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Advanced Bacteriological Studies on Bumblefoot Infections in Broiler Chicken with Some Clinicopathological Alteration

Fatma M Youssef[1*]
Abdelmohsen A Soliman[1]
Ghada A Ibrahim[2]
Hend A Saleh[2]

[1]*Department of Clinical Pathology, Animal Health Research Institute, Ismailia, Egypt*
[2]*Department of bacteriology, Animal Health Research Institute, Ismailia, Egypt*

Abstract

S. aureus is responsible for Bumblefoot and septic arthritis in broilers and layers. The present work is designed to investigate and evaluate the most common bacterial causes of bumblefoot disease and their hematological, biochemical and immunological effects in broiler chicks. For bacteriological examination, one hundred and twenty foot swabs were collected from diseased chicks and investigated for the bacterial causes. Also, blood samples were collected from the same cases for hematological, biochemical and immunological analysis. In this study, *S. aureus* was isolated in 45.8% of diseased broilers. It was isolated either in pure form (18.18%); or in a mixed form with other species like: *E.coli* (58.18%), *Proteus mirabilis* (14.67%) and *Pseudomonas aeruginosa* (9.1%). Molecular characterization of *Coa* and *Spa*genes of *S. aureus* isolates were detected with PCR in 70% and 80%, respectively. Levofloxacin was the highest sensitive antibiotic drug against the isolated species followed by gentamycin, ciprofloxacin and amoxicillin antibiotics. However, most recovered isolates exhibited moderate to high resistance against trimethoprim, oxaciliin, enerofloxacin, tetracycline and erythromycin. For hematological parameters, a significant decrease in RBCs count, Hb concentration and PCV in the diseased broiler chicks indicated anemia. In addition, leucocytosis, lymphocytosis and monocytes is in naturally infected groups were recorded. Also, a significant increase in AST, ALT, uric acid, creatinine, gamma, alpha globulin, IL-6 and TNF-α levels were recorded in naturally infected chicks. Total protein and albumin levels were significantly decreased. The levofloxacine treated groups revealed significant increase in RBCs, Hb, and PCV as well as decreased number of total leukocytic count, lymphocyte and monocyte as compared with infected groups. Levofloxacine also improved the level of liver function, kidney function, IL-6 and TNF. It could be concluded that, levofloxacin represents a promising candidate to treat bacterial infections. Staphylococcal infections are higher in broilers than other organisms. Therefore, the epidemiological importance and good hyagenic measures should be taken in this field to minimize the transmissible diseases of poultry to humans.

Keywords: *S. aureus*, Isolation, PCR, Spa gene, Coagulase, Levofloxacin, Haematological, AST, Antioxidant, IL6, TNF.

Introduction

Bumblefoot is a broad term that includes any inflammatory or degenerative condition of the avian foot. It usually occurs with age (14 -70 days), but most cases occurred around 35 days old [1]. It may range from a very mild redness or swelling to chronic, deep seated abscesses and bony changes [2].

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***Corresponding author:** Fatma Mohamed Yousseff, Department of Clinical Pathology, Animal Health Research Institute, Ismailia, Egypt. Tel: +01025250063; Email: fatmayousseff(at)yahoo.com

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S. aureus infection is one of the important diseases of poultry that are common in commercial broilers and layers. It causes mortality rate (0-15%) and reduces production performance of birds. *S. aureus* is a normal inhabitant of the skin and upper respiratory tract in chickens [3]. In poultry, it has been implicated in arthritis, osteomyelitis, synovitis, cellulites, dermatitis, endocarditis, septicemia, wound infection, ophthalmitis and omphalitis [4].

This disease is most commonly caused by *S. aureus* [5] and sometimes involved with *E.coli* [6] which is of veterinary importance in broilers. In such birds, the most common form of infection involves tenosynovitis (inflammation of tendon sheaths) and arthritis of the hock and stifle joints [7]. Most bacterial pathogens could play a role in the incidence of respiratory disease in domestic poultry species [8]. The respiratory tract infections are of eminent importance in the manufacture of poultry because of high mortality in poorly managed cases [9] which lead to large economic losses [10].

Hematological alterations were recorded the severity of bacterial infection, a significant decrease in RBCs count, Hb concentration and PCV in the affected birds indicate anemia of microcytic hypochromic [11]. Moreover, the biochemical analysis recorded increase in AST and ALT and a significant change in protein. Also, Hypoalbuminemia was observed as well as increase of serum uric acid and creatinine [11].

