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Assessment Of Temperature And Salt Stress Tolerance In Klebsiella Pneumoniae Isolated From Dairy Sources In Karbala Province

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Abstract

Background:

Klebsiella pneumoniae is a significant opportunistic pathogen that poses a public health risk, especially when present in food products such as dairy and meat. Contamination of food with this bacterium can lead to transmission of antibiotic-resistant strains and foodborne illness.

Materials and methods :

A total of 525 samples (dairy and meat) were collected from retail markets in Karbala city, Iraq. Samples were cultured on MacConkey agar and CHROMagar™ Orientation to isolate presumptive K. pneumoniae colonies. Identification was confirmed by PCR targeting the 16S-23S ITS region, yielding a specific product of 130 base pairs. Heat tolerance was evaluated by treating milk samples at 60°C, 65°C, and 70°C for 30 minutes, followed by culturing. Salt tolerance was assessed by incubating bacteria in 0.5%, 1%, and 1.5% NaCl solutions for 1–2 weeks, with subsequent growth checked on Mueller-Hinton agar.

Results:

All cultured samples exhibited characteristic K. pneumoniae colony morphology within 24 hours. PCR amplification confirmed the presence of K. pneumoniae in the dairy and meat samples, with bands observed at 130 bp. Growth was observed in milk samples treated at 60°C and 65°C, but not at 70°C, suggesting thermal elimination at higher temperatures. Short-term salt tolerance was observed at all concentrations, but no viable bacteria were detected after two weeks of incubation.

Conclusion:

Klebsiella pneumoniae was detected in food samples from Karbala, indicating potential contamination risks. The bacterium exhibited limited heat and salt tolerance, supporting the effectiveness of standard pasteurization and preservation methods. Molecular confirmation using ITS-PCR provided reliable identification beyond culture-based techniques.

Keywords: *Klebsiella pneumoniae, PCR, 16S-23S ITS, dairy products, meat contamination, heat resistance, salt tolerance, food safety, Karbala*

INTRODUCTION

The dairy industry is one of the almost all of advanced and profitable food industries. based on the Food and Agriculture Organization (FAO), more than 6 billion people consume milk globally (OECD & FAO, 2020). Statistics indicate that the majority of milk produced is sourced from cows., accounting for approximately 81%, while milk production from goats, sheep, and camels combined only exceeds 4% (Tsakali et al., 2023).Milk occupies a prominent position among other foods, as it is considered the ideal source of fundamental nutrients for humans from infancy to senility (Uddin et al., 2011) This is due to its excellent sensory properties and all the nutrients necessary for rapid growth, as well as its potential ability to prevent or reduce the risk of many illness resulting from malnutrition. However, because of its distinctive properties and composition (such as its richness in nutrients, high moisture content, and pH value), milk can function as not only as a unique growth medium for a diverse range of microbial flora like lactic acid bacteria (LAB) but it can also be a potential transmission source for some pathogens, such as Propionibacterium, Staphylococcus, Streptococcus, Enterobacteriaceae like Klebsiella , and others (Frank, 2013, Quigley et al., 2013).The nature of meat, whether minced or not, makes it susceptible to contamination due to its high moisture and nutrient content, which creates a favorable environment for bacterial growth. K.pneumoniae can be transmitted to meat during various stages, such as slaughtering,

