

Study the Efficacy of Mono and Combination Therapy of Amoxicillin and Levofloxacin against *S.aureus* causing Respiratory Tract Infection in Rabbits

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Abstract

This study was conducted to investigate the difference between the effect of monotherapy by amoxicillin and levofloxacin with their combination in treating respiratory infection induced by *Staphylococcus aureus* in rabbits. For this purpose the study was divided into two parts, *in vitro* and *in vivo*. The *in vitro* included: determination of minimum inhibitory concentration (MIC), inhibition zone, interaction between amoxicillin and levofloxacin. The results of *in vitro* study showed that the MIC was 64 µg/ml, 8µg/ml and 64µg/ml+8µg/ml for amoxicillin and levofloxacin and their combination respectively. The inhibition zone produced by 0.5MIC, 1MIC and 2MIC of amoxicillin, levofloxacin and their combination was concentration dependent, which were 8.66±0.33mm, 14.66±0.16mm and 11.33±0.33mm for amoxicillin, levofloxacin and their combination respectively by using 1MIC. The *in vivo* study included 25 rabbits, five animals kept as negative control without any infection or treatment while the remaining 20 animals were infected with *S.aureus* to induce respiratory tract infection and then divided into four equal groups, first one without treatment (positive control), second and third groups treated with amoxicillin 28.75mg/kg and levofloxacin 24.17mg/kg respectively while the last one treated with combination of half doses of amoxicillin and levofloxacin. In general, the results showed that the levofloxacin was the best in decreasing the deleterious effect produced by *S.aureus* infection followed by combination then amoxicillin, levofloxacin was the superior in decreasing the severity of clinical signs improves the blood picture represented by decreasing the

inflammatory cells like white blood cell and keeping the red blood cells within normal range.

In conclusion, treatment the respiratory tract infection with mono therapy by levofloxacin was better than use it in combination with amoxicillin.

Key words: Amoxicillin, Levofloxacin, Combination, Respiratory tract , *S.aureus*

Introduction

Respiratory infections in pets are a major health concern, making up about 5% of all veterinary cases. These infections can spread quickly and may be fatal. They are also the third most common reason for antibiotic prescriptions in emergency vet visits, accounting for 8.2% of all such prescriptions. Pets with respiratory symptoms can carry harmful microbes that might be resistant to treatment and could potentially be transmitted to humans, causing serious infections. (26).

Staphylococcus aureus is a common bacterium known for causing a range of diseases, including mastitis and respiratory infections. In rabbits, it is a significant pathogen associated with severe respiratory illness and high mortality rates.(23)(6) Research highlights the need for vigilance in

monitoring *S. aureus* in rabbits and the potential for transmission between rabbits and humans. *S. aureus* infections are challenging to treat due to the bacterium's high resistance to multiple antibiotics(5).

The treatment of respiratory infections has become problematic due to the increase in antibiotics resistance. There are increased of the novel bacterial respiratory pathogens that are becoming increasingly challenging to treat, with respiratory tract infections (RTIs) being exacerbated by antibiotic resistance of Gram-positive and Gram-negative bacteria (33,7). Antimicrobial resistance (AMR) is an escalating global crisis with significant economic repercussions. Estimates suggest that the financial impact of AMR ranges widely, from \$3 to \$11 billion up to a staggering \$100 trillion USD. This

underscores the urgent need for alternative or adjusted treatment strategies to address these resistant pathogens effectively. Combination therapy is a strategy for preventing infections caused by resistant pathogens(1)Combining antibiotics holds promise in countering resistance by either enhancing treatment effectiveness or improving infection eradication. Enhanced efficacy can shorten the duration of infection and diminish pathogen numbers, thereby lowering the chance of resistance

Materials and Methods

Bacterial Strain

The tested bacterium *S.aureus* was obtained from department of microbiology/ College of Veterinary Medicine/ University of Baghdad. Overnight (18 hours) cultures of bacterial inoculate were prepared by inoculating a single colony from brain heart infusion broth. Cultures of bacteria on mannitol salt agar, then biochemical test ex, coagulase test, Vitek 2 test.

development. Persistent resistant strains are at risk of evolving further resistance, potentially leading to multidrug resistance (27).Moreover, using synergistic antibiotic combinations can allow for reduced dosages, minimizing toxicity to the host. So, this study designed to evaluate the efficacy of the combination between amoxicillin and levofloxacin in treating respiratory tract infection caused by resistant *S.aureus*(32).

