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Original Article *Pathology*

Evaluation of turkey arthritis syndrome by traditional and advanced diagnostic techniques

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ABSTRACT

Objective: To detect possible causes of arthritis in turkey flocks with emphasis on molecular detection of bacterial pathogens and estimation of their pathology pattern.
Design: Descriptive study
Procedure: A total of 100 arthritic legs from 125 French turkey birds were collected from different farms in Sharkia Governorate. Specimens from joint capsule, tendons, and synovial fluid of leg joints were subjected to bacteriological and direct PCR examinations. Moreover, skin, cartilage of articular surface and epiphysis were undergone pathological examination. Blood samples were collected before scarification for both hematological and biochemical examinations.
Results: Forty percent (40%) bacterial incidence rate including *Staphylococcus spp., E. coli*, *Salmonella spp., Klebsiella spp.,* and Pseudomonas aeruginosa were recorded. Molecular detection of these pathogens revealed rapid and more accurate results of 46%. A high prevalence of multidrug resistance was detected, but all isolates were susceptible to amikacin and florfenicol. Genotyping of isolates revealed the presence of strong virulence markers. Postmortem examinations revealed joints swelling, hyperkeratosis, erosions,and/or ulcerations. Further investigations on positive infected samples revealed a significant decrease in RBCs, Hb, PCV %, lymphocytes, total protein, and albumin ($P<0.05$). However, there was a significant increase in total leukocytic count and heterophile, CRP, ALT, AST, uric acid, creatinine, and total globulin ($P<0.05$). Epidermal leukocytic cells infiltrations, tendonitis, synovium fibrosis, erosions, and/or ulcerations of articular cartilage were recorded.
Conclusion: Turkey arthritis is a serious problem facing turkey flocks in Egypt. Multiplex real-time PCR assay offered an effective alternative to traditional typing methods for the identification of the etiological agents involved in the infectious arthritis in turkey.

Keywords: Turkey, Arthritis, Bacterial pathogens, multiplex real-time PCR, Histopathology

ABBREVIATIONS: PCR: Polymerase chain reaction, CNS: Coagulase -negative staphylococci, RBCs: Red blood cells, Hb: Hemoglobin, PCV: Packed cell volume, CRP: C-reactive protein, AST: Serum aspartate aminotransferase, ALT: Alanine aminotransferase

1. INTRODUCTION

Arthritis is a worldwide welfare issue in poultry production especially turkeys, caused by many bacterial pathogens [1].The most common form of infection involves tenosynovitis (inflammation of tendon sheaths) and arthritis of the hock and stifle joints [2]. The affected joints, usually the hocks, are hot, swollen and painful and affected birds are usually depressed, lameness and reluctant to walk-in tenosynovitis, synovial membranes of tendon sheaths become thickened and edematous, with fibrinous exudate within and around the tendon sheaths leading to reduction of productivity, besides representing a sanitary problem [3]. As most bacterial pathogens could play a role in the incidence of respiratory disease in birds. The respiratory tract infections are of eminent importance in the manufacture of turkeys because of high mortality in poorly managed cases which lead to large economic losses [3].

The most important locomotor affection is septic arthritis which means a microbial infection of one or more joints and characterized pathologically by abscess foci formation either broken with deep ulcer and thickened skin or gangrenous dermatitis. Abscesses contained solidified cheesy-like material with ulcerative epithelial damage, marked hyperkeratosis, and extensive dermal suppuration with the presence of granular basophilic structures microscopically; probable to be bacterial colonies. Extensive fibrous connective tissue proliferation and dermal angiogenesis were also detected [4]. Multifocal degenerative changes in the articular surfaces (fibrosis, metaplasia of cartilage and bone), enlarged joints, thick friable

tissue around the joint and severe articular erosions, a severe proliferation of fibrous connective tissue in /around articular surfaces, severe keratopathy with thick dull yellow exudate around the joints associated with mixed bacterial infection [5].

Also, measurement hematological parameters and CRP play a significant role in the diagnosis of this disease. CRP is synthesized in the liver and its physiologic role is to bind to phosphocholine expressed on the surface of dead or dying (apoptosis) cells to activate the complement / immune system, which enhances phagocytosis by macrophages [6]. Levels of CRP begin to rise within 2 hours. The rapid action of CRP makes it a participant in the acute or first phase of the inflammatory process, which is why it is often called an "acute-phase protein" [7, 8].