Levofloxacin is a member of the fluoroquinolone group which has been extensively used for the treatment of bacterial infections. It is known with its wide antimicrobial activity against both Gram-negative (including *Pseudomonas* species) and some Gram-positive organisms (including *S. aureus*). It also has enhanced activity against *Streptococcus pneumoniae, S. aureus* and *Enterococcus species*, besides its good activity against *Mycoplasma* and *Chlamydia species* [12]. Its pharmacokinetics studies explained its tremendous tissue penetration, large volume of distribution and relatively short elimination half-life in animal body. Takizawa *et al*. (1999) [13] proved that levofloxacin drug was extremely useful in a variety of animal infections including urinary, respiratory, soft tissues, bones and joints. Levofloxacin pharmacokinetics studies had been reported in birds and layers [14]. The oral administration of levofloxacin(10mg/kg) in broiler birds was found safe as non-significant change on hematological or biochemical values were observed [14 and 15]. Thus, the aim of this study was to investigate the bacterial causes of Bumblefoot disease in broiler farms with molecular detection of some genes as well as the hematological, biochemical, and immunological changes accompanied with the disease.

Materials and Methods

History and clinical examination: A total of one hundred and twenty broiler chicks of age (20-35 days) that were reared in broiler farms at Ismailia and Zagazig Governorates in Egypt were used in the present study. They all showed the clinical signs of arthritis, leg affections or Bumblefoot disease and about eighty cases accompanied with some respiratory signs.

Sampling

Bacteriological swabs from (pus or infected lesions) diseased cases showing signs of arthritis or Bumblefoot or leg affections also, lung and liver organs (from those suffering from respiratory signs) were collected for bacteriological examination. In addition, blood samples (anticoagulant blood and serum) were collected from broiler chicks (20 for each)for hematological, biochemical and immunological studies. Also, after 5 days of treatment, another twenty blood samples from the treated broiler chicks were collected.

Bacteriological examination

Isolation and identification of bacterial microorganisms: All sample swabs and organ samples were cultured onto nutrient broth, incubated aerobically at 37°C for 24 hours, then streaked on blood and macConkey agar plates and re-incubated for 24 h at 37°C. Following incubation, the colonies were picked up and sub-cultured till pure growths were obtained. Mannitol salt agar media used for the specific isolation of *S. aureus* with streaking method, then incubated. The hemolysin activity of *S. aureus* and *E.coli* isolate was examined on sheep blood agar medium plates at 37°C [16]. Also, EMB (Eosin Methyline Blue) and Pseudomonas F agar media were used for species specific detection of *E.coli* and *Pseudomonas species.* The purity of the isolated organisms was determined on the basis of their morphological, cultural characteristics and Gram's staining. Biochemical identification of the isolated bacterial species also was done according to [17].

Serological identification of the isolates *E.coli* : *E.coli* isolates were biochemically identified then were subjected to the serological identification according to [18], by using of polyvalent somatic antisera, for application of the slide agglutination test. This work was performed in the serology unit of Animal Health Research Institute (AHRI).

Congo red Binding Assay [19]: *E.coli* positive samples streaked on Congo red agar plates to determine the pathogenicity of *E.coli* isolates**.** Each isolate was streaked on sterile separate plate and kept at 37°C for 24hrs. After 24hrs incubation, the cultures were kept at room temperature for 48 hours. Pathogenic *E.coli* were identified by Congo red positive isolates produced brick red colonies. The non-pathogenic isolates appeared as colorless after 48 hours in room temperature.

Antibiotic sensitivity testing of isolated bacteria: The identified isolates of *S. aureus*, *E.coli*, *Proteus* and *Pseudomonas* species were tested against a panel of nine commonly used antimicrobial agents: Levofloxacin (1 µg), Amoxicillin+clavulanic acid (10 µg), Gentamicin (120 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Tetracycline (10 µg), Enrofloxacin (5 µg), Trimethoprim (30 µg) and Oxacillin (1µg)with the standard Kirby–Bauer disc diffusion method [20]. The results were interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute for antimicrobial susceptibility testing [21]. The susceptibility of identified to isolates resistant to three or more antibiotics were classified as Multidrug Drug Resistance (MDR) strains.

Table 1: Oligonucleotide primer sequences of *coa* and *spa* genes of *S.aureus*.

Target gene	Primer sequence (5'-3')		References
Coa	F	ATA GAG ATG CTG GTA CAG G	Iyer and Kumosani (2011)
	F	GCT TCC GAT TGT TCG ATG C	
Spa	F	TCA ACA AAG AAC AAC AAA ATG C	Wada et al. (2010)
	F	GCT TTC GGT GCT TGA GAT TC	

Molecular detection of *Coa* and *Spa* genes of *S.aureus*

DNA extraction: it was done for ten selected *S.aureus* isolates using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) were performed at RLQP (Reference Laboratory for Veterinary Quality Control on Poultry Production) with some manufacturer's modifications for screening for *Coa,* and *Spa* genes of *S.aureus* isolates. Briefly, 200µlofthe sample suspension was incubated with 10µl of proteinase K and 200 µl of lysis buffer at 56 °C for 10 min. After incubation, 200µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer's recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit. The Oligonucleotide Primers used were supplied from Metabion (Germany) were listed in (Table 1 and 2). For PCR amplification, the primers were utilized in a 25µlreaction containing 12.5µl of Emerald Amp Max PCR Master Mix (Takara, Japan),1µl of each primer of 20 pmol concentrations, 4.5µl of water, and 6µl of DNA template. The reaction was performed in an applied biosystem 2720 thermal cycler.

