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Original Research Article

Isolation, Identification and Antimicrobial Sensitivity of Klebsiella pneumoniae from Bovine Clinical Mastitis Cases

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Abstract: Klebsiella pneumoniae is a ubiquitous pathogen that affects a broad spectrum of hosts, including both animals and humans. It is one of the primary causative agents of clinical mastitis (CM) in dairy cows, contributing significantly to economic losses in the dairy industry, both in Iraq and globally. This study aimed to investigate the role of K. pneumoniae as a causative agent of clinical mastitis in Diwaniyah province, Iraq, and to assess the antibiotic susceptibility patterns of the isolates. A total of 30 milk samples were collected from various locations within Diwaniyah province between November 2024 and February 2025. The samples were transported in a cooled container to the microbiology laboratory at the College of Veterinary Medicine, University of Al-Qadisiyah, for bacteriological analysis. Based on colony morphology and biochemical characteristics, K. pneumoniae was identified in 7 out of 30 milk samples, representing a prevalence of 23.3%. The antimicrobial susceptibility of the isolates was assessed using disc diffusion testing, which revealed that all K. pneumoniae isolates were sensitive to Norfloxacin, Azithromycin, and Ciprofloxacin. However, the isolates exhibited complete resistance to Amoxicillin + Clavulanic acid, Cefixime, Ampicillin, and Cephalosporins. In conclusion, the high prevalence of K. pneumoniae in clinical mastitis cases and the significant antibiotic resistance observed suggest a potential public health risk, particularly through the food chain. These findings highlight the importance of monitoring K. pneumoniae and its resistance patterns to safeguard both animal and human health.

Keywords: *K. Pneumoniae*, Mastitis, Cows, Antibiotic Resistance.

1. INTRODUCTION

Klebsiella species are Gram-negative, facultatively anaerobic, capsulated, rod-shaped bacteria that belong to the *Enterobacteriaceae* family [1]. *Klebsiella pneumoniae*, in particular, is known to cause a variety of zoonotic infections, including those affecting the respiratory and urinary tracts, soft tissues, bloodstream, and mastitis in dairy cattle [2]. The high virulence and increasing antimicrobial resistance (AMR) of *K. pneumoniae* significantly limit the effectiveness of conventional antibiotic therapies, making it a major concern in clinical settings [3]. This pathogen is part of the ESKAPE group of bacteria, which represents a global threat due to the rapid emergence of multidrug-resistant (MDR) strains [4]. Mastitis, an inflammatory condition of the mammary glands, is a prevalent infectious disease in dairy cows, leading to significant economic losses in the dairy industry by reducing both milk yield and quality [5]. Beyond its economic implications, mastitis poses a risk to both human and animal health, as it can serve as a vector for the transmission of antimicrobial-resistant bacteria and cause foodborne illnesses [6]. The misuse and overuse of antibiotics have contributed to the global public health challenge of increasing MDR strains, with *K. pneumoniae* being a notable contributor to this growing issue. Numerous outbreaks of MDR *K. pneumoniae* have been reported, further highlighting its potential to spread resistance genes [7, 8].

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K. pneumoniae is known to harbor a range of mobile AMR genes, which it can transfer between environmental sources, humans, animals, and clinically significant bacteria (9). Studies have shown that the AMR profiles and virulence of *K. pneumoniae* can vary across different geographical regions [10]. Virulence factors, such as siderophore systems and capsular production, are closely linked to the hypervirulent phenotype of *K. pneumoniae*, which enhances its pathogenicity [11]. Furthermore, gene clusters encoding type 1 and type 3 fimbriae, iron acquisition systems, and the type VI secretion system (T6SS) have been strongly associated with the pathogenesis of *K. pneumoniae* in both humans and animals [12]. Among the various environmental niches from which *K. pneumoniae* can acquire AMR genes, animals, particularly livestock, play a significant role [13]. *Klebsiella* species of animal origin are crucial vectors for the dissemination of antimicrobial resistance genes, which can further complicate treatment strategies [14, 15]. Therefore, understanding the prevalence of *K. pneumoniae* in dairy cattle and its associated resistance patterns is critical for managing the spread of resistance and safeguarding both animal and public health [16].The aim of this study is to investigate *K. pneumoniae* as a causative agent of clinical mastitis in dairy cows and to assess the antimicrobial resistance profile of these isolates using in vitro methods.