cutting, transportation, or storage, especially when hygiene and safety procedures are not followed. Contaminated equipment or contact with contaminated hands is also a means of transmitting this bacteria to meat (OBI et al., 2023).Klebsiella pneumoniae is a Gram-negative, facultative anaerobe, non-motile bacterium that typically produces a prominent polysaccharide capsule, contributing to its virulence. As a member of the Enterobacteriaceae family, it is frequently associated with opportunistic infections in humans and can be transmitted through the consumption of contaminated animal-derived food products such as beef, poultry, fish, and dairy. When cultivated on selective media such as MacConkey agar, K. pneumoniae exhibits a distinctive mucoid colony morphology due to its capsule, which plays a protective role against host immune defenses. Additionally, this bacterium is capable of fermenting lactose, a characteristic feature aiding in its laboratory identification. (Wareth, G., & Neubauer, H. , 2021; Lewis et al.,2022). K.pneumoniae exhibits heat resistance through multiple physiological responses, most notably the production of heat shock proteins such as hsp70 and hsp60, which maintain protein stability and prevent damage under heat. This is in addition to the role of regulatory factors such as the rpoS gene, which enhances the bacterial response to stress. Heat resistance is also influenced by external factors such as the physiological age of the bacteria, water activity, the presence of solutes or solid particles, and strain variation (Edmondson et al., 1980).Klebsiella pneumoniae is not commonly considered a foodborne germ, so the majority of foodborne strains studies focus on more common pathogens like Escherichia coli (E. coli), Salmonella, and Shigella. Consequently, there is still a lack of comprehensive data regarding the prevalence of K. pneumoniae in retail food and the traits of these strains, including their virulence properties and antibiotic resistance, even though such information is crucial for evaluating possible public health threats. (Guo et al., 2016; Theocharidi et al., 2022), the study aimed to detection of K.pneumoniae by molecular technique PCR and detection the effects of temperature and freezing on the viability of K.pneumoniae and detect the effect of different salts concentration on viability of K.pneumoniae.

MATERIALS AND METHODS

Material

Samples collection:

This study was done to detection of K. pneumoniae in raw milk and milk by-products in markets of Karbala The work started from October /2024 until April/2025. During this period, about 525 random samples of raw milk, cheese, butter , yoghurt, cream, minced meat and non-minced meat had been collected from five regions of Karbala Province (City center, Al-Hussynia, Al-Hur, Ain Altamor, Al-Hindyia) with 75 samples from each region.

Isolation & identification of K. pneumoniae

Isolation and bacterial identification were carried out using different culture media (MacConkey), CHROMagar Enterobacteria agar and Mueller Hinton agar (Himedia/India). All collected samples were plated on MacConkey agar and then incubated for 24 hours at 37°C. Suspected samples were then cultured on selective medium (CHROMagar Enterobacteria agar) as a preliminary confirmation method.

Molecular detection of Klebsiella pneumoniae

The detection of Klebsiella pneumoniae was carried out using polymerase chain reaction (PCR) table (1). Specific primer sequences targeting conserved regions of the bacterial genome were employed to amplify the desired fragments. The primers were designed based on published nucleotide sequences and were synthesized commercially. DNA was extracted from the bacterial isolates and used as the template in the PCR reactions. Amplification was performed under optimized thermal cycling conditions. The presence of specific PCR products was confirmed by gel electrophoresis, where distinct bands of expected sizes indicated positive amplification. All steps were performed under sterile conditions to prevent contamination, and the results were validated through comparison with known control.

Table 1 : PCR primer with their Nucleotide sequences and amplicon size.

Target Gene	Primer	Sequence	Product size	Reference

K.pneumoniae 16s-23sITS primer	Forward	ATTTGAAGAGGTTGCAAACGAT	130 base pair	Theocharidi et al., 2022
	Reverse	TTCACTCTGAAGTTTTCTTGTGTTC		

Experimental part:

Multidrug resistance via Vitek system

Following the molecular identification of Klebsiella pneumoniae using PCR, bacterial isolates exhibiting multidrug resistance were selected through the VITEK 2 compact system, based on their antibiotic susceptibility profiles. These resistant isolates were subsequently used in thermal and salt tolerance experiments involving sterile milk.

Effect of temperature

To assess thermal resistance, a standardized bacterial suspension was prepared according to the McFarland 0.5 turbidity standard. One milliliter of the bacterial suspension was inoculated into three separate sterile test tubes, each containing 9 mL of autoclaved raw milk. The inoculated tubes were then exposed to different temperature treatments of 60°C, 65°C, and 70°C for 30 minutes using a water bath. After heat exposure, the tubes were allowed to cool at room temperature before being cultured on MacConkey agar to determine the presence or absence of bacterial growth.