Analysis of the PCR Products: The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 20µl of the uniplex PCR products were loaded in each gel slot. A gel pilot 100 bp DNA ladder (Qiagen, Germany, GmbH) and gene ruler 100 bp ladder (Fermentas, Sigma) were used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Inno tech, Biometra) and the data were analyzed through computer software. (Table 1 and 2)

Hematologicalstudies: Determination of the total erythrocytic count, hemoglobin concentration, packed cell volume, total leukocytic count and the differential leukocytic count were determined according to [22].

Serum biochemical analysis: The collected sera were assayed for serum biochemistry. The level of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) according to [23] creatinine [24], uric acid [25], total serum proteins [26], albumin [27] and globulin [28]. These parameters were spectrophotometrically assayed by using semi-automated spectrophotometer (Erba-Chem7, Germany) using commercial kits purchased from (Spectrum, Cairo, Egypt).

Immunological studies

- Protein electrophoresis was done using SDS-Polyacrylamide gel electrophoresis according to [29] in the animal health research institute in the biochemistry department.
- **Determination of interleukin-6:** The collected serum showed 100% cross-reactivity with the ELISA kit, and the limit of serum IL-6 detection was 31.2 pg/ml. ELISA assays were performed according to the manufacturer's protocol.
- **Determination of Tumor Necrosis Factor:** The test was drawn according to [30].

Field treatment: Naturally infected chicks received treatment to farms as the following: Levofloxacin (10 mg/kg b.w) [31], was added to the drinking water. Levofloxacin was purchased from Sanofi Aventis company, and prepared as the following: Grinding of tablets (500 mg) with 10 ml distilled water then drinking to chicks according to dose (levofloxacin 10 mg/kg b.w) as concentrate 0.2 ml per os/chick/once daily) for four successive days. Birds were kept under observation after the end of treatment.

Statistical analysis: The obtained data were statistically analyzed by an ANOVA (one way) variance method considering $P < 0.05$ using MiniTab17© software. The significant differences were taken to Fisher multiple range tests to compare the means [32].

Results

In this study, the observed clinical signs in diseased broiler chicks were: swollen foot pad Figure 1: (1), foot bad dermatitis (2), swelling and inflammation of their hock joint (3), inability to stand (4). Post Mortem examination showed that 45 out of 120cases showed congestion of liver with clear

Figure 1: (1), foot bad dermatitis (2), swelling and inflammation of their hock joint (3), inability to stand (4). Post Mortem examination showed that 45 out of 120cases showed congestion of liver with clear perihepatitis, congestion of lung and pericarditis of heart (5 and 6).

Table 2: Cycling conditions and predicted sizes of PCR products for *Coa*, and *spa* genes of the isolated strains.

Target gene	Initial denaturation	Actual cycles (35) °C/sec			Final extension °C/min	Amplified product Size (bp)
		Denaturation	Annealing	Extension		
Coa	94/10	94/ 60	55/60	72/10	72/10	570
Spa	94/5	94/30	55/30	72/30	72/10	226

Table 3: Bacterial causes for affection with arthritis/ Bumblefoot in chicken cases.

Bacterial species	Cases	Total No. of positive isolates bacteria from 120 samples	
		No	%
S. aureus (pure form)	Bumblefoot	10/120	8.33%
S. aureus mixed with *E. coli*	Bumblefoot with respiratory signs	32/120	26. 67%
S. aureus mixed with *Proteus species*		8/120	6. 67%
S. aureus mixed with *P. aeruginosa*		5/120	4. 17**%**
Total		55/120	45. 8%

Table 4: The results of Serotyping of isolated *E.coli and* Congo red binding test.

E.coli Serotypes	Isolates (32)	Congo red binding test
O_{78}	10	Positive
O_{55}	5	Positive
O_{158}	6	Negative
O_{86A}	5	Negative
O_{1}	4	Positive
O_{11}	2	Negative
Total +ve No. of *E.coli* isolates with Congo red binding test		19

Table 5: Antibiotic susceptibility testing of the isolated species.

