

The Effect of pH Variation on Antibiotic Susceptibility of MDR *Klebsiella pneumoniae* Isolates

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ABSTRACT: Background: *Klebsiella pneumoniae* is a significant opportunistic pathogen responsible for various nosocomial infections in humans. The emergence of multi-drug resistant strains poses a significant challenge in clinical settings, necessitating a deeper understanding of factors influencing antimicrobial resistance. **Objective:** This research aimed to investigate the impact of pH variation on the resistance patterns of multi-resistant *K. pneumoniae* isolated from Iraqi patients with urinary tract infections and wound infections against different antibiotics. **Methods:** Forty *K. pneumoniae* isolates were obtained from urine samples and wound swabs, and their identification was confirmed using the VITEK® 2 compact system and molecular identification of the *rpoB* housekeeping gene. Antibiotic susceptibility testing was performed using the Kirby Bauer's disk diffusion method under varying pH conditions (pH 5, 7, 9, and 11) at 37°C for 18 to 24 hours. **Results:** The study findings indicated that *K. pneumoniae* isolates exhibited differential susceptibility to antibiotics based on pH conditions. Cefotaxime demonstrated increased efficacy under alkaline pH, while tetracycline showed optimal efficacy under acidic conditions. However, ciprofloxacin displayed resistant phenotypes at acidic pH 5 and either resistant or intermediate phenotypes at alkaline pH 9. **Conclusions:** The results suggest a potential influence of pH on the antibiotic susceptibility profiles of *K. pneumoniae* isolates. Understanding the role of pH in antimicrobial resistance can inform strategies for better managing infections caused by multi-resistant pathogens. Further research is warranted to elucidate the underlying mechanisms and implications for clinical practice.

KEYWORDS: Antimicrobial Agents; Antibiotic Susceptibility; *Klebsiella pneumoniae*; Multi-drug Resistance; pH stress

INTRODUCTION

Klebsiella *pneumoniae* (*K. pneumoniae*) is a common Gram-negative opportunistic bacterium of the family Enterobacteriaceae that grows optimally at pH (6-8) and at a temperature of 37°C [1], [2]. It presents as normal flora in the human oropharynx and gastrointestinal mucous membrane, which act as primary reservoirs for *K. pneumoniae* in humans [3], [4]. It causes hospital-acquired infections such as pneumonia, wound infections, and urinary tract infections (UTIs) as well as community-acquired infections such as liver abscess, bone infections, pneumonia, meningitis, and soft tissue infections [5]–[7]. It was considered one of the most serious pathogens incriminated in UTIs, as well as one of the most antibiotic-resistant microorganisms in Iraq [8]. *K. pneumoniae* causes infections with the help of several virulence factors including lipopolysaccharide (LPS), capsular polysaccharide (CPS), adherence factor, and siderophore [9].

Many antibiotics suppress bacterial growth and are still used as first-line treatment for bacterial infectious diseases. Antimicrobial resistance has been recognized to be among the world's three major threats [10], [11]. *Klebsiella pneumoniae* is a rapidly developing multi-drug resistant (MDR) strain that acquires antimicrobial resistance (AMR) more quickly than most bacteria and causes

life-threatening infections, which typically constitute a serious concern among patients because of increased mortality and morbidity risk [2], [7]. Physical parameters such as pH that might affect bacterial growth, antibiotic activity, and Antibiotic resistance factors associated with efflux pumps include biofilm development and quorum-sensing [12]–[14]. In natural habitats, changing the pH by one unit can reduce the metabolism rate of microbial populations by up to 50%, influencing the composition and extracellular enzyme activities [15]. The current research was conducted to determine the influence of varied pH values on the resistance rate of Iraqi *K. pneumoniae* isolates to different antimicrobial agents.

MATERIALS AND METHODS

Bacterial Collection and Growth Conditions

Forty *K. pneumoniae* isolates were acquired from several hospitals in Baghdad/ Iraq and were gathered from clinical sources, including urine samples, and wound swabs from postoperative patients. *K. pneumoniae* isolates were cultured for 24 hours (h) at 37°C on Luria-Bertani (LB) agar, and Mueller Hinton Agar (MHA) (Oxoid/ England), then aseptically adjusted pH values to (5, 7, 9, 11) by adding a few drops of 1 M HCl or 1 M NaOH and measure it by pH meter (LKB/ Sweden) and pour into sterile plates.