Effect of salts

For the salt tolerance assay, the same standardized bacterial suspension was inoculated into sterile milk solutions containing varying concentrations of sodium chloride (NaCl) at 0.5%, 1.0%, and 1.5%. These preparations were incubated under controlled conditions and examined at different time intervals, specifically at 0 hours, 24 hours, 1 week, and 2 weeks. At each time point, samples from the tubes were cultured on MacConkey agar to assess bacterial viability and the ability of K. pneumoniae to grow under saline stress.All experimental procedures were conducted under aseptic conditions to avoid external contamination. Growth on MacConkey agar was evaluated by observing colony morphology and pigmentation to confirm the survival and adaptation of the isolates under tested conditions.

RESULTS AND DISCUSSION

A total of 525 samples were cultured on MacConkey agar and Chromagar, and all showed Klebsiella pneumoniae colony growth within 24 hours. The growth of lactose-fermenting mucous pink colonies occurred on MacConkey agar due to low pH and pigment conversion, while metallic blue colonies appeared on Chromagar due to enzyme interaction with chromogenic substrates.

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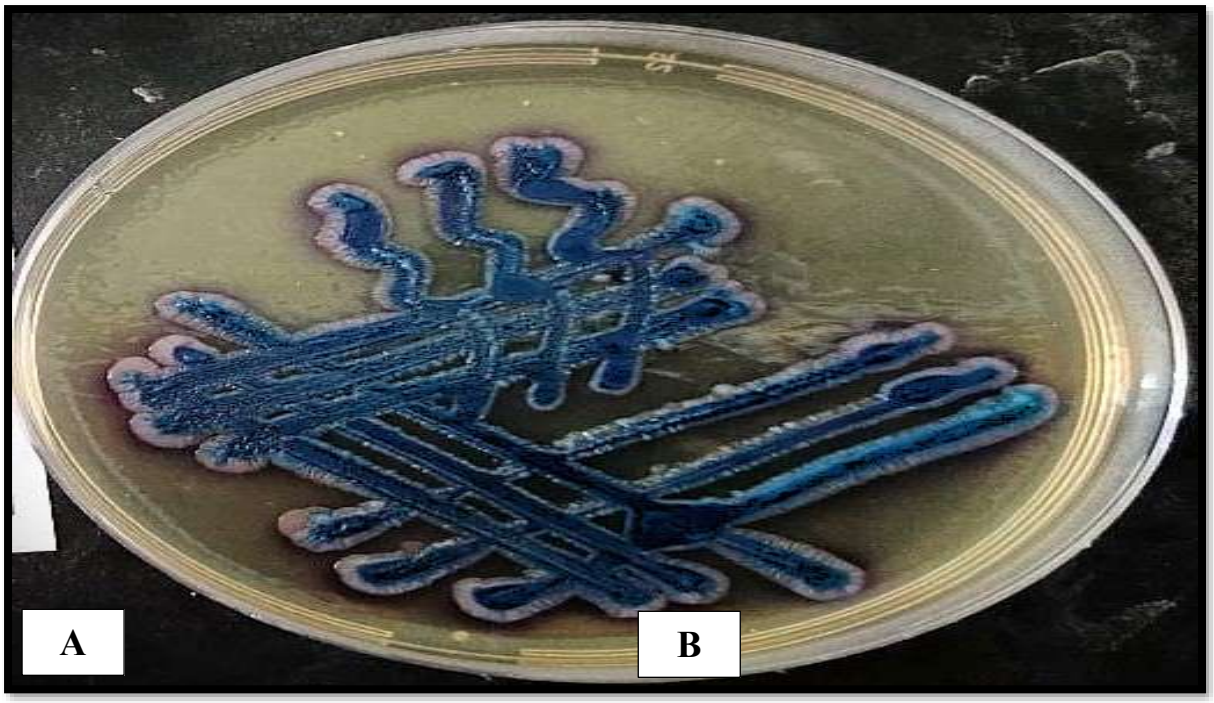
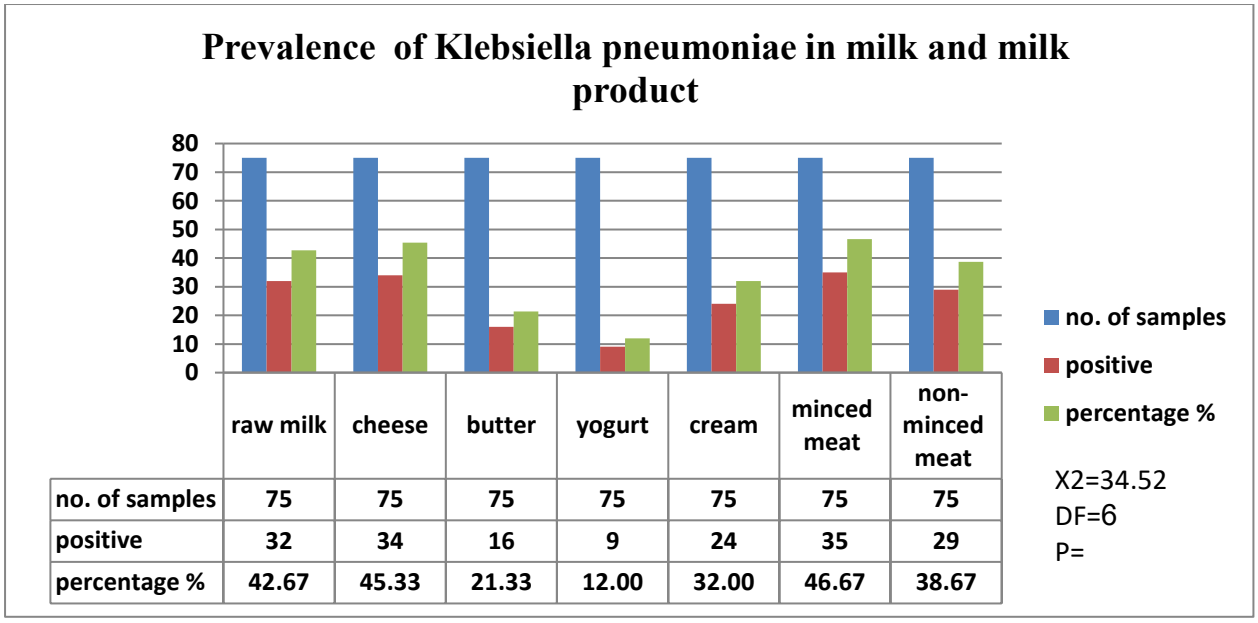