Therefore, the purpose of our study was to validate the recent and traditional techniques in investigation and diagnosis of the microbial communities as well as the hematological, biochemical, and histopathological changes accompanied by turkey arthritis syndrome that unfortunately hasn't received great attention and discussed in very little papers. Additionally, it was crucial to find the drug of choice in light of the random use of antibiotics which resulted in severe and multiple resistant strains to overcome this economic problem.

2. MATERIALS AND METHODS

2.1. Field samples

One hundred and twenty-five French turkey birds (Hybrid Grade Maker, Hybrid Optima, Hybrid Converter and Hybrid XL breeds) of ages varied from 60 to 185 days from both sex suffering from lameness with swollen joints were collected from different farms in Sharkia governorate.

Two blood samples were collected from the wing vein of each bird: the first sample was collected on di-potassium ethylene diamine tetraacetate (EDTA) as an anticoagulant to covering out hematological examination and the second one was collected in a clean, dry centrifuge tube without anticoagulant, left to clot at room temperature then centrifuged at 3000 rpm for 15 minutes. The sera were collected for biochemical studies.

Tissue samples were collected from the arthritic joints of both freshly dead and sacrificed birds under complete septic conditions after the disinfection of the joint externally with alcohol for microbiological examination by traditional methods, direct PCR examination for bacterial pathogens. Finally collected joints tissues were fixed in 10% formalin for histopathological examination.

2.2. Bacterial isolation and identification

Samples were enriched in nutrient broth at 37°C for 24 h then inoculated into Tryptic Soya Broth and Rappaport Vassiliadis (for *Salmonella* enrichment) then subjected to conventional methods for isolation and identification according to methodology recommended by Quinn et al.[9].

2.3. Antibacterial susceptibility testing

Antibacterial sensitivity tests were carried on the obtained isolates using the disk diffusion method on Mueller-Hinton Agar (OXOID), according to the procedure recommended by the Clinical and Laboratory Standards Institute **(**CLSI [10]**)**. All isolates were tested for various routine antimicrobial drugs (OXOID), the tested antibiotics and their concentrations on µg/disk were as following: streptomycin (S; 10), amikacin (AK; 30), novobiocin (NV; 30), amoxicillin-clavulanic acid (AMC; 30), ampicillin (AMP; 10), florfenicol (FLO; 30), neomycin (N; 30), lincomycin (L; 2), doxycycline (DO;30) and colistin (CT;10). The inhibition zones, in millimeters, were measured in duplicate and scored as sensitive, intermediate, and resistant categories following the critical breakpoints recommended by the Clinical and Laboratory Standards Institute.

2.4. Molecular characterization

2.4.1. Genomic extraction

The DNA was extracted from samples using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) according to the manufacturer's recommendations.

2.4.2. Primer specificity

Oligonucleotide primer sets that specifically amplify the target sequences of Virtual primers specificity was checked in a BLAST search available through the National Centre for Biotechnology Information (NCBI), website www.ncbi.nlm.nih.gov ,before they were synthesized at Bio Basic, Canada INC. Further, six reference strains of *S. aureus, coagulase-negative staphylococci, E. coli, Salmonella* spp., *Klebsiella pneumoniae, Pseudomonas aeruginosa* were used for testing the specificity of primers as well as positive controls.

2.4.3. PCR amplification by multiplex real-time PCR direct from samples

The multiplex real-time PCR assay was simultaneously carried out in duplicate for the detection of 6 tested bacterial pathogens. The optimal PCR amplification reaction mixture contained 12.5 master mix (Sensifast Prob, No Rox, Bio-86050), 1 µL of 100 pmole of each primer (Sigma, USA), 1 µL of 50 pmole of each primer, 6.5 µLof template DNA. PCR amplification was performed in one step thermocycler (Germany) with the following cycling conditions at 95 ˚C for 5 min, 95 ˚C for 10 sec, annealing 60 ˚C for 50 sec, and repeated for 40 cycles. Positive controls (reference strains) and negative controls (containing no DNA) were included in each assay run.