Identification of *K. pneumoniae* Isolates

The isolates of *Klebsiella pneumoniae* were identified using the VITEK ® 2 compact system and validated using PCR assay by amplified *rpoB* gene (Housekeeping gene) in DNA thermal cycler (DLab/ China). The boiling method was used for DNA extraction from *K. pneumoniae* isolates [16]. The PCR reaction contains Green Master Mix 2x (12.5µl), nuclease-free water (5.5µl) (Promega, USA), template DNA (5µl), and housekeeping gene (*rpoB*) primers were used in this study [17]: (1µl) of forward primers (5'-GTTGGCGAAATGGCGGAAAAC-3'), (1µl) of reverse primers (5'-ACGTCCATGTAGTCAACCTGG-3'). Thermal cycling consists of a 5 min initial denaturation at 95°C, thirty cycles of (95°C (30 sec) for denaturation, 57°C (30 sec) for annealing, 72°C (45 sec) for extension), and a 72°C (5 min) for final extension.

Determination of the Impact of pH Values on *K. pneumoniae* Antibiotic Susceptibility

The susceptibility of three MDR *K. pneumoniae* isolates (two isolates from urine samples and one isolate from wound swab) to various antibiotics at different pH levels (5, 7, 9, and 11) were tested using Kirby Bauer's disk diffusion method according to the standards set by the Clinical and Laboratory Standards Institute (CLSI) guidelines (with three replicates for each isolate in the experiment) [15], [18]. Overnight culture of *K. pneumoniae* isolates on LB agar, then standardized bacterial suspension from a few colonies of overnight culture, and the correction was set to 0.5 McFarland (1.5×10⁸ CFU/ml). A portion of the bacteria suspension was transferred and evenly distributed over MHA plates with varying pH levels (5, 7, 9, 11) using sterile cotton swabs. Then, the most effective antibiotics discs on *K. pneumoniae* isolates were selected such as ciprofloxacin (5 µg), piperacillin (100 µg), tetracycline (30 µg), cefotaxime (30 µg), amoxicillin-clavulanic acid (20/10 µg) were placed on the inoculated plates and incubated at 37°C for 18-24 h. The diameters of the inhibition zones surrounding the antibiotic discs were measured using a metric ruler in millimeters.

Statistical Analysis

The study's data results were evaluated using Excel Professional Plus 2021 (Microsoft Software, Inc.) for each biological independent triplicate replicate. All statistical analyses have significant $p < 0.05$.

RESULTS AND DISCUSSION

Identification of *Klebsiella pneumoniae* Isolates

Clinical *K. pneumoniae* isolates were identified using colonies' appearance on culture media, microscopic examination, biochemical examinations, and the VITEK ® 2 compact system (BioMérieux/France).

Urine samples were the major source of our isolates (75%), followed by wound swabs (25%), as expressed in Figure 1. The genotypic identification of the isolates by PCR amplification was conducted using the housekeeping *rpoB* gene (599bp) for all isolates (Figure 2). The increased prevalence of *K. pneumoniae* in UTIs might be attributed to the prolonged usage of catheters [19].

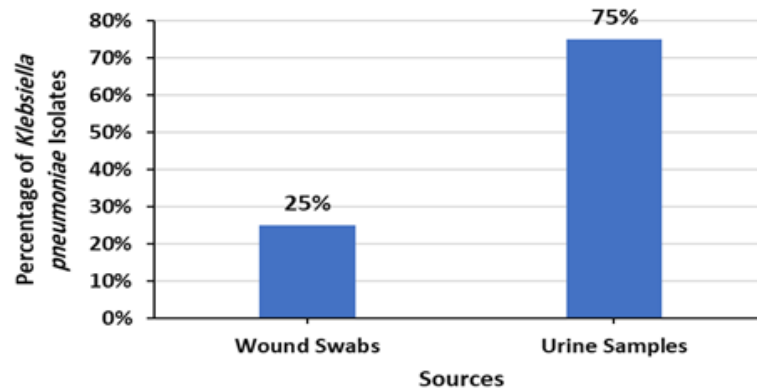


Figure 1. Distribution and percentages of infection sites of clinical *Klebsiella pneumoniae* isolates