Antibiotics	The recovered bacterial isolates			
	S. aureus (55)	*E. coli (32)*	*Pr. Mirabilis (8)*	*P. aeruginosa (5)*
Levofloxacin (1 µg))	S	S	S	S
Amoxicillin+clavulanic acid (10µg)	S	I	S	R
Gentamicin (120 µg)	S	I	S	I
Ciprofloxacin (5 µg)	S	I	S	I
Erythromycin (15 µg)	R	I	S	R
Tetracycline (10 µg)	R	S	S	I
Enrofloxacin (5 µg)	R	I	I	R
Trimethoprim (30 µg)	R	R	R	R
Oxacillin (1 µg)	R	R	R	R

perihepatitis, congestion of lung and pericarditis of heart (5 and 6).

The bacteriological investigation for 120swabs sample from broilers suffering from bumblefoot lesions revealed that *S. aureus* was the most frequent bacterial isolate encountered in this study. The overall percentage of *S. aureus* in this study was 45.8% (55/120). *S. aureus* was isolated in pure form in a percentage of 18.18%(10/55) from leg lesions of bumlefoot diseased cases. In addition, it was isolated in mixed form with other bacterial species from the bumble foot diseased cases and also from lung and liver organs of other cases with respiratory signs: 32/120 were mixed with *E.coli* (26.67%); eight isolates were mixed with *Proteus species* (6.67%) and five with *Pseudomonas aeruginosa* (4.17%) as shown in Table 3.

Serotyping of the yielded *E.coli*isolates revealed that ten isolates were of O_{78}, five of O_{55}, six of O_{158}, five of O_{86A}, four of O_{1} and two ofO_{11} as shown in the table (4). With Congo red testing, serotypes of O_{78}, O_{55} and O_{1}were positive while others didn't give the reaction. The Congo red positive result was indicated by the development of orange or bright red colonies, while the white color indicated as a negative result. (Table 4).

The results of antibiotic sensitivity testing of the recovered isolates ofS. aureus, *E.coli*, P. mirabilis and P. aeruginosain this study developed highest sensitivity level against levofloxacin. Gentamycin, ciprofloxacin, amoxicillin, trimethoprim, oxaciliin, enerofloxacin, tetracycline and erythromycin exhibited showed varying degree from intermediate to resistant as showing in Table 5.

Molecular characterization of coa and spa genes of S. aurues isolates showed that 70% and 80% of the tested isolates which were subjected to conventional PCR reaction were positive and gave clear bands at the specific amplicon size: 570 bp, and 226 bp respectively, as shown in Figures 2 and 3.

The hematological findings of naturally infected broiler chicks in this study are reported in the table 6. The hematological parameters revealed a significant decrease in RBCs count, Hb concentration and PCV in the diseased birds indicated anemia as compared with the control group. Also, a significant increase in total leucocytic counts, lymphocyte and monocyte in diseased groups as compared with control groups. In the levofloxacin treated group, significant increases of RBCs, Hb concentration and PCV levels were

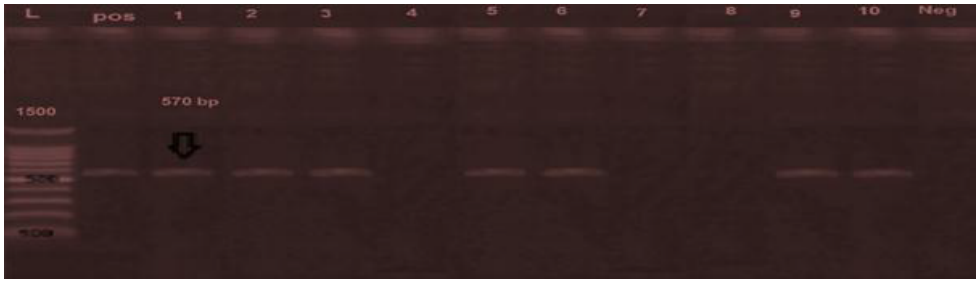

Figure 2: Showed Agarose gel electrophoresis showing positive amplification of the product 570bp fragment of *Coa* gene of *S.aureus* performed with specific primer. L: 100–1500bp DNA ladder; Pos: positive control of *S.aureus*; lanes1-10: 1, 2, 3, 5, 6, 9&10 +ve results of *S.aureus*; lane 4, 7and 8 –ve result; Neg: negative control.