2.4.4. PCR amplification for some bacterial virulence genes by conventional PCR

The multidrug -resistant bacterial isolates were screened for the presence of the major virulence factors with adjusting the final volume of the reaction mixture to 25 µL consisting of 12.5 µL of DreamTaq TM Green Master Mix (2X) (Fermentas, USA), 1 µL of 100 pmole of each primer (Sigma, USA), 5 µLof template DNA and water nuclease-free up to 25 µL. Target genes, primers sequences, amplified product size and cycling conditions were illustrated in Tables 1-3.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in TBE (1x) buffer. For gel analysis, 15µl of the products were loaded in each gel slot. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

2.5. Hematological studies

The RBCs count, Hb concentration **(** gm/dl)**,** PCV (%), and the total and differential leukocytic counts were measured according to Thrall et al. [11]

2.6. Biochemical studies

The CRP was determined by ELISA Kits according to Banerjee *et al.* [12]. AST and ALT activities were measured according to Murray [13] **.** Serum uric acid was determined according to Young [14]**,** serum creatinine level according to Burtis & Ashwood [15], total serum protein was measured according to Doumas et al.[16], albumin was determined according to Drupt [17], globulin was calculated as the difference between total protein and albumin.

***2.7.** Gross lesions and histopathology*

Specimens in the form of several cut sections from the affected joints (stifle joints, hock joints and metatarsal joints) including different parts as; skin, tendons, cartilages, and epiphysis were collected from sacrificed turkey birds suffering from arthritic joints. Specimens were fixed in 10 % neutral buffered formalin for histopathological examination. Paraffin tissue sections of 5-7 micron thick were prepared and stained with hematoxylin and eosin then examined microscopically [18].

2.8. statistical analysis

Data analysis was performed by SPSS version 22 for windows. One-way ANOVA was used to assess hematological and biochemical parameters. Also, the proportion difference between PCR and re-isolation results was done by using medcalc statistical software. Chi-Square Test was used to evaluate if the difference between traditional and molecular assays were statistically significant or not. A $P < 0.05$ value was considered statistically significant.

3. RESULTS

3.1. Postmortem lesions

Various pictures and variable degrees of joints swelling and toes curling were observed in the examined birds. The gastrocnemius tendon sheath along the hock join, the proximal tarsometatarsal joints were the most affected leg parts. Our results revealed skin with hyperkeratosis (thickened, cracked and tough skin) in most cases with ulceration few cases revealed suppuration. Open joints and tendon sheaths with viscid grey to yellow turbid exudate in positive bacterial infection cases were observed, Suspected synovium fibrosis (stretched , decreased size and depressed when compared to normal areas are could be observed in some chronic non-treated cases. Gross lesions associated with different isolated microbes were demonstrated in detail in lesions score **(Table. 4)** and **so**me of these forms of injuries were demonstrated in **Figure 1.**

Table 4. Lesion score of different articular parts in turkey suffering septic arthritis.

Affected parts	Lesions	Types of infection	Lesion Severity
Skin	Dermal hyperkeratosis	All mixed infection with *Staph spp.*	+++
	Subdermal edema	All mixed infection included *Staph* spp.	++
	Heterophile cells Infiltration	All bacterial infection	+++
	Lymphocytic cells infiltration	All pathogens	+++
Tendons and Synovium	Tendonitis	All mixed infection	++
	Fibrosis	All bacterial infection	++
	Heterophile cells Infiltration	All single and mixed infection	+++
Cartilage	Necrosis of chondrocytes	All bacterial infection	++
	Erosions and ulcers	All mixed infection included *Staph* spp.	++
Bone	Decalcification	All bacterial cases	+
	Epiphyseal cyst		
	Heterophile cells Infiltration	All bacterial infection	+++

Severe +++ **Moderate ++** **Mild +**

3.2. The recovery rate of isolation and identification

Bacteriological examination revealed that 40% of collected samples were positive. They were identified as *Staphylococcus aureus* (27%), followed by and coagulase-negative *Staphylococcus* (*CNS*) and *Salmonella* spp with a percent of 15% for each, other bacterial infections were also recorded, but with lower percentages, as shown in **Table 5**.

Regarding molecular examination, 46% of samples were positive for arthritis representing significantly higher percentages than those detected by traditional methods.

Overall, according to PCR results, 13 samples only represent a single infection and the rest represents the mixed ones (**Figure 2**).

