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Clinical and Molecular Study of Upper Respiratory Tract Infections Caused by *Klebsiella Pneumoniae* in Cats

M. J. Jasem [1], A. M. Radhy [2]
[1,2] Department of Veterinary Internal and Preventive Medicine, College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq

ABSTRACT

The study was included clinical and molecular study of *Klebsiella pneumoniae* from upper respiratory tract of cats in 150 (with and without) respiratory signs of (60 local breed and 90 different breed) and both sexes (male70 and female 80) with different ages including more than one year (85) and less than one year (65) attended in veterinary clinics and Baghdad veterinary hospital in Baghdad city at the period from October 2023 to September 2024. The 150 nasal swabs were cultured on macConkey and *Klebsiella* chrom agar, then the bacteria were examined by Gram stain with different biochemical and antimicrobial study using Vitek-2 analysis. In addition, the molecular detection of *16S r RNA* gene of *K. pneumoniae*. The results of clinical study showed increasing with signs of fever (76.2%) and sneezing (61.9%) for *K. pneumoniae*. The total isolation rate was 21 (14%). The DNA of bacteria was extracted and the positive products (21) of *K. pneumoniae* was successfully amplified by using specific primers at 260 bp of *16S r RNA* gene of *K. pneumoniae*. The isolates of *K. pneumoniae* were highly resistant to Ampicillin (71.4%) and Piperacillin (66.6%) while were sensitive (100%) to Ceftolozan. The infection rate of bacteria was significantly increased at age group less than one year and females. A significant increase was observed in Himalaya than other breeds. The highest percentage of infections by *K. pneumonia* appeared in December (76.9%) . The results of sequencing of the (5) isolates of *K. pneumoniae* showed homology with strains in gene bank. The results of phylogenetic study revealed that the local isolates of *K. pneumoniae* of variable origins, these confirm their importance as primary pathogens of many diseases due to their widespread in the environment and their ability to infect humans and different animals.

Keywords: Feline, PCR, phylogenetic, , respiratory infection,Iraq

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INTRODUCTION

Cats are among the most prevalent domestic pets globally, and their capacity for transmitting infectious diseases is widely recognized. The majority of contemporary literature concentrates on skin and soft tissue infections associated with bite and scratch wounds. Moreover, acknowledgement should also be afforded to respiratory diseases contracted from dogs. The respiratory tract infections in cats are highly contagious and easily speared, making the prevention the first and the most important step in avoiding the risks of further complications associated with the inflection (Weese *et al.,* 2015). Respiratory diseases frequently occur in felines, particularly in densely populated environments such as shelters, breeding catteries, and wild cat colonies. Various viruses, bacteria, fungus, and protozoa induce these diseases, adversely affecting feline health. Although immunizations have significantly diminished the occurrence of severe respiratory diseases in felines, they have not eradicated the highly infectious microorganisms responsible for these conditions (Greene, 2012). Symptoms of

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upper respiratory tract infections encompass clear or discolored discharge from the eyes or nose, coughing, sneezing, edema of the mucous membranes surrounding the eyes (conjunctivitis), oral ulcers, lethargy, and anorexia. In infrequent instances, felines may experience respiratory difficulties (Lappin *et al.*, 2017). Different options are available for diagnosis of respiratory tract infections firstly by the isolation of the causative bacteria by culturing on selective media and biochemical testes, polymerase chain reaction (PCR) and Enzyme-Linked Immunosorbent Assay (ELISA) as well as the using of X-rays, Computerized Tomography (CT) scan and Magnetic resonance imaging (MRI) (Greene, 2012). Respiratory tract infections in cats are complex conditions and caused by different types of viruses and bacteria, and typically initiated by stress-induced immunosuppression within infected pets (Dorn *et al.*, 2017). In a study performed in Egypt by Khalifa *et al.* (2021) the most isolated bacterial species from respiratory infections in cats was *Enterobacter cloacae* followed by *Escherichia coli, Klebsiella oxytoca, Klebsiella pneumonia, Citrobacter braakii and Serratia marcescens. K. pneumoniae* are gram-negative, aerobic, and anaerobic developing bacteria from the enterobacteriacea family (Lee *et al.*, 2021). *Klebsiella pneumoniae* is implicated in numerous diseases in humans and animals (Das *et al.*, 2019), including urinary tract, respiratory tract, bloodstream infections, and wound (Aslam *et al.*, 2023). *Klebsiella pneumoniae* also causes meningitis with symptoms including sharp head pain, nausea, dizziness, and impaired memory (Al-samrraee, 2017). It is also present in various environmental sources, including soil, water, and vegetation. (Ibrahim *et al.*, 2019).