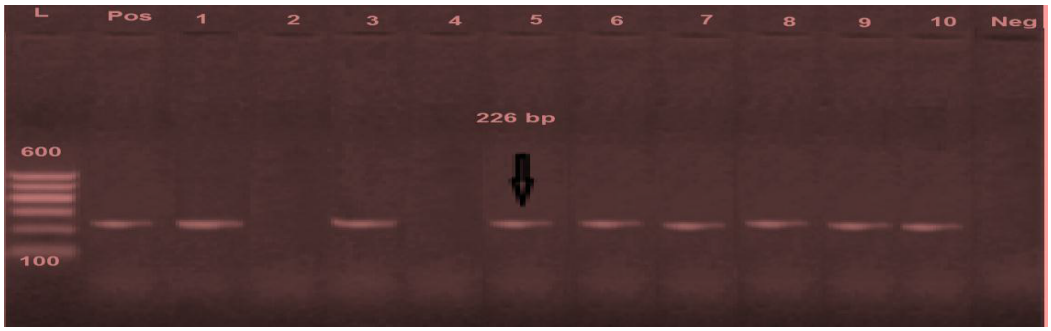

Figure 3: Showed Agarose gel electrophoresis showing positive amplification of the product 226bp fragment of *spa* gene of *S.aureus* performed with specific primer. Lane L: DNA molecular size marker (100-600bp), lanes 1-10:1, 3, 5, 6, 7, 8, 9&10 +ve results of *S.aureus*; lane 2and 4 –ve result; lane (Pos): positive control and lane (Neg): negative control.

Table 6: Hematological parameters in apparently healthy naturally infected and treated broiler chicks in farms. N=20.

Groups/ Parameters	Apparently healthy	Diseased broiler chicks	Treated chicks by levofloxacin
RBCs (10^6/µl)	6.5±0.15^a	3.17±0.2^b	7.1±0.42^a
HB (Gm %)	11.5±0.3^a	7.1±0.22^b	10.82±0.43^a
PCV (%)	35.0±0.43^a	21.3±0.66^b	33.1±0.6^a
WBCs (10^3/ µl)	9.95±0.17^b	13.55±0.2^a	9.86±0.22^b
Heterophile (10^3/ µl)	5.50±0.32^a	3.50±0.22^b	5.20±0.28^a
Lymphocyte (10^3/ µl)	4.00±0.15^b	8.50±0.13^a	4.20±0.33^b
Monocyte (103/ µl)	0.35±0.07a	1.40±0.06a	0.32±0.05a
Eosinophile (103/ µl)	0.10±0.11a	0.15±0.06a	0.14±0.04a

Table 7: Some biochemical and immunological parameters in apparently healthy naturally infected and treated broiler chicks in farms. N=20.

Group /Parameters	Apparently healthy	Diseased broiler chick	Treated broiler chicks by levofloxacin
ALT (u/l)	9.50±0.08^b	13.40±0.11^a	9.23±0.09^b
AST (u/l)	117.5±0.3^b	152.3±0.4^a	111.33±0.32^b
Uric acid (mg/dl)	8.0±0.4^b	11.40±0.32^a	8.7±0.5^b
Creatinine (mg/dl)	1.50±0.02^b	2.00±0.01^a	1.62±0.06^b
T. protein (gm/dl)	0.01^b	8.35±0.12^c	10.36±0.09^a
Albumin (gm/dl)	5.93±0.05^a	3.50±0.01^b	6.31±0.06^a
Globulin (gm/dl)	3.11±0.04^b	4.85±0.05^a	4.05±0.2^b
χ γλοβυλιν (γμ/δλ)	1.92±0.01^b	2.50 ±0.01^a	2.00±0.03^b
βγλοβυλιν (γμ/δλ)	0.19±0.14^a	0.20±0.19^a	0.22±0.15^a
α γλοβυλιν (γμ/δλ)	1.50±0.03^b	2.20±0.05^a	1.60±0.2^b
IL-6 (pg/ml)	122.82±2.5^b	139.2±2.2^a	126.4±3.2^b
TNF-α (pg/ml)	48.27±0.56^b	66.3±0.84^a	53.45±0.92^b

Values with superscripts a, b and c differ significantly (P<0.05) in a row

shown in compared with the diseased group and they were closely related to an apparently healthy group.

In addition, the results of some biochemical and immunological parameters in naturally infected and treated broiler chicks are revealed in the table 7. A significant increase in (AST & ALT), uric acid, creatinine, an elevation in the alpha and gamma globulins. Hypoproteinemia, hypoalbuminemia and significant increase in total globulin in diseased group were recorded as compared with control group. The levofloxacin treated group revealed an improvement in the levels of total protein, albumin, gamma, and alpha globulin as compared to the diseased group. In the present study, the significant increase ofIL-6 and TNF-αin serum was observed in diseased birds when compared with treated birds. The levofloxacin treated group revealed an improvement in the levels of IL-6 and TNF-αin serum.

Discussion

Lameness or leg weakness is symptomatic words describing a condition resulting from several causes either bacterial or viral. It is adversely affected by the poultry performance and may increase morbidity and mortality levels in broiler flocks. Purulent arthritis, bacterial chondronecrosis with osteomyelitis were considered the most common causes of lameness in commercial broilers [33].