Table 1. Primers and probes sequence for real-time PCR amplification

Microorganism	Oligo name	Sequence 5″-3″
E. coli	*E. coli.F*	ATCGTGACCACCTTGATT
	E. coli.R	TACCAGAAGATCGACATC
	E. coli.Pro.	HEX-CATTATGTTTGCCGGTATCCGTTT- BHQ2
S. aureus	*S.aureus.F*	ATCGTGACCACCTTGATT
	S.aureus.R	TACCAGAAGATCGACATC
	S.aureus.Pro	HEX-CATTATGTTTGCCGGTATCCGTTT-BHQ1
Pseudomonas	*Pseudomonas.F*	CCTGACCATCCGTCGCCACAAC CGCAGCAGGATGCCGACGCC
	Pseudomonas.R	FAM-CCGTGGTGGTAGACCTGTTCCCAGACC-BHQ1
	Pseudomonas.Pro	
CN.staph	*CN.staph.F*	GGGTGACTAACAGTGGA
	CN.staph.R	GCGGATCCATCTAAGTGA
	CN.staph.Pro.	FAM-GGATAATATATTGAACCGCA-BHQ1
Salmonella	*Salmonella.F*	GGAGTATGGTTGCAAAGCTGA
	Salmonella.R	GGTGAGGTTTCCCGTGTTG
	Salmonella.Pro	Cy5-AAGGAATTGACGGAAGGGCA-BHQ1
Klebsiella	*Klebsiella.F*	TCCGGGATGCGTGACGTTGC
	Klebsiella.R	TGCTGCGCGAATGAAGACGA
	Klebsiella.Pro	FAM-TCATGGAGAATCGCTGGGGGAAAGC-BHQ1

Table 2. Primers sequence of the virulence markers of each pathogen

Microorganism	Target gene	Primers sequences	Length of amplified product (bp)	Reference
Salmonella	***mgtC***	TGA CTA TCA ATG CTC CAG TGA AT ATT TAC TGG CCG CTA TGC TGT TG	677	Huehn et al.[46]
	sopB	TCA GAA GRC GTC TAA CCA CTC TAC CGT CCT CAT GCA CAC TC	517	
Staphycocci	***spa***	TCA ACA AAG AAC AAC AAA ATG C GCT TTC GGT GCT TGA GAT TC	226	Wada et al. [47]
	icaA	CCT AAC TAA CGA AAG GTA G AAG ATA TAG CGA TAA GTG C	1315	Ciftci et al. [48]
E. coli	***ompA***	AGCTATCGCGATTGCAGTG GGTGTTGCCAGTAACCGG	919	Ewers et al. [49]
	neuC	GGTGGTACATTCCGGGATGTC AGGTGAAAAGCCTGGTAGTGT	670	
P. aeruginosa	***aprA***	GTCGACCAGGCGGCGGAGCAGATA GCCGAGGCCGCCGTAGAGGATGTC	993	Sabharwal et al. [33]
	tox A	GGAGCGCAACTATCCCACT GGTAGCCGACGAACACATA	150	
Klebsiella spp.	***magA***	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1283	Compain et al. [50]
	rmpA	CATAAGAGTATTGGTTGACAG CTTGCATGAGCCATCTTTCA	461	

3.3. Antibiotic susceptibility results

The tested pathogens showed different percentages of resistance and susceptibility to different antibiotics. All isolates revealed absolute resistance to amoxicillin-clavulanic acid and novobiocin. However, the isolates showed the ultimate susceptibility to amikacin and florfenicol, (**Table** 6).

Moreover, the multidrug -resistance phenomenon was observed through almost isolates including 23% of *Staphylococci* species (10/43), 40% *E. coli* (4/10), 26% Salmonella (4/15), 66.6% *Klebsiella* spp. (2/3) and 50 % *P. aeruginosa* (1/2).

3.4. Virulence determinants of the obtained multidrug- resistant isolates

The results indicated a clear abundance of these virulence genes, which were detected in 18 of 21 analyzed isolates (85.71%). Regarding Salmonella isolates, 50% of isolates were harbored *mgtC* and the rest exhibited another tested gene (*sopB*). Among tested staphylococci species, all tested isolates harbored *icaA* and all examined *S. aureus* carried *spa* gene. Analyzing the PCR profiles of 4 *E. coli* isolates revealed that the variations in genotypes to *ompA* and *neuC* genes where only one of them harbored both genes and the rest carried *ompA* gene alone. Furthermore, *aprA* and *tox A* genes were simultaneously present in all tested *P. aeruginosa* isolates. Among the genes coded virulence profile of tested *Klebsiella* isolate, *magA* was only detectable and yielded a 1,283 bp specific sequence (**Figure 3**).

