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BLOOD PICTURE AND DNA DAMAGE CAUSED BY *KLEBSIELLA PNEUMONIA* IN ALBINO MALE RABBITS

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ABSTRACT

Klebsiella is an encapsulated bacillus, a member of the family Enterobacteriaceae. Klebsiella can cause various illnesses, including pneumonia, bloodstream infections, urinary tract infections, and meningitis. Klebsiella has emerged as a nosocomial pathogen, particularly in premature infants and intensive care units (ICUs). This experiment aimed to investigate the toxic effects of *K. pneumoniae* on blood pictures and DNA structure. Thirty (30) male rabbits were divided into three groups. 1st group (GI) was given 1 milliliter /animal an oral dose of phosphate buffer saline (PBS) by a stomach tube as a control group for 60 days. The 2nd group (GII) was given one dose a week of 1 milliliter containing viable *K. pneumoniae* (1 X 10^6 CFU/ml) orally by stomach tube for 60 days. The 3rd group (GIII) was given the same dose twice a week. After 60 days, blood samples were collected from the heart and divided into 2 parts: 1st part for hematological analysis and 2nd part for detection of DNA damage using comet assay. The results of the hematological analysis showed significantly decreased RBCs, Hb and PCV, and considerably increased WBCs, lymphocytes, monocytes and neutrophils in the 2nd and 3rd groups, compared with the control group. In contrast, the comet assay showed DNA damage in the WBC of the different groups. The mean ± SE of tail length, tail intensity, tail moment and % DNA in the tail showed significant increases in the 2nd and 3rd groups, compared with the control group.

Keywords: Klebsiella pneumonia, rabbits, blood picture, DNA comet assay.

INTRODUCTION

The genus *Klebsiella* belongs to the tribe Klebsiellae, a member of the family Enterobacteriaceae. The organisms are named after Edwin Klebs, a 19th-century German microbiologist (1834–1913).

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Klebsiella pneumoniae was first described as an encapsulated bacillus by Carl Friedländer in 1882. He isolated it from the lungs of patients with pneumonia, particularly those who were immunocompromised, such as individuals suffering from chronic diseases or alcoholism, and named it Friedländer's bacillus (Calfee, 2017).

Klebsiella has emerged as a significant nosocomial pathogen in neonatal units. Nosocomial Klebsiella infections are also remarkably troublesome, particularly in premature infants and intensive care units

(ICUs). Pediatric patients are easily colonized by Klebsiella spp., with intestinal and oropharyngeal colonization serving as the main reservoirs for nosocomial outbreaks. *K. pneumoniae* has been reported as a prominent cause of infections in individuals with indwelling urinary catheters (Haryani *et al.,* 2007; Gorrie *et al.,* 2017).

Vegetables may be a source of contamination with *K. pneumoniae*. Raw vegetables are often eaten in salads and other meals. *Klebsiella pneumoniae* is usually present in the oral cavity, skin, and intestine, and is also prevalent in healthcare environments and medical equipment (Abu-Zaid *et al.,* 2016).

K. pneumoniae which causes nosocomial and community-acquired infections, normally resides in the human intestines, where it is not contagious. However, if it enters other parts of the body, Klebsiella can cause various illnesses, including pneumonia, bloodstream infections, urinary tract infections, and meningitis in the nervous system (Li *et al.,* 2019).

MATERIALS AND METHODS

Ethical approval

Ethical approval was granted through the local committee of animal care and use at the College of Veterinary Medicine within the University of Baghdad (number 2539 on 15/ 11/ 2023) before starting this study.

Isolation of *Klebsiella pneumoniae* from human samples*:*

Isolation from urine, blood and stool specimens.

1- Urine: 5-10ml, urine samples were collected in sterile tubes, and transported to the laboratory, Department of Medicine; College of Veterinary Medicine; University of Diyala, Iraq. The samples were centrifuged at (3000 rpm for three minutes), floating was neglected, and a part of the sediment was taken by loop and cultured on media (MacConkey, Nutrient & EMB) for 24 hours at 37 °C in the incubator.