2. MATERIAL AND METHODS

2.1 Samples Collection

Samples of current study collected during period November 2024 -June2025). A 30 milk samples from cows with clinical mastitis were collected from various region in Al-Diwaniyah city / Iraq .Samples of milk, about 10 mL in volume, were aseptically collected in sterile plastic tubes. After being submerged in an antiseptic solution, each cow teat was cleaned with 70% alcohol and dried with a separate paper towel. After the initial 2-3 squirts were removed to remove contaminating microorganisms from the teat canal, a single milk sample was taken. To isolate and identify *K.pneumoniea*, samples were collected aseptically, kept on ice, and transported to the microbiolgy lab in college veterinary medicine within 1-2 hours of collection.

3.2 Isolation *K Pneumonia*

One ml of collected samples (Milk) was pre-enriched in 9 ml of Nutrient broth incubated at 37°C for 24 hrs. Then the isolated *K. pneumonia* was isolated *by* a loopful from the incubated culture and streaked on MacConkey agar plates then incubated for 24 hr at 37ºC, suspected colonies (rounded, pink colonies) subculture on EMB agar subculture on blood base agar and CHROM agar Orientation at same incubation circumstances, identification of isolates based on the morphology of the colonies, coloration in addition to biochemical tests [17].

3.3. Bacterial Identification

3.3.1 Microscopic Examination and Biochemical Tests for Isolates

For the microscopic examination, one isolated colony was transferred to a microscope slide, fixed, and stained using Gram stain to observe cell shape and arrangement. The results were compared with the method outlined in reference [18]. To identify the bacterial species, several biochemical tests were performed, relying on the differences in biochemical activities among various bacteria. The Catalase test was conducted by transferring a small amount of bacterial growth to a clean glass slide and mixing it with 3% H_2O_2; the production of gas bubbles indicated a positive result [19]. For the Urease production test, uncontaminated colonies were streaked onto urea agar slants and incubated at 37°C for 24 hours. A color change in the media from yellow to pink indicated a positive result. The Indole test was performed by inoculating P.W. media with bacterial growth, followed by incubation at 37°C for 48 hours. After adding 5 drops of Kovac's reagent, the formation of a crimson circle at the broth's top indicated a positive result. The Voges-Proskauer test involved inoculating bacterial colonies in MR-VP broth tubes, incubating them at 37°C for 24 hours, and then adding 0.6 mL of alpha-naphthol (reagent A) and 0.2 mL of 40% KOH (reagent B). Pink color development after 15 minutes indicated a positive result, associated with partial hydrolysis of glucose to acetone or acetyl-methyl-carbinol. In the Methyl-Red test, small colonies were injected into MR-VP broth tubes and incubated at 37°C for 24-48 hours, followed by the addition of 5 drops of MR reagent. The appearance of a red color indicated a positive result. For the Citrate Utilization test, bacterial colonies were streaked onto a slant of citrate medium, incubated for 24 hours at 37°C, and a color change from green to blue indicated a positive result. Lastly, the Triple Sugar Iron (TSI) test involved inoculating TSI agar by stabbing through the center of the medium and streaking the surface. After incubating at 37°C for 18-24 hours, the results were observed to identify fermentation patterns [20].