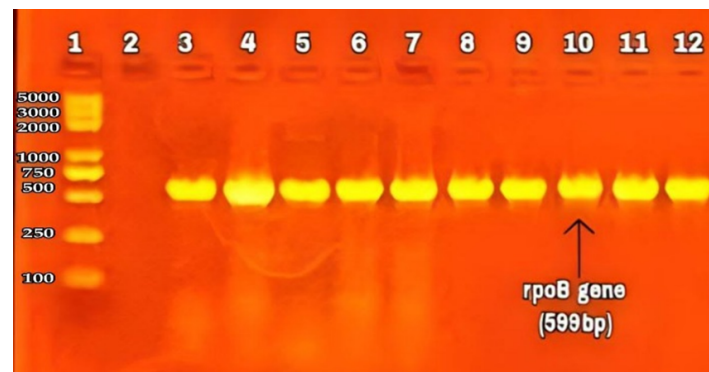


Figure 2. *Klebsiella pneumoniae* isolates were identified by amplification of the *rpoB* gene (Housekeeping gene), Lanes 1-12, as follows. A 100-5000 bp DNA ladder; a negative control and the *rpoB* gene (599 bp) in isolates' gDNA were numbered from 3 to 12

Antibiotic Susceptibility Test

The current research observed that *K. pneumoniae* isolates were resistant to all antimicrobial agents used in this study at neutral pH 7, while cefotaxime exhibited increased activity at alkaline pH levels (9, 11). Tetracycline demonstrated enhanced antibacterial efficacy in acidic pH, but piperacillin showed increased zone of inhibition ranges at pH (5, 9) but remained resistant. The pH stress (5, 9, 7, 11) did not affect the amoxicillin-clavulanic acid antibiotics. Ciprofloxacin displays resistant phenotypes at acidic pH 5 and resistant or intermediate phenotypes at alkaline pH 9 as shown in Figure 3 and Table 1.

The emergence and spread of MDR *K. pneumoniae* recently have attracted the scientific community's interest [20]. Several factors such as temperature, pH, nutrition availability, and salinity influence not only bacterial growth and survival but also their pathogenicity. For example, the pH regulates metabolisms of microbial, interactions, the redox processes thermodynamics, and virulence gene expression [12], [21].

The current study has demonstrated that MDR *K. pneumoniae* is more susceptible to tetracycline at an acidic pH because low external pH conditions that promoted proton influx induce antibiotic tolerance, whereas *K. pneumoniae* is more susceptible to cefotaxime antibiotics at an alkaline pH. After all, high external pH conditions decreased proton influx and caused an increase in antibiotic sensitivity of bacteria to some antibiotics [22].

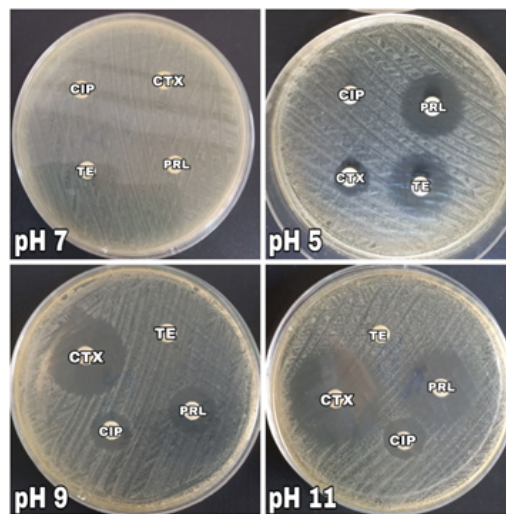


Figure 3. *Klebsiella pneumoniae* isolates antibiotic Susceptibility test at different pH (5, 7, 9, and 11) to cefotaxime (CTX 30 µg), ciprofloxacin (CIP 5 µg), tetracycline (TE 30 µg), and piperacillin (PRL 100 µg) antimicrobial agents

Table 1. Antibiotic susceptibility of *Klebsiella pneumoniae* for various antimicrobial agents at different pH levels utilizing Kirby Bauer’s disk diffusion method