2- Blood samples from 125 patients, 2-4 ml, from patients up to ten years of age, and 5–10 ml, from patients, of more than ten years of age, in a special container with BHI broth for enhanced growth, then put in a bacterial or alert apparatus to detect the presence of bacterial infection. After that, a drop was taken by a syringe and put on a culture media plate, then incubated overnight in an incubator at 37 °C (analysis done in the microbiology department in Al-Batol hospital). The isolated bacteria were subjected to automated identification using the VITEK-2 system (Biomeriux, Germany) (Radhakrishnan, 2022). Vitek-2 is a smart colorimetric method used for the identification of clinical isolates by a computerized microbiology program system (Aziz *et al.,* 2014).

3- Stool specimens: 2 grams of formed stool or 2 mL liquid stool (preferred specimens); were collected in a sterile, clean, and dry plastic jar. Using an applicator swab, a small amount of feces was gathered, from areas with visible blood or mucous, if present. They were cultured over the surface of nutrients, MacConkey, Sabouraud dextrose agar by streaking method, incubated all media at 36°C in an incubator for 24 hours (overnight) (WHO, 2010).

Preparation of the experimental dose:

The infective dose of *Klebsiella pneumoniae* required to induce infection in experimental animals was determined using the McFarland method. The mixture was shaken well, placed in a screw-capped test tube, and stored in a dark place at 4ºC. Before use, the solution was mixed well to compare it with bacterial turbidity, as it provides a turbidity equivalent to 1 x 10^6 CFU/ml (McFadden, 2000; Andrews, 2001).

Lab animals

This study was conducted in the Faculty of Veterinary Medicine at the University of Diyala, in the animal house for the

Department of Internal Medicine and Preventive, after the adaptation period (2 weeks), A total of 30 male rabbits were divided into three groups: 1st group (GI): given (1 milliliter /animal) an oral dose of phosphate buffer saline (PBS) by a stomach tube as a control group for 60 days. 2nd group (GII) was given one dose a week, and 3rd group (GIII) was given twice a week of 1 milliliter containing viable *K. pneumoniae* (1 X 10^6 CFU/ml) orally by stomach tube for 60 days.

Collection of blood samples:
After 60 days of the experiment, blood samples were collected from the heart to be used for hematological analysis and comet assay for detection of DNA damage.

1- For hematological analysis:
Blood was collected from each rabbit into an EDTA tube, and the analysis was performed at the Veterinary College, University of Baghdad, and the complete blood count (CBC) was performed using an auto-hematology analyzer, including red blood cells (RBCs) count, hemoglobin (Hb) concentration, packed cell volume (PCV), white blood cells (WBCs) count, lymphocytes, monocytes, and neutrophils).

2- For DNA comet assay:
The lymphocytes were isolated from the heparinized whole blood as described by Zizzadoro *et al.* (2002) as follows:

1. The blood was centrifuged at 1000 rpm for 15 min.
2. The buffy coat was collected in 10 ml centrifuge tubes and diluted with 5 ml of PBS (cell suspension).
3. Five ml of the diluted cell suspension was layered on 3 mL of ficoll-isopaque separation fluid (lymphoprep has a density of = 1.077 g/ml).
4. The tubes were centrifuged at 2000 rpm for 30 min. at 4 ºC.
5. After centrifugation, the mononuclear cells were visible as a cloudy band between the PBS and lymph prep layers.
6. The band was collected in a 10 ml test tube, and the cells were suspended in 5 mL of PBS.
7. The tube was centrifuged at 2000 rpm for 5 min (first wash), the supernatant was discarded, and the cells were re-suspended in 5 ml of PBS (this step was repeated twice).
8. The suspension was centrifuged at 1000 rpm for 10 min. The supernatant was discarded.
9. The precipitated cells were re-suspended in 1 mL of PBS solution and used in the planned experiments.