3.4 Antibiotic Susceptibility Test

The antimicrobial susceptibility test was conducted following the guidelines of the European Committee on Antimicrobial Susceptibility Testing and the Clinical and Laboratory Standards Institute (84). Initially, 3 to 4 well-isolated colonies of similar morphological type were selected and suspended in 5 mL of sterile saline. The suspension was mixed thoroughly and adjusted to match the turbidity of the 0.5 McFarland standard. A sterile swab was then submerged in the bacterial suspension, transferred, and evenly spread onto Mueller-Hinton Agar (MHA) plates. After inoculation, the plates were left to sit for 10 minutes. Antimicrobial discs were then placed onto the surface of the inoculated MHA using sterile forceps, and the plates were incubated for 24 hours at 37°C. The isolates were classified as Sensitive (S), Intermediate (I), or Resistant (R) to each

antibiotic, based on the diameter of the inhibition zones surrounding the discs, which were measured and recorded using a ruler [21].

3. RESULTS

3.1. Prevalence Rate of K Pneumonia

The Results of bacteria isolated from 30 milk samples showed that 7 samples belonged to *K pneumoniae*, representing 23.3% of the milk samples, as shown in Table (3-1).

Table 3-1: Isolation rate of *K. pneumonia* from milk

Sample type	No sample	Positive sample	Percentage
Milk	30	7	23.3%

3.2. Morphology and Biochemical Characterization for *K. Pneumoniae*

The results of sample showed different morphological characteristics of bacteria on different media, after incubation at 37 °C for 24hours. On MacConkey agar colonies were appeared are pink(lactose fermenter) and appear large, round, and mucoid as shown in Figure 3-1.**A**.while on CHROM agar Orientation the colonies appeared as round, metallic blue. (Figure 3-1.B). Isolated bacteria were appeared under light microscopic lenses gram-negative rods. The Biochemical identification of *K pneumoniae* showed that bacteria were Gram –ve, Rod, Catalase positive, citrate utilization positive, voges-proskauer test Urease positive, Indole negative, TSI A/A gas,-H_2S & methyle red as shown in Table (3-2)

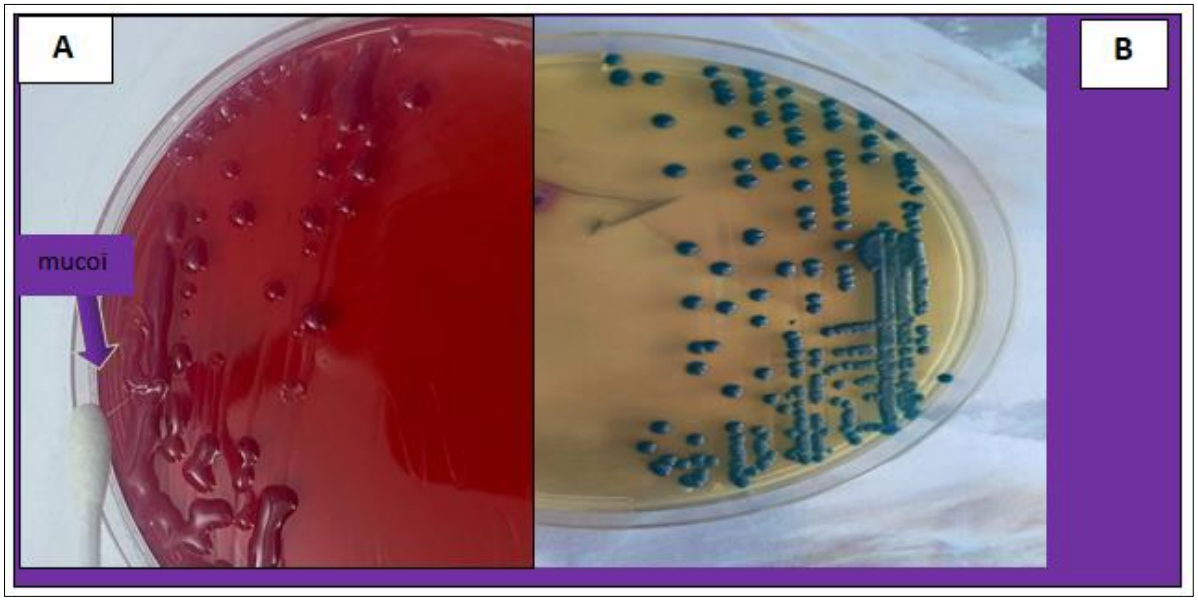

Figure 3-1: A. ***K. pneumoniae*** on MacConkey agar Pinke-mucid colonies
B. Chrom agar Orientation Metallic blue colonies

