bp; lanes (1-23) visualized under the UV light

Milk and milk products including cheese are complex ecosystems for numerous pathogens including bacteria. As shown in Table 2, *K. pneumoniae* was isolated from 23% (23/100) of cow milk samples and 20% (10/50) of local cheese samples. However, the microorganisms can contaminate the cheese through shared contamination pathways originating from an infected cow's udder or from unhygienic handling equipment and the environment itself. The number of isolates in local soft cheese was lower than the number isolated from cows' milk. This level is higher than the results previously reported by Khalil *et al*. (2021) in cheese and Dhay and Maytham, (2023) in local white soft cheese and local cream. In contrast, high isolation rates were reported by Enferad and Mahdavi, (2020) in raw cow milk and raw and mastitic milk, respectively. Another study by Gelbíčová *et al*. (2020) found that *K. pneumoniae* has been identified not only on hands of the personnel but also in the environment of cheese processing and an opened packaging of milk powder. The current results indicate that cow's milk and its products are important sources for *K. pneumoniae* isolation.

String test

In the present study, hypermucoviscous *K. pneumoniae* was examined with the string test in 33 isolates (Figure 3 A & B). It was found in 2 (6.06%) isolates of the local soft cheese. It was later found that 1 of 2 isolates contained the *rmpA* gene, which is the regulator of mucoid phenotype A. This result is lower than that reported by AlKhafaji *et al*. (2024) and Taha *et al*. (2024). Initially, it was a common misconception that the trait of a hypermucoviscosity is specifically linked to hvkp strains. It was later clear that some non hvkp strains possess this characteristic (Russo *et al*., 2018). These observations are consistent with our results.

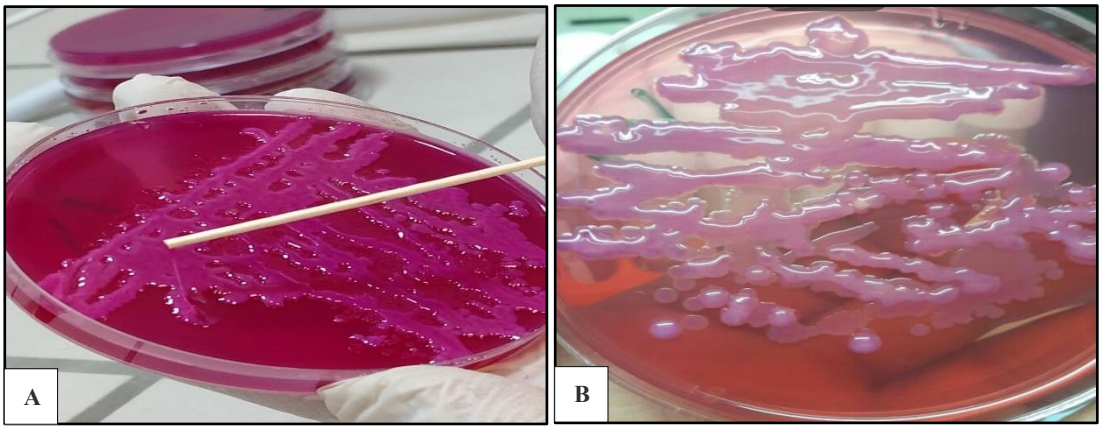


Figure 3: The colony morphology of *K. pneumoniae* on the McConkey's agar. A: Positive string test, the length of mucus filaments is longer than 5 mm. B: Large mucoid, pink color and convex surface colonies

Molecular detection of the K2 serotype and *rmpA* of hvkp in local cheese

A few studies reported the presence of K1 and K2 hvkp strains in food (Zhang *et al*., 2018). Nevertheless, there were no previous studies, to our knowledge, of a K2/rmpA-positive hvkp in Iraqi dairy products. This study showed that K2 hvkp isolates can be transmitted through the local soft cheese, where 1/33 (3.03%) of the K2 hvkp isolates contained the *rmpA* gene. In contrast, Gelbíčová *et al*. (2020) did not identify K1and K2 serotypes in the processed cheese plant. A study was isolated the K2 serotype from raw and ready-to-eat retail food, while the *rmpA* gene was not detected. However, the difference in results may be associated with origins and geographic distributions of the isolates. The *rmpA* is an essential virulence gene that has been closely related with hypermucoid phenotype and highly virulent strains (Yanping *et al*., 2023).