Counting and viability assessment of Lymphocytes:
Counting the cells was performed before the experiment, according to Porakishvili et al. (2004). Cells were examined under a phase contrast microscope (10 X magnification) to monitor cells, and then cells were collected by centrifugation at 2000 rpm for 5 min. and re-suspended with 5 mL of complete RPMI medium. 10µl of cell suspension was mixed with 90µl of trypan blue for 3 minutes, and a drop was allowed to spread under the coverslip of a hemocytometer. Then the viability was determined according to the following equation:

Viability (%) =Total Number of Living Cells/ Total Number of Cells x 100.

At the same time, the number of lymphocytes was counted by a light microscope, and the cell concentration was adjusted to 1 x 10^6 cells/ml.

Comet assay
Comet assay was done according to Boutet-Robinet *et al.* (2021) and Morsy *et al.* (2024).

A- Reagent Preparation:
Reagents marked with an asterisk should be prepared immediately before use.

1. **1X PBS, Ca++, and Mg++ free**
   10X PBS was diluted with deionized water to prepare 1X PBS and stored at room

temperature (10X PBS is available from Trevigen).

2. **Lysis solution:**
For up to 10 slides (2 samples per slide) were prepared:

- Lysis solution 40 mL.
- DMSO (optional) 4 mL.
- Should be kept cool to 4°C, or on ice, for at least 20 minutes before use. The addition of DMSO is optional and is required only for samples containing heme, such as blood cells or tissue samples. The buffer formulation is proprietary.

B. LM Agarose:
LM Agarose was used once it was molten. The cap was loosed to allow for expansion, then heated in a 90–100°C water bath for 5 minutes, or until the agarose was molten. The bottle was placed in a 37°C water bath for at least 20 minutes to cool. The LM Agarose remained molten at 37°C for sample preparation indefinitely. The LMA agarose formulation is proprietary.

C-SYBR Green staining solution:
The diluted stock is stable for several weeks when stored at 4°C in the dark.

- 10,000X SYBR Green in DMSO 1 µl.

D-Alkaline Unwinding Solution, pH>13 (200 mM NaOH, 1 mM EDTA):
Gloves should be worn when preparing and handling the alkaline unwinding solution.

Per 50 ml of Alkaline Solution combine:

- NaOH Pellets 0.4 gm
- 200 mM EDTA 250 µl
- DH_2O 49.75 ml.

The solution was warmed during preparation. The solution was allowed to cool at room temperature before use.

E-Alkaline electrophoresis solution pH >13 (200 mM NaOH, 1 mM EDTA) for the Comet Assay ES system:

A stock solution of 500 mM EDTA was prepared. For 1 liter of electrophoresis solution:

- NaOH pellets 8 gm.
- 500 mM EDTA
- DH_2O (after NaOH is dissolved) added to 1 liter of freshly made solution was recommended for use and kept cool at 4°C.

F- Assay Protocol:

G-Alkaline Comet assay:

1- Cells were combined at 1 x 10^5 /ml with molten LM Agarose (at 37°C) at a ratio of 1:10 (v/v) and immediately pipetted 50 µl onto Comet Slide. If necessary, the side of the pipette tip was used to spread agarose/cells over the sample area to ensure complete coverage of the sample area. If the sample is not spreading evenly, the slide was warmed at 37°C before application.

2- Slides flat was placed at 4°C in the dark (e.g. place in refrigerator) for 10 minutes. A 0.5 mm clear ring appears at the edge of the Comet Slide area. Increasing the solidification time to 30 minutes improves adherence of samples in high-humidity environments.

3-Slides were immersed in 4°C lysis solution for 30-60 minutes. For added sensitivity or convenience, it should be incubated overnight at 4°C.

4-Excess buffer was drained from slides and immersed in freshly prepared alkaline unwinding solution, pH>13. 5. Slides were immersed in an alkaline unwinding solution for 20 minutes at room temperature or 1 hour at 4°C, in the dark.

6. Electrophoresis: alkaline electrophoresis solution was added, and slides were placed in an electrophoresis slide tray (slide labeled adjacent to black cathode) and covered with slide tray overlay. The power supply was adjusted to 21 volts and voltage was applied for 30 minutes.