Figure 1 :(A) Metallic blue rounded and large colonies of Klebsiella pneumonia on chrome agar and (B) Pink , mucoid colonies on MacConkey agar



	raw milk	cheese	butter	yogurt	cream	minced meat	non-minced meat
no. of samples	75	75	75	75	75	75	75
positive	32	34	16	9	24	35	29
percentage %	42.67	45.33	21.33	12.00	32.00	46.67	38.67

Figure (2): Prevalence of Klebsiella pneumoniae in milk and meat product

Molecular techniques for detection of Klebsiella pneumoniae

Detection of Klebsiella pneumoniae was performed using a PCR assay targeting the **16S-23S internal transcribed spacer (ITS) region**, producing a specific amplicon of approximately **130 base pairs**. The PCR was conducted on DNA extracted from **dairy products and meat samples** collected from various markets in **Karbala city**. Prior to molecular confirmation, presumptive colonies were initially isolated using **MacConkey agar** and **CHROMagar™ Orientation**, which are selective media for Enterobacteriaceae.

As shown in the gel electrophoresis image (Figure 3), positive amplification was observed in multiple samples, indicated by the presence of clear bands at approximately 130 bp. A DNA ladder was used in the first lane for fragment size estimation. The consistent presence of bands in the expected region confirms the successful amplification of the target sequence from K. pneumoniae DNA. No bands were detected in the negative control, indicating the absence of contamination

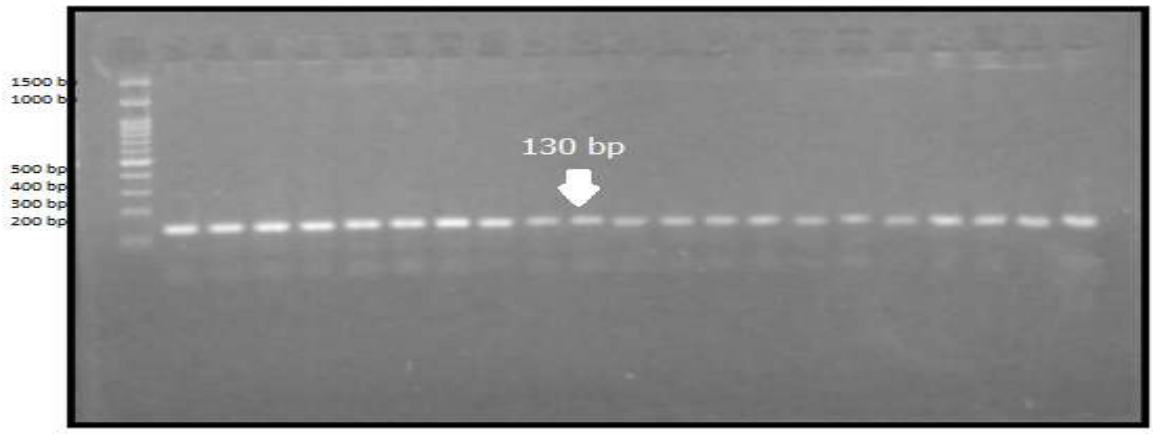


Figure (3): gele electrophoresis of 16S-23S internal transcribed spacer (ITS) region, producing a specific amplicon of approximately 130 base pairs in Klebsiella pneumonia isolated from dairy and meat product

Effect of Temperature:

The results of the experiment showed that Klebsiella pneumoniae bacteria grew after 24-hour incubation on MacConkey plates cultured with milk samples treated at 60°C and 65°C, with the characteristic pink bacterial colonies appearing on the culture media. In contrast, no bacterial growth was observed on the plate cultured with the milk sample treated at 70°C, indicating complete elimination of the bacteria at this temperature during the 30-minute treatment period.

Table (2): Effect of temperature on K. pneomniae

Temperature	Duration	Result
60	30 min	Growth
65	30 min	Growth
70	30 min	No-growth

Effect of Salt Concentration:

After incubation for one day in saline solutions, followed by culture on Mueller Hinton agar, the results showed bacterial growth at all concentrations (0.5%, 1%, and 1.5% NaCl).

After incubating the tubes for a full week in saline conditions, the bacteria adapted to the 0.5% concentration, showing only growth upon re-culture on Mueller-Hinton agar medium. In contrast, no growth was observed at the 1% and 1.5% concentrations after re-culture.

After two weeks of incubation, no growth was observed at all concentrations upon re-culture.

Table (3):Effect of Salts on K. pneomniae

Concentration	After one hour	After one day	One weeks	Two weeks
0.5	Growth	Growth	Growth	No-growth
1	Growth	Growth	No-growth	No-growth
1.5	Growth	Growth	No-growth	No-growth