In the present study, the observed clinical signs of diseased birds were swollen hock joint, keel bone, unable to stand, foot bad dermatitis, reluctance to move, gradual emaciation and finally death. The same signs were recorded with [34,35]. Postmortem examination of naturally infected chickens showing swelling of joints filled with inflammatory exudates, arthritis of the hock, stifle, as well as congestion of internal organs. These results agreed with revealed that arthritis of the hock, stifle, coxofemoral joints and vertebral osteomylitis. Youssef and Dalia, (2012) [35] reported the postmortem examination of naturally infected chickens showed swelling and necrosis of the comb, necrosis and congestion of liver, spleen, kidney and lungs. Other birds had affected swollen joint filled with inflammatory exudates.

Staphylococcus is normal inhabitants of the skin and mucous membranes and common organisms in environments where poultry were hatched, reared or processed. Most *Staphylococcus* species are considered to be normal flora, but when a breakdown in the natural defense mechanism of host occurs, a pathogenic infection will raise leading to decrease in the rates of weight gain, egg production and great economic losses in broiler farms [35].

According to the bacterial investigation results of bumblefoot or arthritic broiler and or respiratory diseased cases, *S. aureus* was the most frequent bacterial isolate encountered in the recent study (45.8%) among 120 diseased examined cases either in pure or mixed form. Similar results were achieved with [36,37] who detected the prevalence of septic arthritis caused by *S. aureus* within the range of 50.98% collected from different broiler chicken farms with

symptoms of arthritis. Meanwhile, higher prevalence ratio of *S. aureus* was recorded up to 81% from hock joint. Also, (Feizi *et al.,* 2012) [38] stated that a high prevalence ratio (85.71%) of septic arthritis due to *S. aureus* in broilers in Iran. Differently, lower isolation rate of *S. aureus* (36.6%) of arthritic broiler joints in Sharkia Governorate [39].

The most frequent sites of *S. aureus* infection in poultry are bones, tendons, sheaths and joints, especially tibiotarsal and stifle joints [36]. Arthritis was claimed to bad management, dietary and traumatic factors within the bird's enclosure, including improper perching, poor hygiene, piercing on the bottom of the foot and leg fractures [40]. Moreover, pneumonia caused by *S. aureus* represents a serious complication in individuals with cystic fibrosis and patients affected by immunosuppressive therapy [41].

In addition, mixed cultures of *S. aureus* with other bacterial species were also isolated. The results indicated that 32 isolates were mixed with *E.coli* , 8 isolates were mixed with *Pr. Mirabilis* and fivemixed with *Pseudomonas* as shown in table 3. In the same way [42] recovered that four positive *S. aureus* isolates out of sixteen isolates in pure cultures, 10 isolates were mixed with *E.coli* and 2 isolates were mixed with *Proteus mirabilis*. Similar results of mixed infection of *S. aureus* with *E.coli* (28.6%), or *Salmonella* (1.3%), or *Pseudomonas* (1.95%) *and Pasteurella multocida* (1.95%)were isolated from arthritic broiler cases [43]. In addition, (Pleydell *et al*., 2007 and Lutfulkabir, 2010) [44,45] stated that the recovered isolates of *Staphylococcus spp.,* from the infected bone of broilers; were mixed with other bacteria like *E.coli, Mycobacterium avium, Salmonella spp.,* and *Enterococcus*.

Serotyping of the examined *E.coli* isolates clarified that the most prevalent serogroups of *E.coli* isolates were O_{78} followed by O_{55}, O_{158} then O_{86A}, O1 and O_{11} as shown in the table 3. Nearly similar results were detected by [46] reported that serotype O_{78} was the most common (28.2%) from diseased broiler chicken. Also, [43] Labdah *et al*., (2015) classified seventy *E.coli* isolates into 6 different serotypes: O_{78} (30), O_{125} (14), O_{55} (16), O_{166} (6) and O_{146} (4).

As regards to Congo red binding activity as a pathogencity factor of *E.coli* , 19/32 (59.4%) isolates gave positive result while the other serotypes did not bind Congo red dye up to 48 hours post inoculation. These results were similarly to [47] who observed that 20 isolates of 56 (35.7%) were Congo red positive and 36 isolates (64.3%) were negative from broiler chickens in Egypt. Also, [48] showed that 32 out of 53 *E.coli* isolates (60.4%) were Congo red positive.