7- Excess electrophoresis solution was drained gently immersed twice in dH_2O for 5 minutes each, then in 70% ethanol for 5 minutes.

8- Samples were dried at 37°C for 10-15 minutes. Drying brings all the cells in a single plane to facilitate observation. Samples were stored at room temperature, with desiccant prior to scoring at this stage.
9- One hundred µls of diluted SYBR Green was placed onto each circle of dried agarose and stained for 30 minutes (room temperature) in the dark. The slide was gently tapped to remove excess SYBR solution and rinsed briefly in water. The slides were dried completely at 37°C.
10- Slides were viewed by fluorescence microscopy (SYBR Green's maximum excitation/emission is 496 nm/522 nm. Fluorescein filter is adequate).

Analysis:

The way of quantification was by using the image analysis software, Comet Score. The analysis software calculated different parameters for each nucleus. Four parameters were estimated to indicate DNA migration: tail length, tail intensity, tail moment and tail DNA%. Figure (3-5) (Humadi, 2019).

RESULTS

Hematological analysis:

The hematological analysis showed a significant decrease of RBCs, Hb and PCV in 2nd and 3rd groups, compared with the control group (Table 1). The analysis showed a considerable increase of WBCs, lymphocytes, monocytes, and neutrophils in the 2nd and 3rd groups, compared with the control group.

Table 1: Effect of *K. Pneumonia* on complete blood counts in albino rabbits in different groups.

Parameter	G1	G2	G3
RBCs (10^5 cells/ul)	8.73±0.19a	7.19±0.35b	6.68±0.23b
Hb (g/dl)	15.09±0.85a	12.68±0.22b	10.61±0.90c
PCV (%)	40.03±1.25a	33.48±12.28b	32.52±1.80b
MCV	61.36±0.12a	61.43±0.18a	61.49±0.25a
MCH	20.06±0.04a	18.60±0.08b	17.76±0.19c
MCHC	32.55±0.08a	31.70±0.15a	29.65±0.25b
Platelets (10^3cells/mm^3)	238±0.9a	192±0.1b	198±1.2b
MPV (FL)	7.55±0.15ab	7.30±0.45ab	7.12±0.35b
RDW	17.60±0.25b	17.94±0.45a	16.85±0.10c
PDW	16.65±0.33a	16.85±0.11a	16.55±0.45a
WBCs (10^3 cells/ mm^3)	12.85±0.15c	13.19±1.25b	16.70±1.20c
Heterophiles	22.65±1.45c	24.70±2.10b	29.10±1.20a
Lymphocytes	55.12±5.22c	60.25±4.20b	64.19±5.95a
Monocytes	11.5±0.35c	12.7±0.12b	15.1±0.90a

Values are expressed as means ± SE with different superscript letters, are significantly different in blood parameters ($P \leq 0.05$).

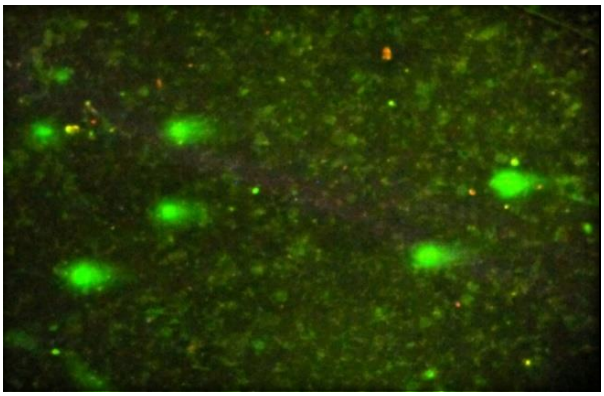
DNA damage:

The results of the comet assay of score rate for damage of lymphocytes in different groups are indicated in Table 2.