Table 3-2: Biochemical test for *K. pneumoniae* isolates

No.	Biochemical test	Resulte
1	**Catalase test**	+
2	**Urease test**	+
3	**Indole test**	-
4	**Citrate utilization test**	+
5	**TSI test**	Acid/acid/gas +/H_2S -
6	**Methyl red test**	-
7	**Voges-proskauer test**	+

3.3. Antibiotic Sensitivity Tests

The results of antibiotics susceptibility test showed that *K. pneumoniae* were resistant 100% to Tetracyclin, Amoxicillin + clavulanic acid, Cefixime, Ampicillin and Cephalosporin and susceptible 100% to Norfloxisin, Azithromycin and Ciprofloxacin, 71.4% to Chloramphenicol, While only 14.2% from isolates was sensiteve for Doxycyclin. As shown in Table (3-3), Figure (3-2).

Table 3-3: Antimicrobial susceptibility patterns of *K. pneumoniae* (*N*=7)

No.	Antibiotic /disc load (µg)	abbreviations	Antibacterial susceptibility		
			S	I	R
1	Norfloxisin	NOR	7/7(100%)	-	-
2	Amikacin	AK	2/7(28.5%)	-	5/7(71.4%)
3	Azithromycin	AZM	7/7(100%)	-	-
4	Ciprofloxacin	CIP	7/7(100%)	-	-
5	Doxycyclin	DO	1/7(14.2%)	6/7(85.7%)	
6	Tetracyclin	TE	-	-	7/7(100%)
7	Amoxicillin + clavulanic acid	AMC	-	-	7/7(100%)
8	Cefixime	CFM	-	-	7/7(100%)
9	Ampicillin	AM	-	-	7/7(100%)
10	Cephalosporin	CTX	-	-	7/7(100%)

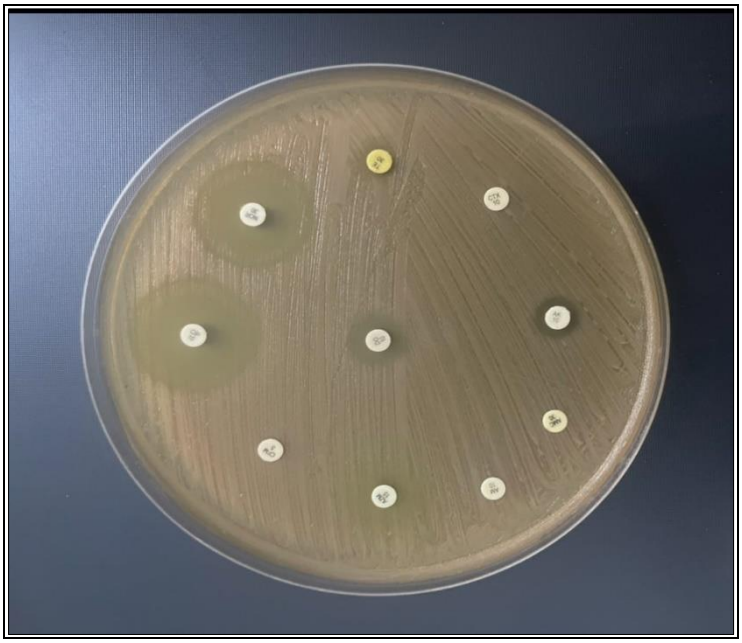

Figure 3-2: Antibiotic sensitivity test by Kirby-Bauer disc diffusion method

4. DISCUSSION

4.1. Colonies Characteristic

K. *Pneumoniae* is considered one of the most important opportunistic pathogen in animals causing mainly respiratory affection and mastitis. In current study, the samples were examined for presence of *k pneumonia* isolates using bacteriological methods, the obtained isolation rate are nearly similar to that recorded by [22], who examined samples from wild animals. In addition [23], detected the same cultural characters of klebsiella, recorded that the isolated bacteria were cultured on blood agar and MacConkey agar for 24 h, and grew well. Large, regular, round, smooth, raised, moist mucoid. There was no hemolysis on the blood agar and the colonies were smooth, moist, ivory and raised round colonies, while large, pink, regular, mucoid, round, smooth, and raised colonies grew on MacConkey agar. All isolates was positive for catalase, urease, vogus proscaur and citrate utilization and negative for indole test this result agreement with [24].