Identification of bacteria *S.aureus* by vitek 2 system

The vitek 2 is an automated set to identify microbial and antimicrobial susceptibility system this device include 48 biochemical tests in chart used to diagnostic bacteria and accuracy reach to 98% diagnostic (25).

Minimum inhibitory concentration

The broth macrodilution method was employed to assess the minimum inhibitory concentration (MIC) of various antibacterials against *S.aureus* following CLSI guidelines (9). Bacteria were cultured in Mueller-Hinton broth for 24 hours, and the suspension was

adapted to a 0.5 McFarland standard (approximately 1.5×10^8 CFU/ml). A two-fold serial dilution of amoxicillin, levofloxacin, and their combination was then applied to each tube of the culture. The tubes were incubated for 24 hours at 37°C. After incubation, visual inspection for turbidity was conducted. Cloudiness indicated that the antimicrobial concentration was insufficient to inhibit bacterial growth. The MIC was determined as the lowest concentration of the antibacterial agent that prevented visible growth.

Determination of the inhibition zone

The agar well diffusion method (10) was utilized to evaluate the antibacterial activity of amoxicillin, levofloxacin, and their combination against *S.aureus*. First, 500 mL of sterile Mueller-Hinton agar was combined with 5 mL of a standardized bacterial suspension (1.5×10^8 CFU/mL) of the tested bacteria. This mixture was poured into sterile Petri dishes, with each dish receiving 25 mL of the inoculated agar. After allowing the agar to solidify for 10 minutes, three wells, each 6 mm in diameter, were created in the agar of each plate. The wells were then filled with 100 μ L of solutions at three

different concentrations: 0.5 MIC, 1 MIC, and 2 MIC of amoxicillin, levofloxacin, and their combination. The plates were left at room temperature for 2 hours to facilitate diffusion, followed by incubation at 37°C for 24 hours. Antibacterial activity was assessed by measuring the diameter of the inhibition zones around each well in millimeters, with measurements taken in triplicate for each concentration.

Study the interaction between levofloxacin and amoxicillin against *S.aureus*

The experiment adhered to established guidelines and used levofloxacin and amoxicillin in Mueller-Hinton broth at concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 μ g/mL. Solutions were added to a 96-well plate, 180 μ L of bacterial solution followed by 20 μ L of a 10^6 CFU/mL suspension and incubation at $35 \pm 2^\circ\text{C}$ for 3 hours. Afterward, 22 μ L of 10% resazurin dye was added and the plates were incubated for 2 more hours. Bacterial growth was indicated by red or rose color, while blue indicated no growth. The fractional inhibitory concentration (FIC) index was calculated as $\text{FICI} = (\text{MIC of compound A in combination} / \text{MIC of compound A alone}) + (\text{MIC of compound B in$

combination / MIC of compound B alone). $FICI \leq 0.5$ denotes synergy, 0.5-4 indicates no interaction, and $FICI > 4$ signifies antagonism(19).

Induction of the respiratory infection

Following a 14-day adaptation period, 25 rabbits were inoculated with a pathogenic strain of *S.aureus*. The inoculum was administered into each nare using an insulin syringe without a needle. Immediately after inoculation, the rabbits' heads were held upright for 2 minutes to ensure the inoculum reached deep into the nasal cavity. The rabbits were then monitored for clinical signs and mortality. Daily body temperatures were recorded using a digital thermometer inserted rectally. After that the infected rabbits were divided randomly into four equal groups, the first group was not receiving any treatment and was kept as positive control, the second group was treated with amoxicillin (28.75mg/kg B.W.), the third group was treated with levofloxacin (24.17mg/kg B.W.) while the last group was treated with half dose

of amoxicillin. + half dose of levofloxacin. All treatments was administrated orally twice daily for 10 days from the infection. Control negative group no infect and no treat.