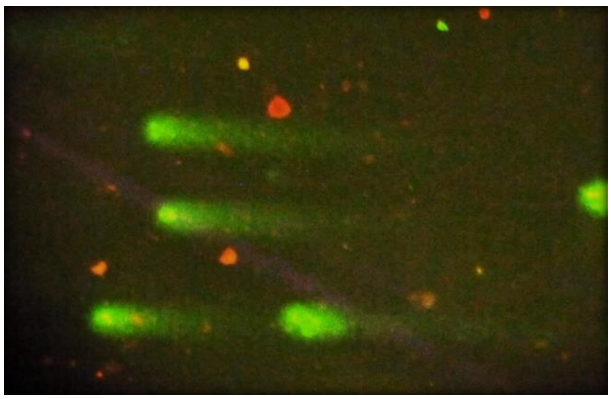
Table 2: Scores mean DNA damage % of lymphocytes of male albino rabbit groups.

Score means %Groups	No damage (ND) %	Low damage (L) %	High damage (HD)%	Sum %
1st group (Control)	47.11%[a]	43.97%[a]	8.92%[c]	100%
2nd group	39.19%[b]	36.62%[b]	24.19%[b]	100%
3rd group	34.67%[c]	33.02%[b]	32.31%[a]	100%

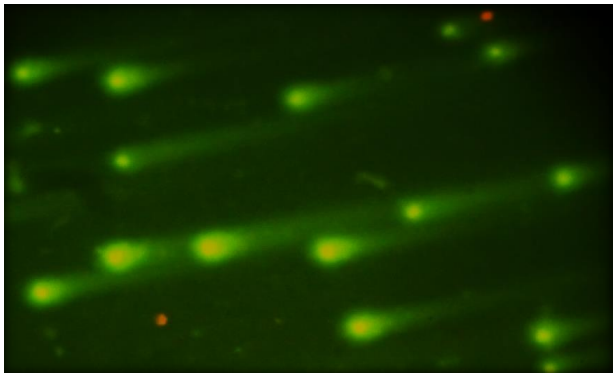
The significantly different ($P<0.05$).



very low damage in the 1st group (Control)



Moderate damage in the 2nd group



High damage in the 3rd group

The comet assay of the control group showed very low damage. The intact DNA which showed no tail indicating no damage. It was the highest in the control group, and significantly decreased in the 2nd and 3rd groups. The high DNA damage showed a significant increase in the 2nd and 3rd groups, compared to the control group.

The way of qualification by using the image analysis software comet score program, showed the results in the below table.

Table 3: The quantification of DNA damage using image analysis software, comet score program, in albino male rabbit groups.

Groups	Tail length M ± SE	Tail intensity M ± SE	Tail moment M ± SE	% DNA in tail M ± SE
1st group (C)	29. 95± 12.3^{c}	175 ± 10.2^{c}	0.76 ± 5.2^{c}	0.88 ± 1.4^{c}
2nd group	185± 11.3^{b}	2186± 445^{b}	33.85± 0.3^{b}	7.20±1.7^{b}
3rd group	476± 55.2^{a}	3820 ± 128^{a}	89.25± 5.4^{a}	16.68 ± 0.6^{a}

Means significantly (P<0.05)
The mean ±SE of tail length, tail intensity, tail moment and % DNA in the tail. A significant increase in the 2nd and 3rd groups, compared to the control group.

DISCUSSION

This experiment aimed to investigate the effects of *K. pneumoniae* on blood pictures and damage to DNA.

Hematological assay:
There are very rare or limited studies on the effects of K. pneumoniae on the blood picture. In the present study there was a significant decrease in RBCs, Hb, and PCV. Whereas the WBCs, lymphocytes, monocytes, and neutrophils increased significantly. These results are in line with (Munther, 2018) who showed a significant decrease in RBCs and an increase in WBCs after infection by *K. pneumoniae* in rabbits.

Toxic substances usually have similar effects, where an alteration of blood pictures (RBCs & Hb) was shown after exposure to the toxic dipyrone (Hameed *et al.,* 2019). Other substances affect the WBCs, where a significant increase in WBC count after infection of guinea pigs by toxic substance (Dioxin) (Humadi *et al.,* 2020). Likely, some substances affect both RBCs and WBCs, such as cypermethrin, where RBCs, PCV and Hb concentration decrease and WBC counts increase (Orun *et al.,* 2014).