DISCUSSION

A total of 525 samples were cultured on MacConkey agar and CHROMagar™ Orientation, and all exhibited growth characteristic of Klebsiella pneumoniae within 24 hours. On MacConkey agar, lactose-fermenting, mucoid pink colonies were observed, attributed to acid production lowering the pH and inducing pigment conversion. On CHROMagar, metallic blue colonies developed as a result of specific enzymatic interactions with chromogenic substrates, These characteristics are consistent with previous studies (Kumar et al., 2020; Neumann et al., 2023; Till, 2013; Chaturvedi and Banashankari, 2017). The PCR assay targeting the ITS region between 16S and 23S rRNA genes proved to be a reliable molecular

method for confirming the presence of K. pneumoniae in both dairy and meat products. This finding underscores the importance of accurate identification of this opportunistic pathogen, particularly in food items that may serve as reservoirs for antimicrobial-resistant strains. The use of CHROMagar™ and MacConkey agar enabled preliminary differentiation of lactose-fermenting colonies, which was subsequently confirmed by PCR.These results are consistent with previous studies that have emphasized the utility of ITS-PCR in differentiating Klebsiella species from other closely related Enterobacteriaceae (Brisse et al., 2004; Osman et al., 2014). The high prevalence of K. pneumoniae in food samples in this study may reflect environmental contamination or poor hygiene during food processing and handling, which poses potential public health risks (Zheng et al., 2017).Continued surveillance and implementation of molecular diagnostics are recommended to monitor the occurrence of K. pneumoniae in food sources, especially in light of its rising resistance to multiple antibiotics. he differential growth patterns of Klebsiella pneumoniae following heat treatment of milk samples demonstrate the bacterium's partial thermal tolerance and highlight the importance of effective pasteurization. The survival of K. pneumoniae at **60°C and 65°C for 30 minutes**, as evidenced by the appearance of characteristic pink colonies on MacConkey agar, aligns with earlier findings that some strains of this pathogen can withstand sub-pasteurization temperatures due to protective mechanisms such as capsular polysaccharides and biofilm formation (Migeemanathan et al., 2022). In contrast, **complete inhibition of bacterial growth at 70°C** indicates that this temperature is sufficient to inactivate K. pneumoniae under the conditions tested, consistent with standard thermal inactivation kinetics for Gram-negative bacteria in dairy products.These findings emphasize the risk of consuming inadequately heated or raw milk, particularly in regions where informal or home-based milk processing is common. Moreover, heat resistance in K. pneumoniae has recently been linked to adaptation under sublethal stress conditions, which could increase virulence and antibiotic resistance traits (Zhou et al., 2023). Given the emerging global concern regarding foodborne transmission of multidrug-resistant K. pneumoniae strains, strict adherence to thermal processing standards is imperative (Theocharidi et al., 2022). Future studies should investigate whether extended exposure times at lower temperatures could achieve similar bactericidal effects, or if combination treatments (e.g., heat and pressure or antimicrobial additives) offer improved control in dairy safety protocols.The ability of Klebsiella pneumoniae to survive in varying salt concentrations over time reflects its adaptive response to osmotic stress. Initial growth at 0.5%, 1%, and 1.5% NaCl concentrations after 24 hours indicates short-term tolerance to moderate salinity, consistent with its classification as a facultative halotolerant bacterium (Goraya et al., 2022; Al-Isawi et al., 2020). However, after one week of incubation, bacterial survival was only observed at 0.5% NaCl, suggesting an upper limit to sustained salt tolerance and possible stress-induced metabolic adjustment. The absence of growth at higher concentrations (1% and 1.5%) at this stage indicates cellular damage or inability to maintain osmotic homeostasis beyond the initial adaptation phase. By the second week, complete loss of viability at all concentrations upon re-culture highlights the cumulative impact of prolonged hyperosmotic stress, likely resulting in membrane destabilization, disrupted ion transport, and inhibited replication. These findings underscore the importance of time-dependent stress in microbial survival studies. The osmotic sensitivity shown here contrasts with certain environmental or hospital-derived K. pneumoniae strains that possess enhanced stress resistance mechanisms, such as compatible solute synthesis or upregulation of osmoprotective genes (Patel et al., 2021; Wang et al., 2022). In conclusion :this observation reinforces the idea that although K. pneumoniae can transiently endure osmotic pressure, long-term exposure to even moderate salt levels may effectively limit its viability, a concept relevant to both food preservation and infection control strategies.

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Conflict of Interest
The author(s) declare that there is no conflict of interest regarding the publication of this research.

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