Here, the presence of hvkp in local soft cheese may be due to several factors, including the use of raw milk or milk from animals infected with sub-clinical mastitis that contain large numbers of bacteria. Otherwise, perhaps, the reason is exposure of milk to insufficient thermal processing in most home-made cheese. In addition, marketing in unsanitary environments and direct exposure to weather conditions contribute to a significant increase in the microbial load. Although the prevalence of hvkp in our samples was low (1/150, 0.67%), the detection of a single MDR K2 strain underscores the potential for contamination and justifies more extensive monitoring. Figures 4 and 5 reveal the presence of the partially amplified *K2wzy* gene as well as *rmpA* gene of *hvkp*.

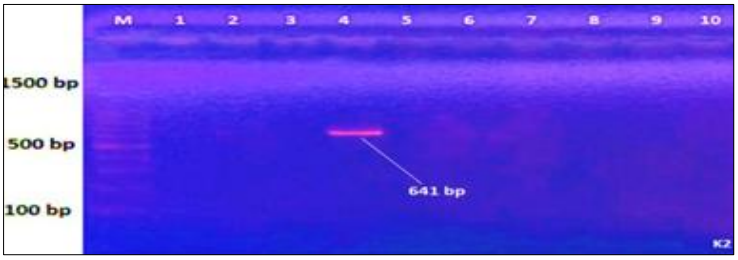


Figure 4: Gel electrophoresis shows a band of approximately 641 bp, which represents the partially amplified *K2wzy* gene of hvkp. M: DNA ladder, lane 4: DNA from local soft cheese

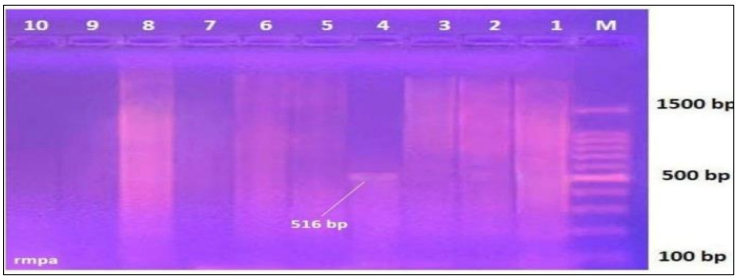


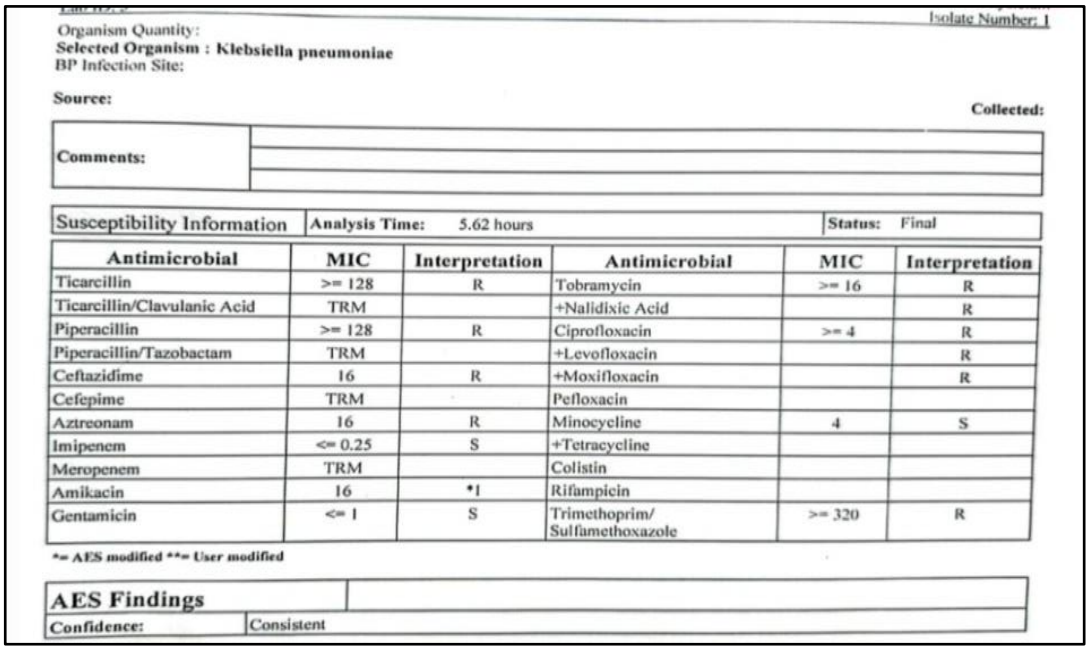
Figure 5: Gel electrophoresis shows a band of approximately 516 bp, which represents the partially amplified *rmpA* gene of hvkp. M: DNA ladder, lane 4: DNA from local soft cheese