MATERIALS AND METHODS

Bacteriological examination: The nasal swabs were streaked on MacConkey agar and HiCrome *Klebsiella* Selective agar base (Hamad *et al.*, 2022), then incubated aerobically at 37°C for 24 hours. The expanding colonies underwent ocular inspection to assess their dimensions, morphology, and pigmentation. The suspected colonies were analysed using Gram staining and biochemical assays with the Vitek 2 compact system (Sadeq and Lafta, 2024).

Antimicrobial susceptibility test:
Antimicrobial susceptibility test of *K. pneumoniae* was performed using the vitek system accuracy and fully automated system. The antibiotics used are Piperacillin/tazobactam, Amikacin, Cefazolin, Ceftazidime, Cefepime, Imipenem, Gentamicin, Ciprofloxacin, Levofloxacin, and Tigecycline (Majeed and Al-Aubydi , 2019 ; Mustafa and Abdullah , 2020).

PCR assay for detection of *fim* gene

DNA extraction: The genomic DNA was extracted according to gDNA bacteria extraction kit.

Primers: The identity of *K. pneumoniae* isolates was confirmed using specific primers for the *16S rRNA gene*: F-5'-GCAAGTCGAGCGGTAGCACAG-3' and R-5'-CAGTGTGGCTG GTCATCCTCT C-3' The product size was 260 pb, as designed by (Tayebeh *et al.*, 2016). The purity and concentration of extracted DNA were measured using a Nanodrop spectrophotometer, with results displayed on a digital screen.

Gel electrophoresis for check extracted DNA: The final PCR products were assessed using 1% gel electrophoresis (agarose gel), loading dye and UV imaging (Voytas , 2000).

Termo-cycler program: The process of DNA amplification was executed using a thermal cycler, beginning with an initial denaturation step at 94°C for 5 minutes. This was followed by 35 cycles consisting of denaturation at 94°C for 40 seconds, annealing at 61°C for 40 seconds, and extension at 72°C for 40 seconds. The final extension occurred at 72°C for a duration of 10 minutes.

Sequencing: The PCR results from five samples of *K. pneumoniae* were dispatched to Macrogen-Korea for sequencing. The sequences were analysed via the UPGMA algorithm (Sneath and sokal , 1973). The phylogenetic tree was constructed based on evolutionary distances calculated using the Maximum Composite Likelihood approach (Tamura and Kumar, 2004), employing substitutions per site as units for sequencing polymorphism. Evolutionary studies were

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performed using MEGA6 (Tamura *et al.*, 2013).

Statistical analysis: The Statistical Analysis System was conducted using the SPSS program (2020), employing odds ratios and risk factors to analyze and estimate significant differences in all study data (IBM Crop, 2011). The statistical test to estimate significant differences between clinical signs variables was used Chi-square at level ($P \leq 0.05$), while for assess the significant differences between other variables used Odds ratio at confidence interval (CI) 95%.

Ethical approved: This experiment was conducted in compliance with institutional rules of the University of Baghdad´s College of Veterinary Medicine´s, Animal care and use committee Iraq project number: pg. 2237. The herd s´ owners gave their verbal agreement before samples were taken.

RESULTS AND DISCUSSION

Clinical study of cats: The results of clinical signs were showed increasing with fever (76.2%) and sneezing (61.9%), followed by coughing (26.6%) and other clinical signs as show in (Table 1), *K. pneumonia*e has been documented to colonies companion animals and induce extra intestinal illnesses, including upper respiratory tract infections (Martin *et al.*, 2016). The infection in cats are characterized by serous to mucopurulent nasal discharge, sneezing , epistaxis and conjunctivitis (Lappin *et al.*, 2017) as well as stertorous breathing , coughing, lethargy and respiratory distress (Aslam *et al.*, 2023).