In this study, as shown in table 3, a lower isolation rate (6.67%) of *Pr. Mirabilis* in mixed culture with *S. aureus* was recorded. In the same manner, [49,43] Hassan *et al*., 2012 mentioned that *Pr. mirabilis* was isolated from 18 months old hens with Bumblefoot and it was able to re-induce the disease experimentally when inoculated into the footpad of chickens. In the same table, a mixed infection with P. aeruginosa was isolated in 4.17% (5/120) of all examined samples. This result nearly similar to (Hebat-allah, 2004) recorded *Ps. aeruginosa* from 3.3% of examined broilers. It is the most common avian pathogens which produce a variety of toxins and enzymes that may contribute to pathogenicity [50,51]. John Barnes (1997) [52] stated that *Pseudomonas* infection might occur due to either skin wounds or inadequate hygiene like (contaminated vaccines, contaminated syringe needles during egg dipping or egg inoculation, the use of contaminated premises from infected flock).

The occurrence of antimicrobial resistance in staphylococci of poultry origin has increased over time in poultry farms due to the frequent use of antimicrobial agents in poultry husbandry. Indeed, antimicrobial agents have been administered for many years, not only to control and prevent disease, but also for growth promotion and improved feed conversion efficiency. The results indicated moderate to high resistance of most antibiotics (trimethoprim, oxaciliin, enerofloxacin, tetracycline and erythromycin) against *S. aureus, E.coli,Pr. Mirabilis* and *Ps.aeruginosa* isolated strains were recorded. However**,** levofloxacin developed the highest sensitivity rate against all isolated bacteria followed by gentamycin, ciprofloxacin, and amoxicillin with varying degrees of sensitivity as shown in the table 5. Levofloxacin is a fluoroquinolone with improved activity in vitro against Gram-positive bacteria including *S. aureus* [53]. Also, (Rohner *et al*., 1992) [54] showed that this group exhibited an additive effect in combination with standard anti-staphylococcal therapy in severe *S. aureus* infections in some experimental studies. Levofloxacin, the isomer of the race mate ofloxacin, has a high level of in vitro activity against *S. aureus* [55] and demonstrated good activity in foreign body experimental models [56]. Based on pharmacokinetic parameters, when levofloxacin of a dose of (10 mg/kg b.w); was given intramuscularly/orally every 24 h in turkeys, it maintains effective plasma concentrations with bacterial infections [31].

The virulence of *S.aureus* is complex. It depends on an array of virulence genes which are clustered into 2 categories: cell surface associated (adherence) and secreted (exotoxins) genes [57]. *Coa* gene (or coagulase) is a major determinant factor for the identification of *S. aureus* strains. It is an extracellular protein that has traditionally been used to differentiate coagulase positive Staphylococcus from the less virulent coagulase-negative Staphylococcus [58].

In this study, the amplified product of the coagulase gene in all examined *S. aureus* strains was cleared at 570 bp. It was recovered in 70% of the examined isolates in this study. In addition, S*pa* gene which encodes for protein A; is mostly used for typing of *S. aureus* [59]. Protein A is an important exo-protein virulence factor which enables the microbe to evade host immune responses. Its molecular weight of 42KD. It is covalently anchored to the peptidoglycan of *S. aureus*. About 90% of protein A is found in the cell wall and the remaining 10% is free in the cytoplasm of bacteria. In MRSA strains of *S. aureus*, protein A is unable to adhere to the cell wall and therefore is released into the media (secretary protein) [60]. In this study, it was found in 80% of the examined isolates. Vintov *et al.* (2003) [61] cleared that the polymorphic *coa* and *spa* genes could be used to investigate the diversity of *S. aureus.*

The obtained results of hematological parameters revealed a significant decrease in RBCs count, Hb concentration and PCV in the diseased birds indicated anemia that was correlated with the severity of infection of bacterial organisms. These results were in accordance with [62,11]. Also, a significant increase in total leucocytic counts, lymphocyte, and monocyte between infected and control groups was recorded. Similarly, Sakiniene *et al*. (1999) [63] reported that *S. aureus* infection could lead to leukocytosis and [64] Lucke *et al.* (2003) recorded a significant increase in total leucocytic count in infected animals. In the levofloxacin treated group, significant increases of RBCs, Hb concentration and PCV levels were shown in compared with the diseased group and they were closely related to an apparently healthy group. Our results indicated a significant decrease in leukocyte in the treated group when compared with the diseased group. These results indicated the levofloxacin antibacterial effect on microorganisms [12,65]. Lee et al., (2017) [66] showed that the administration of 5mg/kg of levofloxacin seems to be effective in killing *E.coli.*

S. aureus secretes proteins which inhibit complement activation, lyes neutrophils, neutralizes antimicrobial peptides and reduces the effectiveness of neutrophil. It could survive in phagosomes, express polysaccharides and proteins which inhibit opsonization by antibody and complement, and it's cell resistant to lysozyme. Furthermore, *S. aureus* has several types of super antigen that corrupt the normal humoral immune response, resulting in immunosuppression [67]. A characteristic manifestation of *S. aureus*-caused pneumonia is the intense host inflammatory response characterized by a rapid and excessive recruitment of neutrophils to the site of infection [68].