Antibiotic resistance of hvkp isolate from local cheese

As shown in Table 3 and Figure 6, the hvkp isolate exhibits resistance to antimicrobial agents from at least six different classes including: penicillin, cephalosporin III/IV, monobactam, quinolone, fluroquinolones and sulfonamides, but intermediate to aminoglycosides (amikacin).

Table 3: Pattern of resistant, intermediate and susceptible isolates of hvkp isolate recovered from local soft cheese

Classes of Antibiotics	Types of Antibiotics	Results of AST Cards		
		Susceptible	Intermediate	Resistant
Penicillin	Ticarcillin			R
	Piperacillin			R
Cephalosporin III/IV	Ceftazidime			R
Monobactam	Aztreonam			R
Carbapenem	Imipenem	S		
Aminoglycosides	Amikacin		I*	
	Gentamycin	S		
	Tobramycin			R
Quinolone	Nalidixic acid			R
	Moxifloxacin			R
Fluroquinolones	Ciprofloxacin			R
	Levofloxacin			R
Tetracycline	Minocycline	S		
Sulfonamides	Trimethoprim/Sulfamethoxazole			R



Isolate Number: 1

Organism Quantity:
Selected Organism : Klebsiella pneumoniae
BP Infection Site:

Source:

Collected:

Comments:

Susceptibility Information | **Analysis Time:** 5.62 hours | **Status:** Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ticarcillin	>= 128	R	Tobramycin	>= 16	R
Ticarcillin/Clavulanic Acid	TRM		+Nalidixic Acid		R
Piperacillin	>= 128	R	Ciprofloxacin	>= 4	R
Piperacillin/Tazobactam	TRM		+Levofloxacin		R
Ceftazidime	16	R	+Moxifloxacin		R
Cefepime	TRM		Pefloxacin		
Aztreonam	16	R	Minocycline	4	S
Imipenem	<= 0.25	S	+Tetracycline		
Meropenem	TRM		Colistin		
Amikacin	16	*I	Rifampicin		
Gentamicin	<= 1	S	Trimethoprim/ Sulfamethoxazole	>= 320	R

*= AES modified **= User modified

AES Findings

Confidence: Consistent

Figure 6: Vitek®2 Compact report on the identification and susceptibility information of hvkp isolated from local cheese

The extensive use and repeated exposure to large group of antimicrobial agents can trigger the emergence of new multidrug resistance (MDR) phenotypes (Yanping *et al*., 2023). The hvkp isolate of our study showed resistance to ten antimicrobial agents and revealed intermediate susceptibility to one antimicrobial agent. This level of resistance and intermediate resistance referred to as MDR (Magiorakos *et al*., 2012). The hvkp isolate was resistant to piperacillin, and susceptible to imipenem. Zhang *et al*. (2018) reported that 16.1% of the 62 of strains from retail foods were resistant to piperacillin but all strains were susceptible to imipenem. In addition, the isolation was resistant to aztreonam, ceftazidime, levofloxacin, and ciprofloxacin. In addition, the hvkp isolate was resistant to trimethoprim/sulfamethaxazole, nalidixic acid and amikacin. It is reported that 6, 2 and 1 of 97 *K. pneumoniae* isolated from food were resistant to trimethoprim/sulfamethaxazole, nalidixic acid and amikacin, respectively, and all isolates were susceptible to gentamicin. The hvkp isolate also showed resistance to tobramycin. Azwai *et al*. (2024) reported that 4 (33.3%) isolates from different food items were resistant to tobramycin. This study results are similar to the findings of Rodrigues *et al*. (2021). In this study, high resistance rate to antibiotics by the local cheese isolates signals an urgent call for strict monitoring of milk products in Baghdad.