Table 1. The clinical signs shown in examined cats

Clinical Sign	*K. pneumoniae* N= 21 (%)	Chi-square Value	p-value
Fever	16 (76.2%)	0.0	1.0
Sneezing	13 (61.9%)*	5.79	0.0161
Coughing	7 (33.3%)	3.88	0.049
Mucopurulent nasal discharge	12 (57.1%)	0.05	0.8232
Mandibular lymphadenomegaly	0 (0.0%)	13.72	0.0002
Mucopurulent ocular discharge	10 (47.6%)*	3.32	0.049
Gurgling respiratory sound	0 (0.0%)	9.72	0.0018
Dyspnea	7 (33.3%)	1.59	0.2077
Cyanosis of mucous membranes	0 (0.0%)	2.87	0.0901

Chi-square test was used to analyze the difference in the frequency of each clinical sign between the two infections. A p-value less than 0.05 was considered statistically significant.

Isolation and identification: Out of 21 nasal swabs were positive to *K. pneumoniae*, the colonies of isolates appeared on MacConkey agar appear as large ,smooth colonies with a moist, mucoid texture (Figure 1), while positive culture on and HiCrome *Klebsiella* Selective agar base appear as purple color (Figure 2). These results are agreed with (Safika *et al.*, 2022) who detected the isolates of *K. pneumonia* depending on bacteriology culture, Gram staining and biochemical analysis. The current results agreed with what was noticed by (Quinn *et al.*, 2011). Safika et al., (2022) conducted a study to examine the occurrence of multidrug-resistant *K. pneumoniae* in sputum and laryngeal swabs from cats hospitalized at a veterinary clinic in Bogor, Indonesia, where the bacteria were isolated and identified through macroscopic, microscopic, and biochemical methods, these results were disagreed with (Ramadhan *et al.*, 2021) in Indonesia who reported a high isolation rate (84.48%) of *K. pneumoniae* from sputum and laryngeal swabs of cats that were hospitalized at the veterinary clinic. The current findings in contrary to those by other researchers in China (Zhang *et al.*, 2022) who recorded a low isolation rate 4.6% and 5.4% respectively of *K. Pneumoniae* from nasal swab of cats.

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Figure 1. Positive culture of *K. pneumonia* on MacConkey agar appear as large ,smooth colonies with a moist and mucoid texture

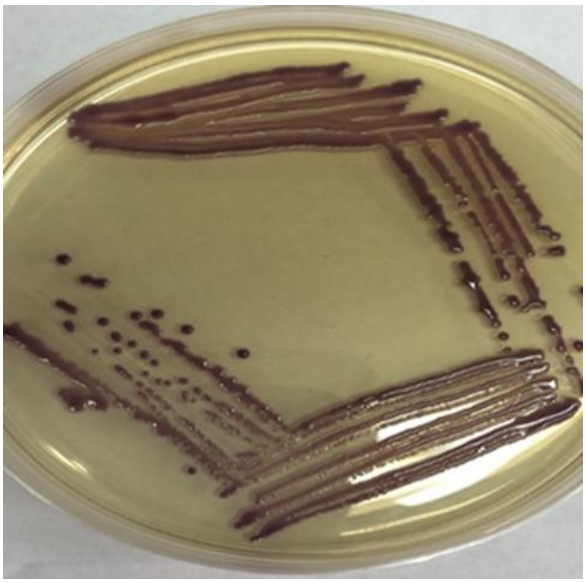

Figure 2. Positive culture of *K. pneumonia* on HiCrome agar with purple color

Antimicrobial susceptibility testing:

The present findings revealed that the isolates of *K. Pneumoniae* were resistant (100%) to Ampicillin and Piperacillin, while the isolate were highly susceptible to Ceftolozane (100%) and Cefotaxime (87%). This results show in (Table 2). Recently *K. pneumoniae* has swiftly evolved into a multidrug-resistant (MDR) organism (Parisi *et al* ., 2015). The present findings revealed that the isolates of *K. pneumoniae* were resistant (100%) to Ampicillin and Piperacillin, while the isolate were highly susceptible to Ceftolozane (100%) and Cefotaxime (87%). The current results demonstrated that the isolates of *K. pneumoniae* were resistant to more than ten types of antibiotics and it is considered multidrug resistant (MDR) bacteria. Numerous instances of multidrug-resistant *K. pneumoniae* have been identified in various regions globally, including Tunisia (Messaoudi *et al.*, 2009), South Africa (Fielding *et al.*, 2012) and China (Guo et al., 2016). The *K. pneumoniae* isolates have genes for extended-spectrum beta-lactamases (ESBL). Mutations in the gene encoding Penicillin Binding Protein (PBP) that result in beta-lactam resistance (Lee et al., 2021). Ramadhan et al., (2021) in Indonesia noticed the highest level of resistance of *K. pneumoniae* isolated from sputum and laryngeal of cats were to Ampicillin (76%) followed by Oxytetracycline (72%) and Tetracycline (68%), Enrofloxcin (52%) and Gentamicin (44%).At the same line (Zhang *et al.*, 2022) in China found the isolates of *K. pneumoniae* were extremely resistant to Amoxicillin – Clavulanate (89.9%) and Trimethoprim – Sulfamethoxazole (77.1%). In study performed in south Kores, (Hong *et al.*, 2019) stated the *K. pneumoniae* obtained from pets (dogs and cats) possesses a resistance gene to the Cephalosporin class of medicines.

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Table 2. The proportion of K. pneumoniae (sensitive and resistant)

Antibiotic	Isolation	Sensitive	Intermediate	Resistant
Ampicillin/sulbactam	21	3(14.2%)	3(14.2%)	15 (71.4%)*
Piperacillin/ sulbactam	21	7(33.3%)	0.(0%)	14 (66.6%)
Cefotaxime	21	18 (85.7%)	0.(0%)	3(14.2%)
Ceftazidime	21	15 (71.4%)*	0.(0%)	6(28.5%)
Ceftazidime/avibactam	21	18 (85.7%)*	0.(0%)	3(14.2%)
Ceftolozana/ tazobactam	21	21(100%)*	0.(0%)	0.(0%)
Cefepime	21	14(66.6)*	0.(0%)	7(33.3%)
Imipenem	21	4(19%)	13(61.9%)*	4(19%)
Meropenem	21	15 (71.4%)*	0.(0%)	6(28.5%)
Gentamicin	21	14(66.6)*	0.(0%)	7(33.3%)
Ciprofloxacin	21	15(71.4)*	6(28.5%)	0.(0%)
Tigecycline	21	0(0%)	0.(0%)	0.(0%)
Colistin	21	0(0%)	13(61.9%)*	8(38%)
Trimethoprim/ sulfamethoxazole	21	14 (66.6%)*	0.(0%)	7(33.3%)
		Chi: 4.66; df:1 (P: 0.03) significant	Chi: 4.709; df:1 (P: 0.03) significant	Chi: 4.7; df:1 (P: 0.033) significant
		OR: 4 (CI:1.10-14.4) significant	OR: 4.063 (CI:1.15-14.8) significant	OR: 4.06 (CI:1.11-14.8) significant

Molecular detection of *K. pneumoniae* by using *16s rRNA* gene: The overall isolation rate for *K. Pneumoniae* was documented as 14% (21/150) using conventional PCR method, (Figure 3). The present study revealed that the total isolation rate of *K. Pneumoniae* from nasal swabs of cats was 14% using Vitek-2 system and conventional polymerase chain reaction. The present results revealed to a successful amplification of (*16S rRNA*) gene of *K. pneumoniae* by PCR at 260bp. These results in accordance with (Zhang *et al.*, 2022) in China who detected the isolates of *K. pneumoniae* from nasal swabs and various types of samples of cats by the same gene. In Bazile, (Hayakawa Ito de Sousa *et al.*, 2021) confirmed (*16S r RNA*) gene of *K. pneumoniae* in a case of cats with urinary tract infection. (Aslam *et al.*, 2023) in Malaysia confirmed the presence of (*16S r RNA*) of *K. pneumoniae* by PCR in (34) feline cases with acute and chronic respiratory signs.