Antimicrobial agent	Isolates code No. 1				Isolates code No. 2				Isolates code No. 3			
	pH7	pH5	pH9	pH11	pH7	pH5	pH9	pH11	pH7	pH5	pH9	pH11
Tetracycline (TE 30 g)	R=0	I=15 mm	R=0	R=0	R=0	R=0	R=0	R=0	R=0	S=16 mm	R=0	R=0
Cefotaxime (CTX 30 g)	R=0	R=11 mm	S=34 mm	S=36 mm	R=0	R=0	R=0	R=0	R=0	R=14 mm	S=32 mm	S=30 mm
Amoxicillin-clavulanic acid (AMC20/10 g)	R=0	R=0	R=0	R=0	N/A	N/A	N/A	N/A	R=0	R=0	R=0	R=0
Piperacillin (PRL100 g)	R=0	15 mm	20 mm	24 mm	R=0	9mm	R=0	2mm	R=0	R=0	R=0	R=0
Ciprofloxacin (CIP 5 µg)	R=0	R=0	R=7 mm	R=12 mm	R=0	R=0	I=15 mm	R=0	R=0	R=0	R=0	R=0

Each isolate has three repeats. S, I, and R denote resistance, intermediate, and susceptibility of bacteria to the antimicrobial agents, respectively, and N/A denotes not applicable. The diameter ranges of the inhibition zone, were measured in millimeters using a ruler. Based on the Clinical & Laboratory Standards Institute (CLSI) M100 32nd Edition 2022, the piperacillin had no Zone diameter values.

In another study, the result of the disc diffusion test has shown the greatest effects of tetracyclines against staphylococci and *Escherichia coli*, all exhibiting antibacterial intermediate or resistant phenotypes at alkaline pH 8, and sensitivity at acidic pH 5 [18]. These findings strongly suggest the possibility that the pH can affect the ability of some antibiotics to reach their target site in bacteria cells, as well as varying pH levels can significantly affect the efficacy of antibiotics and the antibiotic susceptibility of *K. pneumoniae* by modulating the activity of virulence mechanisms, including efflux pumps, quorum sensing, and biofilm formation. Therefore, it is important to take the pH changes into account when assessing how well antibiotics work against *K. pneumoniae* and its ability to resist treatment. In another study, ciprofloxacin demonstrated greater antimicrobial effects at pH levels of 7, 8, and 9, against *P. mirabilis* 33877, *K. pneumoniae* 33443, *K. pneumoniae* 33163, as well as *E. coli* 33504 strains using the minimal inhibitory concentration test [13]. Overall, studying how pH levels affect sensitivity might provide insights into how resistance develops in *K. pneumoniae*. If pH levels influence how bacteria react to antibiotics it could play a role in resistance development. It helps develop strategies to prevent or combat resistance. This exploration deepens our understanding of the relationship among bacteria, antibiotics, and environmental factors, for the management of infections caused by pathogens.

CONCLUSION

Environmental conditions, such as pH can greatly impact the sensitivity of *K. Pneumoniae* to antimicrobial agents such as cefotaxime, ciprofloxacin, tetracycline, and piperacillin. This influence is believed to be tied to alterations in the structure of the cell wall and membrane. The acidity levels of urine and skin may affect the antibiotic efficacy of certain antimicrobial agents against MDR-bacteria found in wounds and urinary tract infections. Recognizing the relationship between pH levels and antibiotic effectiveness can aid in choosing the antibiotics, for treating *K. Pneumoniae* infections.

SUPPLEMENTARY MATERIAL

None.

AUTHOR CONTRIBUTIONS

Writing—review, editing, and validation Fatima J. Hassan; Methodology and supervision, Mohammed F. Al-Marjani, and Intesar N. Khelkal; analysis, Amira A. Moawad.

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DATA AVAILABILITY STATEMENT

None.

ETHICAL APPROVAL

All experiments involving human samples and bacteria collection were ethically authorized in accordance with the Helsinki Declaration by all participants with biosafety requirements at the Biology department, College of Science/ Mustansiriyah University, and the ethics committees at the Ministry of Health in Baghdad, Iraq (Ethical approval ref.BCSMU/1221/0002M).

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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