Blood sampling

The blood collection was done to estimate some blood parameters (RBC and WBC). The collection was done at 3, 7 and 14 days after inducing infection. Blood samples were obtained via cardiac puncture from each rabbit by using disposable syringe (1 ml) from all animals. Blood samples were collected in test tubes with anticoagulant and stored until used.

Results

Identification of *S.aureus*

Based on the data obtained from Vitek 2 system the tested bacterium was *S.aureus* figure (1).

bioMérieux Customer:		Microbiology Chart Report		Printed October 30, 2023 8:27:21 PM CDT													
Patient Name: Dr. Abdulwahab			Patient ID: 98														
Location:			Physician:														
Lab ID: 98			Isolate Number: 1														
Organism Quantity:																	
Selected Organism : Staphylococcus aureus																	
Source:			Collected:														
Comments:																	
Identification Information		Analysis Time: 5.82 hours		Status: Final													
Selected Organism		95% Probability		Staphylococcus aureus													
ID Analysis Messages		Bionumber:		030412063363231													
Biochemical Details																	
2	AMY	-	4	PIPLC	-	5	dXYL	-	8	ADH1	+	9	BGAL	+	11	AGLU	-
13	APPA	-	14	CDEX	-	15	AspA	-	16	BGAR	-	17	AMAN	-	19	PHOS	+
20	LeuA	+	23	ProA	-	24	BGURr	-	25	AGAL	-	26	PyrA	+	27	BGUR	-
28	AlaA	-	29	TyrA	-	30	dSOR	-	31	URE	-	32	POLYB	+	37	dGAL	+
38	dRIB	+	39	ILATk	+	42	LAC	-	44	NAG	+	45	dMAL	+	46	BACI	-
47	NOVO	-	50	NC6.5	+	52	dMAN	+	53	dMNE	+	54	MBdG	+	56	PUL	-
57	dRAF	-	58	O129R	+	59	SAL	-	60	SAC	+	62	dTRE	+	63	ADH2s	-
64	OPTO	+															

Figure 1: Vitek 2 system show the tested bacterium is *Staphylococcus aureus*

Determination of Minimum inhibitory concentration (MIC) of the amoxicillin and levofloxacin against S.aureus

Results of determination the lowest concentration of the amoxicillin and levofloxacin that inhibit *S.aureus* growth were 64µg/ml and 8µg/ml respectively figure (2) and (3) , which seem very high as compared with breakpoints fixed by CLSI 2020 (9), that means this bacterium resistant to these antibacterials.



Figure 2: Determation of MIC for amoxicillin against *staphylococcus aureus* which equal 64µg/ml

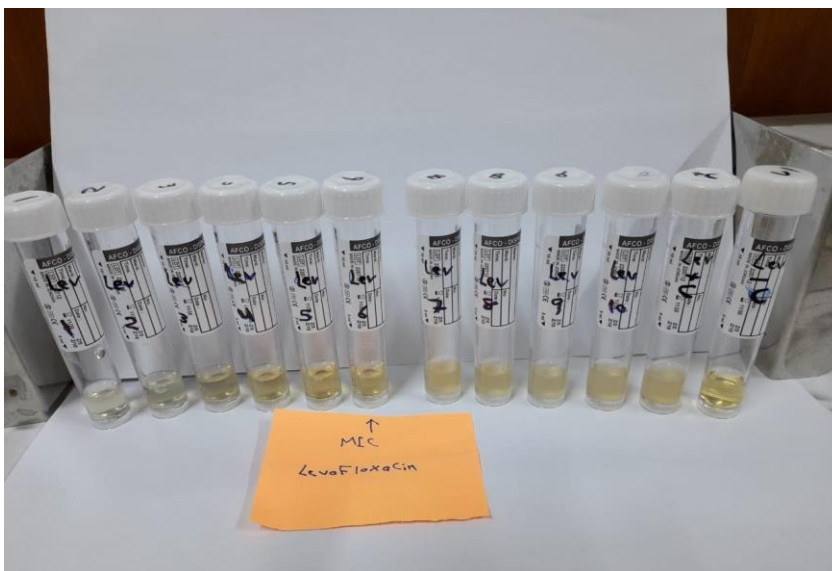


Figure 3: Determation of MIC for levofloxacin against *staphylococcus aureus* which equal 8µg/ml.