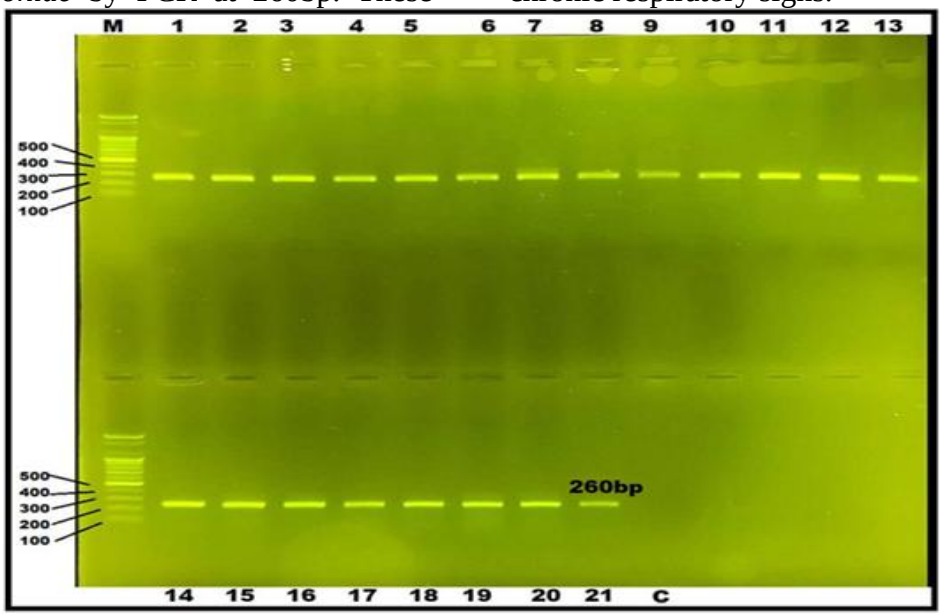

Figure 3. Gel electrophoresis (1.2%) 80 mAm, 100 vol. with red safe stain show the result of amplification of 260 bp of 16s rRNA gene of *K. pneumonia*

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Sequence of *fim* gene: The findings of the current investigation indicated that the genetic sequences of local isolates of *K. pneumoniae* from nasal swabs of cats originated from several countries, including India , China, South Africa, Pakistan, United Kingdom, Thailand, Uzbekistan, USA, Sweden, Spain, Germany, Brazil, Taiwan, Switzerland with a similarity of 99%, (Figure 4).