Humadi & Qaisei (2019) suggested that these changes of parameters in case of the toxic effect of acrylonitrile due to free radicals. The increased total leukocyte counts in G2 and G3 can be attributed to the capsular material that forms a thick bundle of fibril structures covering the bacterial surface in massive layer to protect the bacteria from phagocytosis by macrophages and prevent killing by bactericidal serum factors (Lin *et al.,* 2015; Rahman, 2018)

The current results showed very low damage of lymphocyte DNA in the control group. While there was severe damage of lymphocyte DNA in the 2nd and 3rd groups compared with the control group.

The above results may indicate that these inflammatory cells might release reactive oxygen species (ROS) that may be destroyed on the basis of the chromosomal strands leading to DNA damage and chromosomal aberration. Highly affected tissues due to double dose weekly of viable *Klebsiella pneumoniae,* which causes a fast defect. These results agree with (Humadi, 2019) who showed that DNA damage is detected in different organs and free radicals' production

by the inflammatory cells. The continued oxidative stress can lead to chronic inflammation and the development of different dangerous diseases.

The recruited inflammatory cells to the inflamed area might stimulate respiratory oxygen burst in these cells. They are the main source of reactive oxygen species, and under hypoxic conditions, these cells produce nitric oxide, which generates reactive nitrogen species (RNS) (Poyton et al., 2009).

CONCLUSION

The infection by *K. pneumoniae* is critical and leads to an alteration in blood profile (WBC and RBC) and DNA damage.

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تغيرات الصورة الدموية وتلف الحمض النووي بواسطة بكتيريا الكليبسيلا الرئوية في ذكور الأرانب البيضاء

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ان الكليبسيلا الرئوية تعتبر احد اجناس العائلة البكتيرية المعوية Enterobacteriaceae ، وهي نوع من انواع البكتيريا التي يمكنها البقاء على قيد الحياة مع أو بدون الأكسجين، وهي غير متحركة، ولها شكل قضيبي، وتصنف على أنها ذات صبغة سالبة الجرام. تم استخدام ٣٠ ارنب ذكر ابيض اللون في التجربة وتم تقسيمها الى ٣ مجاميع: المجموعة الاولى وهي المجموعة الضابطة حيث تم اعطاء محلول ملحي phosphate buffer saline عن طريق الفم بجرعة ١ ميليليتر ولمدة ٦٠ يوم، المجموعة الثانية تم اعطاء جرعة ١ ميليليتر يحتوي على الكليبسيلا الرئوية ($1 X 10^6$ CFU/ml) مرة واحدة بالأسبوع عن طريق الفم ولمدة ٦٠ يوم، المجموعة الثالثة تم اعطاء جرعة ١ ميليليتر يحتوي على الكليبسيلا الرئوية ($1 X 10^6$ CFU/ml) مرتين بالأسبوع عن طريق الفم ولمدة ٦٠ يوم، بعد انتهاء ال ٦٠ يوم من التجربة تم سحب عينات الدم من القلب مباشرة وتم تقسيمها الى قسمين القسم الاول لغرض اجراء تحليل الصورة الدموية والقسم الثاني لغرض اجراء اختبار المذنبcomet assay . النتائج في اختبار تحليل الصورة الدموية اظهرت انخفاض معنوي في RBC ، Hb ، PCV وكذلك اظهرت ارتفاع معنوي في WBC ، Lymphocytes ، Monocytes ، Neutrophil في المجموعة الثانية والثالثة مقارنة بمجموعة الضابطة، اما بالنسبة لاختبار comet assay فقد اظهرت جميع المجاميع تلف في خلايا اللمفوسايت وكذلك ارتفاع معنوي في طول الذيل، كثافة الذيل، لحظة الذيل والنسبة المئوية لتلف الحمض النووي في الذيل في المجموعة الثانية والمجموعة الثالثة مقارنة مع المجموعة الضابطة.

كلمات مفتاحية: الكليبسيلا الرئوية، الارانب، الصورة الدموية، اختبار DNA comet assay