Determation of the inhibition zone

Results of Determation the zone of growth inhibition by using three concentrations of amoxicillin (0.5MIC,

1MIC and 2MIC) against *S.aureus*, showed different activities on the Mueller Hinton agar which were 6.66 ± 0.16 mm, 8.66 ± 0.33 mm and 11.33 ± 0.33 mm respectively. In concern to levofloxacin,

the inhibition zones were 9.66 ± 0.33 mm, 14.66 ± 0.16 mm and 18.33 ± 0.33 mm by using (0.5MIC, 1MIC and 2MIC) respectively, whereas the zones that resulted from the combination of half concentration of 0.5MIC, 1MIC and 2MIC of amoxicillin and levofloxacin were 7.66 ± 0.33 mm, 11.33 ± 0.33 mm and 16.33 ± 0.33 mm respectively. All these above results are displayed in figures 4-

6. Levofloxacin exhibited superior antibacterial activity as compared to amoxicillin and the combination and there was significant difference ($P < 0.05$) between all groups and concentrations. Significant differences were found between different concentrations and treatments, with a least significant difference (LSD) of 0.90. Values are means \pm standard error (SE), with $N = 3$.

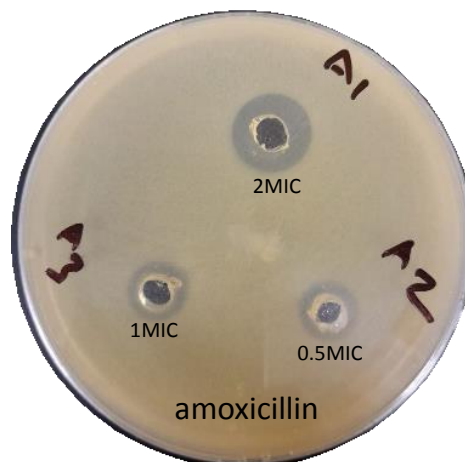


Figure 4: Inhibition zone produced by different concentrations of amoxicillin (0.5MIC, 1MIC and 2MIC) against *Staphylococcus aureus*

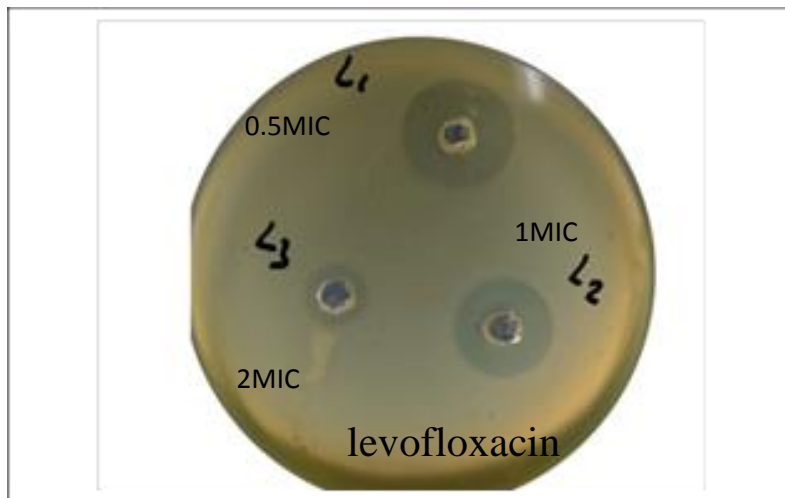


Figure 5: Inhibition zone produced by different concentrations of levofloxacin (0.5MIC, 1MIC and 2MIC) against *Staphylococcus aureus*