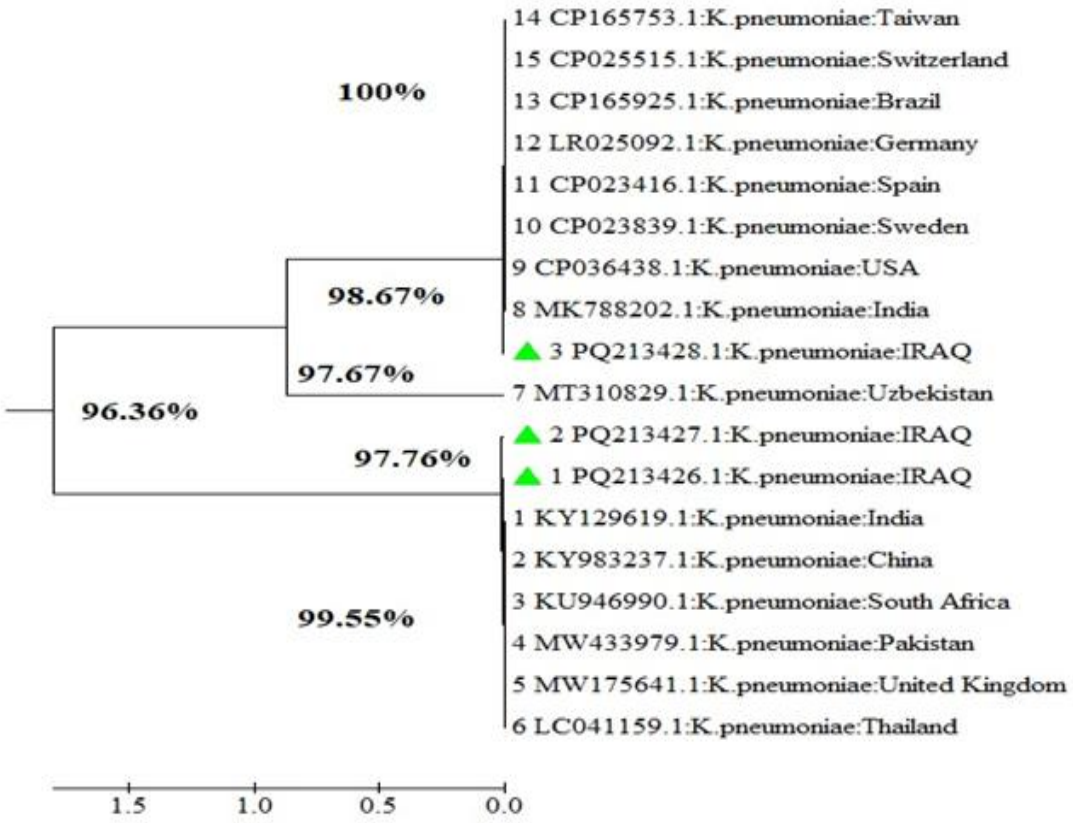

Figure 4. Phylogenetic tree of *K. pneumonia*

Prevalence of *K. pneumonia* according to age, sex, breeds and months: Age: The present findings showed that the infection rate of isolation of *K. pneumoniae* was highly reported at age group less than with one year (16.9%) as compared age group more than one year 11.7%; (Table 3). The present study demonstrated the distribution of infection with *K. Pneumoniae* in both age groups of the study, Although it was higher in age group less than one year (16.9%) which may be due to low immunity, severity and virulence factors of bacteria, overcrowding with bad management (Greene , 2012). *K. pneumoniae* is an opportunistic infection that colonies the skin, upper respiratory tract, and digestive system (Holt *et al.*, 2015). Additionally, it is a principal cause of diseases in neonates, the elderly, and immunocompromised humans and animals (Wyres *et al.*, 2020).

Table 3. Prevalence of *K. pneumonia* according to age of infected cat

Age/year	No. of tested cats	No. of positive cats for *K. pneumoniae* isolates	percentage
≤ 1	65	11	16.9%
> 1	85	10	11.7%
Total	150	21	OR(CI%) 1.5 (0.67-3.33)

Non-significant

Sex: The infection rate of isolation of K. pneumoniae was increased significantly in female cats (20%) as compared with male (7.1%), (Table 4). The infection rate of *K. pneumoniae* showed a significant variation according to the sex , a high infection rate were recorded in females (20%), these results

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were corroborated by (Zhang *et al.*, 2022) in China, who discovered that the isolation rate of *K. pneumoniae* was substantially greater in females. These findings may be due to the physiology of the females during pregnancy and location period as well as the hormonal and immunological effects.

Table 4. Prevalence of *K. pneumonia* according to sex in infected cats

Sex	No. of tested cats	No. *K. pneumoniae* isolates	percentage
Males	70	5	7.1%
Females	80	16	20 %*
Total	150	21	OR(CI%) 3.32 (1.33-8.26)

***Significant**

Breeds: In our study, we focused in five breeds in Baghdad city, local (stray), Himalya , Shirazi, Scottish and Chinchilla. The isolation rate of *K. pneumoniae* were increased in stray cats 9 (15%) compared with another breeds and there are revealed non-significant variation among the different breeds of cats as shown in (Table 5). The infection rate of *K. pneumoniae* in present study were increased in local (stray) cats as compared with the other breeds of cats found with respiratory signs, particularly the stray cats often have high prevalence rates for most infectious agents that are associated with direct contact with other cats as well as they don't subject to vaccination programs against any pathogens. There is no a viable study in the world regarding with the isolation rates of *K. pneumoniae* in relation to cat breeds.

Table 5. Prevalence of *K. pneumonia* according to breeds

Breeds of cats	No. of tested cats	No. of cats positive for *K. pneumoniae*
Local (stray)	60	9(15%)
Himalya	26	4(15.3%)
Shirazi	24	3(12.5%)
Scottish	20	3(15%)
Chinchilla	20	2(10%)
Total	150	21
		OR(CI%) 1.58 (0.67-3.72) Non-significant

Months: The isolation rate of *K. preumoniae* was increased in December (76.9%) and November; While the negative results were recorded in other months of the study as show in (Table 6). The climatic effect on the rate of infection with *K. pneumoniae* was obviously observed, a significant high infection rates were recorded in cold months (December, November, October and January) respectively, this reveals that *K. pneumoniae* is stable and has ability for remaining long period in the environment. More over the reproduction of cats increased actively at cold environment, so that high number of susceptible new born kittens found at this period which leads to increase the rate of infection with bacteria (Greene, 2012). *K. pneumoniae* is an increasingly significant bacterial pathogen capable of inducing severe organ dysfunction and life-threatening diseases.