The increase in serum AST and ALT levels in this work could be due to liver damage produced by the infected bacteria. Campbell and Coles, (1986) [69] mentioned that the increased of the activity of AST had been associated with hepatocellular damage in birds. Also, they reported elevation of ALT in birds which were infected with bacteria. Our result agreed with [70,71] who observed a significant increase in (AST & ALT) in chicken infected with *E.coli* . The significant change in total protein and albumin in the present work could be due to liver and kidney damage which could be associated with bacterial infection. Similar findings were previously mentioned by [69,71-73].

Bacterial toxins increase the capillary permeability and permitted the escape of plasma proteins into tissue resulting in hypoproteinemia [74]. An elevation in the alpha and gamma globulins usually indicated an activation of the immune system which is due to infection or inflammatory diseases [75] and could be associated with bacterial septicemia or chronic infection [74-76]. An increase in beta and gamma globulin in chickens were recorded in an experimentally infected chickens with *E.coli* and *S. aureus* [76]. The levofloxacin treated group revealed an improvement in the levels of total protein, albumin, gamma, and alpha globulin as compared to the diseased group.

The increase in uric acid and creatinine could be due to the effect of the microorganisms and its toxins on the kidneys. Our results are completely agreed with [11,71,72,77] who reported highest creatinine and urea levels in case of the renal disease. Uric acid and creatinine were significantly increased in respiratory affection birds. This increase might be attributed to the increased protein catabolism, febrile respiratory diseases, impaired cardiac function and decreased renal blood flow [78].

Interleukin-6 is one of the most pleiotropic interleukins released at sites of injury or infection [79]. Some investigators reported that IL-6 could activate osteoclasts which increase the damage of joints during the arthritic process [80]. Increased serum IL-6 levels had been observed throughout the course of *S. aureus* arthritis in several animal models. Interleukin-6 is produced by many different cell types and acts on B-lymphocytes, T-lymphocytes, hepatocytes, hematopoietic progenitor cells, and cells of the central nervous system [79]. In the present study, the significant increase ofIL-6 and TNF-αin serum was observed in diseased birds when compared with treated birds, which was similar to the reports by (Zhou *et al*., 2007) [79] in a model of staphylococcal arthritis. However, the *S. aureus*-infected birds treated with levofloxacin showed reduced levels of IL-6 in serum, as compared with the diseased birds. Zhou *et al*., (2007) [79] showed that IL-6 activities and concentrations were reduced ($P < 0.05$) in the serum of infected broilers treated with levofloxacin compared with birds injected only with *S. aureus*.

Tumor necrosis factor-α (TNF--α) is the major macrophage-derived cytokines present in the rheumatoid joint and both induce the synthesis and secretion from synovial fibroblasts of matrix-degrading proteases. The purification and cloning of a molecule called "cachectin", which causes wasting in chronic diseases, was subsequently found to be identical to TNF-α. In general, the systematic inflammatory response is considered to be mediated through the interactions among cytokines, including the activation of tumor necrosis factor (TNF) and the corresponding receptors and neuroendocrine pathways (Woodcock and Morganti-Kossmann, 2013). TNF-α is produced primarily by macrophages, a lesser extent by lymphocytes, monocytes and macrophages, but a number of non-immune cell types, including fibroblasts, neurons, keratinocytes and smooth muscle cells, also produce TNF. TNF-α acts as a key intermediary in the local inflammatory immune response and is an acute-phase protein that initiates a cascade of cytokines. Furthermore, high levels of TNF result in increased vascular permeability, thereby recruiting macrophages and neutrophils to the site of injury and/or infection (Esposito and Cuzzocrea, 2009 and Woodcock and Morganti-Kossmann, 2013).

Levofloxacin drug, a member of the fluoroquinolone family, possesses excellent potent activity against a wide microbial spectrum. Hu *et al*., (2002) [65] showed that Levofloxacin was effective for the control of avian staphylococcus is especially when was administered in the drinking water. It had also been demonstrated that the levels of tumor necrosis factor-α (TNF-α) and IL-6 in serum were associated with the severity of the infectious process. Additionally, IL-1β and TNF-α could induce chondrocytes

and synovial macrophages to release a high production of IL-6locally in bone, which consequently increases damage to joints during the arthritic process [79].

Conclusion

Staphylococcal infections in poultry are high especially in Bumblefoot disease or arthritis disease. Hence, due to its epidemiological importance, the measures must be taken in the field to minimize the zoonotic disease transmissible from poultry to humans. The hematological, biochemical and immunological analysis (IL-6 and TNF) could reflect the pathogenesis of the microbe. Levofloxacin treatment succeeded in the treatment of the disease in the broiler farm.

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