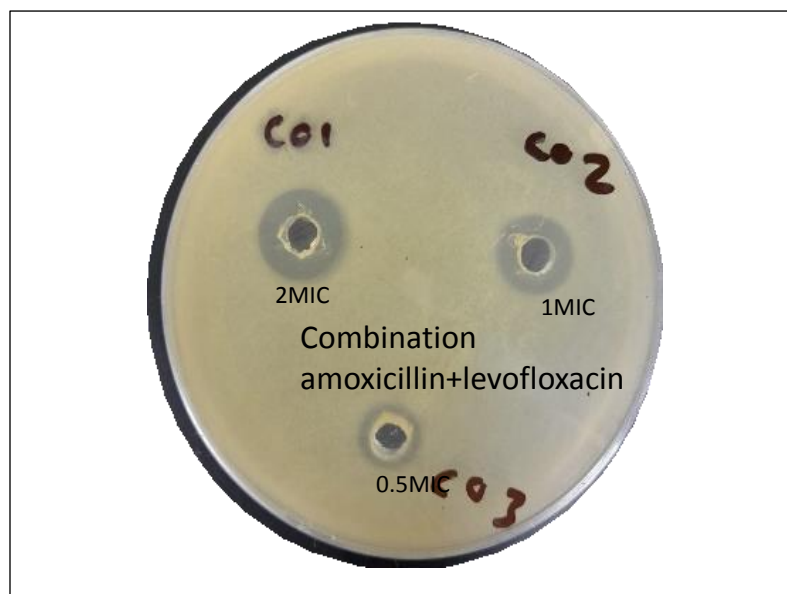


Figure 6: Inhibition zone produced by different concentrations of combination amoxicillin + levofloxacin (0.5MIC, 1MIC and 2MIC) against *Staphylococcus aureus*

Study the interaction between levofloxacin and amoxicillin against *Staphylococcus aureus*

The results of this test showed that the MIC of the combination between amoxicillin and levofloxacin was 64µg/ml and 4µg/ml respectively.

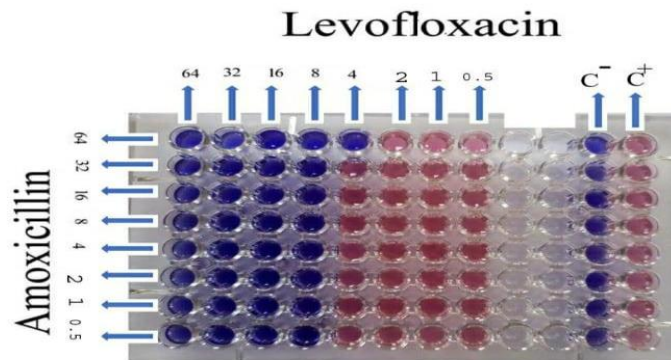


Figure 7: Checker board showing the MIC of the combination between amoxicillin and levofloxacin is 64µg/ml and 4µg/ml respectively, blue color no bacterial growth, while the red color indicate growth.

FICI value of 1.5 indicated an indifferent interaction between amoxicillin and levofloxacin when used in combination.

Two days after rabbits were infected with *S.aureus* they showed signs like fever, loss of appetite, coughing, sneezing, rapid breathing, and nasal discharge. These symptoms were similar to those reported in previous studies. (4)

Levofloxacin was the most effective in reducing fever in rabbits infected with *S. aureus*. By three days, all infected rabbits had higher temperatures than the healthy controls. By seven days,

temperatures remained high in the untreated group but decreased significantly in the levofloxacin group. Differences between groups were mostly significant, except between the negative control and levofloxacin, and between amoxicillin and combination therapy.

After 14 days, temperatures in all infected groups had decreased compared to day three, but the drop was not significant in the untreated and amoxicillin groups.

The control negative group remained at 38.92°C, while the control positive started at 40.14°C. Levofloxacin-treated rabbits'

temperatures dropped from 39.56°C to 38.58°C, amoxicillin-treated rabbits' from 39.88°C to 39.40°C, and combination treatment from 39.66°C to 39.24°C. Significant differences were noted between treatments, with results presented as means \pm standard error and a least significant difference (LSD) of 0.33, based on 5 rabbits per group.