4.2 K. Pneumonia Prevalence Rate

In the present study the prevalence rate of *K. pneumonia* 7/30 (23.3%) this result agreement with result recorded by (25) who founded *K. pneumoniae* at rate (20.16%). while in the current study was 23.3%, while the percentage of K pneumonia isolation was less than recorded by (26) who recorded 27% and (27) isolated K. pneumonia with an incidence (54.5%) (90) who isolated *K .pneumoniae* (85.7%). On the other hand, both (91,92& 93 recorded in their studies a lower isolation rate than the current study, where the isolation rate was (11.9%,16.3% &18.7%). respectively. *Klebsiella* is usually referred to as particularly aggressive and is prone to cause severe clinical mastitis, which responds poorly to treatment and as a consequence, infections tend to be severe and long lasting with a fatal outcome [28].

4.3. Antibiotic sensitivity of *K Pneumoniae*

Bovine mastitis is a serious concern in the dairy industry worldwide and invariably requires treatment with antimicrobials [29]. Monitoring antimicrobial resistance in bacteria causing mastitis holds significant

clinical and public health importance, as antimicrobial therapy is commonly employed for the prevention and control of mastitis. Unfortunately, despite the use of optimal antimicrobial treatments, failures in achieving bacteriological cures are frequent, and improperly processed milk contaminated with drug-resistant pathogens can serve as a carrier posing a risk to human health [30]. Antibiotic sensitivity test helps to understand the resistance and susceptibility of bacteria towards a particular drug and thus helping in the choice of drug to be used for treatment. Overuse and misuse of antimicrobial agent led to an increased antimicrobial resistance around the world leading to treatment failures in infectious diseases of human and animal. Thus, 10 antibiotics were chosen for the study according to their common use in research in veterinary practice to investigate the susceptibility of *K pneumonia* isolates. In the present study all isolates had resistance 100 % for tetracycline this result disagreement with [97], who recorded in your study 72% from isolates had high sensitivity to tetracyclin while in another study recorded in brazillin by [31], 22.5% was resistance for tetacyclin (32) founded 19% from isolates resistance for tetracycline, while a study conducted in Libya (100) Found a high sensitivity rate 66.6% for tetracycline. this mean current study recorded higher resistance rate for tetracycline &Current study reported all isolates was resistance for Amoxicillin + clavulanic acid while [33], reported in his study only 7.1% resistant for same antibiotics (34) recorded 66.6% sensitive for Amoxicillin + clavulanic acid. A 71.4% from isolates sensitive for Amikacin and 100% resistance for ampicillin results nearly from the results recorded by [33-35]. All isolates was sensitive for ciprofoxicin this result agreement with study reported in Libya by [36]. The widespread use of tetracycline in the veterinary sector and agriculture for its broad spectrum of activity has increased the bacterial community's tolerance to this compound [37, 38].

5. CONCLUSIONS

The findings of this study indicate that Klebsiella pneumoniae is one of the most common causative agents of clinical mastitis and a significant foodborne pathogen that can be transmitted to humans through the consumption of raw milk. In terms of antimicrobial susceptibility, all strains of K. pneumoniae in the current study exhibited complete sensitivity (100%) to Norfloxacin, Azithromycin, and Ciprofloxacin. However, the investigation also revealed that the majority of K. pneumoniae isolates were completely resistant (100%) to Tetracycline, Amoxicillin + Clavulanic acid, Cefixime, and Ampicillin within the study area.

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