Registration of the K2 serotype and the *rmpA* gene of hvkp strains in GenBank

Depending on the partial amplification of the *K2wzy* gene of hvkp strains and the *rmpA* gene of hvkp strains by PCR followed by nucleotide sequencing, these sequences were registered within NCBI, as written in Table 4.

Table 4: Registration of the K2wzy gene of hvkp strains and rmpA of hvkp strains in GenBank

No.	Gene	Name of the Isolate in GenBank	Accession Numbers
1	*K2wzy*	*Klebsiella pneumoniae* strain Rahma IQ3 1175 DNA sequence	PX055694.1
2	*rmpA*	*Klebsiella pneumoniae* strain Rahma IQ7 509 DNA sequence	PX055698.1

Conclusions

This study indicates that foodborne *K. pneumoniae* exhibited the virulent K2 serotype of hvkp and had MDR in local cheese samples. Therefore, this work might be the first report of a K2/rmpA-positive hvkp in Iraqi dairy products. The string test can serve as a useful preliminary screening tool for the hypermucoviscous phenotype, but positive results must be confirmed by PCR for definitive hvkp-associated genes like *rmpA*. The potential health risks posed by such isolates should not be underestimated, and it underscores the importance of monitoring and controlling the spread of these bacteria, particularly in the field of food safety and public health.

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الكشف الجزيئي عن النمط المصلي K٢ لجراثيم الكلبسيلا الرئوية شديد الضراوة في الجبن الطري المحلي

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المستخلص

الخلفية: تُشكل الكلبسيلا الرئوية (*K. pneumoniae*) تهديدًا متزايد الأهمية للصحة العامة منذ ظهور السلالات شديدة الضراوة. هدفت هذه الدراسة إلى تقييم وجود الكلبسيلا الرئوية شديدة الضراوة في الحليب ومنتجاته. **المنهجية**: تم فحص مئة عينة من الحليب البقري وخمسون عينة من الجبن الطري المحلي من مناطق ريفية مختلفة وأسواق محلية في مدينة بغداد. في البداية تم استُخدام اختبار قاعدة آجار الكلبسيلة الانتقائية HiCrome™ واختبار الإندول من أجل تحديد البكتيريا. وقد جرى التحقق من صحة العزلات بواسطة نظام Vitek®2 Compact وتضخيم التسلسل الجزيئي لجين الحمض النووي الرايبوزي الريبوسومي S١٦ الذي تم تصميمه باستخدام قاعدة البياناتNCBI Genbank، متبوعًا بتسلسل جزئي للنيوكليوتيدات. **النتائج**: شخصت الكلبسيلا الرئوية في ثلاث وثلاثين عينة (٢٢٪). أشار تفاعل البوليميراز المتسلسل إلى أن عزلة واحدة (٣,٠٣٪) من الجبن الطري المحلي تحتوي على النمط المصلي K2 من الكلبسيلا الرئوية شديدة الضراوة مع منظم النمط الظاهري المخاطي وتتميز بدرجة عالية من المقاومة للأدوية المتعددة. وقد سُجّلت عزلة النمط المصلي K2 وجين منظم النمط الظاهري المخاطي في "المركز الوطني لمعلومات التكنولوجيا الحيوية" تحت رقمي الوصول (PX055694.1) و(PX055698.1) على التوالي. **الاستنتاجات**: أظهرت نتائجنا أن الجبن الطري المحلي يمثل مصادرا محتملا لسلالة الكلبسيلا الرئوية شديدة الضراوة، لذا هناك حاجة إلى تحسين استراتيجيات المكافحة والوقاية خاصة في مجال منتجات الألبان.

الكلمات المفتاحية: الكلبسيلا الرئوية، النمط المصلي k2، الجبن الطري المحلي