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Table 6. Prevalence of *K. pneumonia* according to months

Month	No. of tested cats	No. of positive for *K. pneumoniae*
January	13	3(23%)*
February	13	1(7.6%)
March	13	0(0%)
April	12	0(0%)
May	12	0(0%)
June	12	0(0%)
July	12	0(0%)
August	12	0(0%)
September	12	0(0%)
October	13	2(15.3%)
November	13	5(38.4%)*
December	13	10(76.9%)*
Total	150	21
		OR(CI%)
		3.43 (1.45-8.11)
		*Significant

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دراسة سريرية وجزيئية لالتهابات الجهاز التنفسي العلوي التي تسببها الكليبسيلا الرئوية في القطط

مروة جمال جاسم ، اماني محمد راضي
فرع الطب الباطني والوقائي/ كلية الطب البيطري/ جامعة بغداد

المستخلص

تضمنت الدراسة التشخيص السريري والجزيئي لبكتريا الكلبسيلا الرئوية من الجهاز التنفسي العلوي للقطط في 150 (مع وبدون علامات تنفسيه) ولسلالات محليه (60) وسلالات مختلفة (90) ولكلا الجنسين (70 ذكر و 80 انثى) وبإعمار مختلفة متضمنه اكثر من سنه (85) واقل من سنه (65) من العيادات البيطرية والمستشفى البيطري في مدينة بغداد للفترة من تشرين الأول 2023 ولغاية أيلول 2024. تمت زراعة (150) مسحه انفية على وسط المكونكي والكروم الخاص ببكتريا الكلبسيلا الرئوية ثم فحصت البكتريا بصبغة جرام والاختبارات الكيموحيوية المختلفة واختبار الحساسية بوساطة تحليل الفايتك للبكتريا المعزولة. بالإضافة الى التشخيص الجزيئي لجين **(16SrRNA)** العام لبكتريا الكلبسيلا الرئوية .أظهرت نتائج الدراسة السريرية زيادة باعراض الحمى (76.2%) والعطاس(61.9%) لبكتريا الكلبسيلا الرئوية. كانت معدلات العزل الكلية لبكتريا الكلبسيلا الرئوية 21(14%). تم استخلاص الحامض النووي والنواتج الموجبة (21) للكبسيلا الرئوية تم تضخيمها بنجاح باستخدام البريمرات الخاصة وبحجم **(260bp)** لجين **(16SrRNA)** لبكتريا الكلبسيلا الرئوية. كانت العزلات الكلبسيلا الرئوية عالية المقاومة للامبسيلين (71.4%) و ببيراسلين (66.6%) بينما كانت حساسة (100%) للسفتولوزان. ازدادت معنويا معدلات الإصابة للبكتريا في المجاميع العمرية اقل من سنة والاناث. وقد لوحظ زيادة معنوية لمعدل الإصابة في سلالة الهملايا من السلالات الاخرى. ظهرت اعلى نسبة إصابة ببكتريا الكلبسيلا الرئوية في شهر كانون الأول (76.9%). أظهرت نتائج التسلسل القواعد النيتروجينية للعزلات الخمسة لبكتريا الكلبسيلا الرئوية تماثلا مع العزلات الموجودة في البنوك الجنية العالمية. اما نتائج دراسة الشجرة الجينية اشارت ان العزلات المحلية هي من أصول مختلفة مما يؤكد على أهميتها كمسببات مرضية أولية للعديد من الامراض بسبب انتشارها الواسع في البيئة ولها القدرة على إصابة الانسان والحيوان.

الكلمات المفتاحية: القطط، تفاعل البوليميراز المتسلسل، الشجرة الجينية ، إصابة تنفسية ، العراق