The study tracked white blood cell (WBC) counts in rabbits infected with *S.aureus* and treated with levofloxacin, amoxicillin, or both over 3, 7, and 14 days. The control negative group showed WBC counts of $7.04 \times 10^9/L$ at 3 days, $7.35 \times 10^9/L$ at 7 days, and $6.65 \times 10^9/L$ at 14 days. The control positive group had higher counts, starting at $10.33 \times 10^9/L$ and increasing to $11.96 \times 10^9/L$ by day 14. Levofloxacin treatment reduced counts from $7.17 \times 10^9/L$ to $5.68 \times 10^9/L$. Amoxicillin treatment showed counts decreasing from $9.87 \times 10^9/L$ to $7.89 \times 10^9/L$. The combination treatment resulted in counts dropping from $8.70 \times 10^9/L$ to $6.15 \times 10^9/L$. Significant differences between groups were observed, with a least significant difference (LSD) of 1.21.

Results of this test showed there was significant elevation in WBCs count at three days post infection in all infected

groups except in levofloxacin which was very closed to control group. After 7 and 14 days post infection there was gradual decreasing in WBCs count in all infected groups except positive one which showed significant raise ($P < 0.05$) as compared with all groups, and the levofloxacin was the best in lowering these cell with significant difference ($P < 0.05$) only in comparing with amoxicillin in these periods .

Number of Red Blood Cells (RBC)

The study measured red blood cell (RBC) counts in rabbits infected with *S. aureus* and treated with levofloxacin, amoxicillin, or both at 3, 7, and 14 days. The control negative group had RBC counts of $5.62 \times 10^{12}/L$ at 3 days, $5.77 \times 10^{12}/L$ at 7 days, and $5.07 \times 10^{12}/L$ at 14 days. The control positive group started at $5.73 \times 10^{12}/L$, dropped to $4.53 \times 10^{12}/L$ by 7 days, and slightly increased to $4.91 \times 10^{12}/L$. Levofloxacin-treated rabbits had counts of $5.66 \times 10^{12}/L$, increasing to $5.78 \times 10^{12}/L$, then decreasing to $5.44 \times 10^{12}/L$. Amoxicillin-treated rabbits had counts starting at $4.97 \times 10^{12}/L$ and falling to $4.48 \times 10^{12}/L$. The combination treatment group had counts of $5.99 \times 10^{12}/L$, rising to $6.38 \times 10^{12}/L$, and then dropping to $5.09 \times 10^{12}/L$. Significant differences were

found, with a least significant difference (LSD) of 1.00.

According to the results there was no significant difference in RBC number among periods of study except between 3 and 7 days in positive control and between 7 and 14 days in combination group which revealed in both groups reduction in RBC number. In concern to the comparison between groups, the results showed no any significant difference between them after 3 and 14 days post infection, in contrast, there were substantial differences ($P < 0.05$) among bunches after 7 days post infection, in which the combination group showed the significant higher number of RBC in comparison with positive and amoxicillin groups, followed by group that treated with levofloxacin which showed significant difference just in comparison with positive control.

Discussion

According to the results of the minimum inhibitory concentration and inhibition zone produced by amoxicillin and levofloxacin, the tested bacterium considered resistant to these antibacterials, because the obtained data very high as compared with the values range of the susceptible isolates, in case

of amoxicillin the $MIC \geq 2 \mu\text{g/mL}$ classified as resistant bacterium (29), whereas no any recorded data about inhibition zone. In concern to levofloxacin the breakpoint values of the resistant are $MIC \geq 4 \mu\text{g/mL}$ and zone of inhibition $\leq 15 \text{ mm}$ (9).

Amoxicillin inhibit penicillin-binding protein (PBPs) competitively, and these proteins are blamable for transpeptidase and glycosyltransferase reactions which principal on cross-linking of D-aspartic and D-alanine acid in the cell wall of bacteria, so without these (PBPs), bacteria produce autolytic enzymes and are incapable to build or repair the wall, causing its death (28) In this study the resistance of *S.aureus* to amoxicillin may be mediated by production of β -lactamase which destroy the structure of β -lactam and diminish the activity of this antibacterial (17). Furthermore, the (PBPs) form a new or different type of these sites, which stimulates the bacterial wall to develop tolerance (2)

Levofloxacin targets DNA gyrase enzyme which introduces negative supercoils into chromosomal DNA as well as topoisomerase IV which causes

decatenation of the chromosome after replication. Resistance of this isolate to levofloxacin may be due to mutational changes to the topoisomerases that decrease efficiency of this binding, or belong to high expression of endogenous efflux pump (12, 13).

An FICI value of 1.5 suggests an indifferent interaction between amoxicillin and levofloxacin, meaning their combined antimicrobial effect is not greater than the sum of their individual effects. Values of 0.5 ~ 4 indicate no interaction, while less than 0.5 shows synergy and more than 4 indicates antagonism. The combination does not exhibit synergy nor antagonism but rather acts independently. These findings have clinical implications for the use of amoxicillin and levofloxacin in combination therapy against *S.aureus* (34) While each antibiotic has its own MIC, suggesting varying degrees of effectiveness, their combination at the tested concentrations does not enhance antimicrobial activity of amoxicillin beyond what is achieved individually where the MIC stilled as it 64µg/ml.

Increasing body temperature (fever) is due to the body's immune

response as a result to bacterial infection and the ability of these cells to produce endogenous pyrogenes, These pyrogenes signal the hypothalamus to raise the body's temperature, resulting in fever. This fever response is a protective mechanism aimed to hindering bacterial growth, as many bacteria thrive at normal body temperatures. Therefore, fever serves as a beneficial response orchestrated by the immune system to combat bacterial infections effectively (18)

Results of the blood cells count showed that the total WBCs were elevated in infected groups after three days post infection but with variable degree, and this normal result in case of bacterial infection as recorded by many researchers. (3) showed there was significant elevation in WBC number in rabbits infected with *S.aureus*. Similarly, reported the infection with this bacterium caused significant increase in WBCs count in animals(20).

Inflammation of the respiratory tissues occurs when *S.aureus* infects the upper airway, damaging the mucosal lining of epithelial cells. This damage triggers an immune response crucial for controlling pathogen spread, initiating

tissue repair, and maintaining pulmonary homeostasis(22). During the inflammatory response, neutrophils are rapidly produced and migrate early to the site of infection. Guided by gradients of pro-inflammatory mediators, these cells exhibit increased stiffness and adhesion molecule expression, which facilitates their retention in pulmonary capillary beds and migration to infection or injury sites in the lungs (32). Neutrophils combat pathogens by engulfing them, releasing toxic substances, and secreting enzymes that degrade foreign materials or damaged tissue components .Additionally, neutrophils can kill pathogens through the extrusion of neutrophil extracellular traps (NETs) and phagocytosis . Thus, neutrophils serve as a primary defense mechanism against lung infections . Beyond their traditional roles, recent studies have also highlighted their involvement in tissue repair. (8).

After 7 and 14 days post-infection, all treated groups showed reduced WBC counts due to the antibacterials' actions, which inhibit bacterial growth and inflammation. In contrast, the positive control group had high inflammatory cell counts, likely

due to the severity of the infection, which correlates with pulmonary disease severity (13). The lack of improvement in the positive control may be due to *S.aureus* affecting pulmonary surfactant protein A (SP-A), which it cleaves with staphopain A (ScpA), reducing SP-A's antimicrobial properties and facilitating lung colonization (16). SP-A helps in bacterial aggregation, phagocytosis, and inhibition of bacterial adherence to epithelial surfaces (28,29).

RBC counts declined in the untreated infected group (positive control), the changes were not severe, possibly due to the short study duration. Significant declines typically require prolonged infection or inflammation (11). Additionally, cytokines can alter iron transport and storage in macrophages, resulting in decreased plasma iron and increased sequestration within macrophages. Persistent immune stimulation and inflammatory mediators further restrict iron availability in the mononuclear phagocyte system (MPS), leading to functional iron deficiency (23).

Conclusion

Results of this study demonstrated that the single or monotherapy of levofloxacin was effective than amoxicillin alone or as combination in diminishing the signs of infection in rabbits caused by *S.aureus*.

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