Table 3: PCR amplification and cycling protocol of virulence genes of bacterial pathogens

Thermal profile	*Salmonella*		*Staphycocci*		*E.coli*		*P. aeruginosa*		*Klebsiella* spp.	
	mgtC	*sopB*	*Spa*	*icaA*	*ompA*	*neuC*	*aprA*	*tox A*	*magA*	*rmpA*
Initial denaturation	94ºC 5 min	94ºC 5 min	94˚C 5 min	94°C 5 min	94°C 5 min	94°C 5 min	95˚C 2 min	95˚C 2 min	95˚C 15 min	95˚C 15 min
Denaturation	94ºC 30 sec	94ºC 30 sec	94˚C 30 sec.	94°C 30 sec	94°C 30 sec	94°C 30 sec	95˚C 40 sec	95˚C 40 sec	94˚C 30 sec	94˚C 30 sec
Annealing	58ºC 45 sec	58ºC 45 sec	55˚C 30 sec	49°C 1 min	55°C 40 sec	60°C 40 sec	50˚C 1 min	65˚C 1 min	55˚C 30 sec	55˚C 30 sec
Extension	72ºC 45 sec	72ºC 45 sec	72˚C 30 sec	72°C 1 min	72°C 45 sec	72°C 45 sec	72˚C 2 min	72˚C 2 min	72˚C 30 sec	72˚C 30 sec
Final extension	72ºC 10 min	72ºC 10 min	72˚C 7 min	72°C 12 min	72°C 10 min	72°C 10 min	72˚C 10 min	72˚C 10 min	72˚C 7 min	72˚C 7 min
Amplification cycles	35	35	35	35	35	35	30	30	35	35

Table 6. Antibiograms of different bacterial pathogens

Microorganism	Number of resistant isolates to each antimicrobial agent									
	S	AK	NV	AMC	AMP	FLO	N	L	DO	CT
S. aureus (27)	10	0	27	27	11	0	10	11	9	12
CNS (15)	5	0	15	15	5	0	4	5	5	5
E. coli (10)	7	0	10	10	4	0	3	4	4	4
Salmonella (15)	6	0	15	15	6	0	6	7	6	6
Klebsiella (3)	2	0	3	3	1	0	2	2	1	2
P. aeruginosa (2)	1	0	2	2	1	0	1	1	1	1
Total (72)	31	0	72	72	28	0	26	30	26	30
% Resistant*	43.05	0	100	100	39.4	0	36.6	41.6	36.6	41.6
% Intermediate	38.88	0	0	0	16.66	0	12.5	44.44	44.44	30.55
% Susceptible	18.07	100	0	0	43.93	100	50.9	13.9	18.95	27.84

***** The percentage of the total number of isolates resistant, intermediate, or susceptible for a particular antimicrobial is indicated in the last three rows below each antimicrobial.

Table 5: Incidence rate of bacterial pathogens by traditional and molecular assays.

Bacterial pathogens	Conventional culture results	PCR results
S. aureus	27	31
CNS	15	19
E. coli	10	12
Salmonella species	15	17
Klebsiella	3	4
P. aeruginosa	2	7
Total	72*	90*

*: Indicates significant variations (*P* value < 0.05)

3.5. Hematological parameters measurements

In the present study, the hematological parameters showed a significant decrease in RBCs count, Hb concentration and PCV% in the diseased birds. The leukogram of infected turkeys showed leukocytosis associated with heterophilia , monocytosis, and lymphopenia, non-significant change in basophils, and eosinophils (**Table 7**).

3.6. The biochemical parameters

The obtained results of the biochemical analysis in **Table 8** revealed an increase in CRP, AST, and ALT activities. Also, a significant decrease in protein hypoproteinemia was detected. Hypoalbuminemia was observed as well as an increase of total

globulin, serum uric acid, and creatinine in diseased turkeys compared to the control group.

Table 7. Hematological parameters (mean± SE) in turkey birds affected by infected group compared with control group (N= 5)

Parameter	Non-infected	Infected
RBCs x 10^6/ul	2.2±0.11	1.22±0.08***
HB gm/dl	9.69±0.34	7.52±0.43**
PCV %	30.08±1.05	22.78±1.43**
WBCs x10^3 /UL	6.33±0.37	7.8±0.30*
Heterophil %	31.69±0.84	47.2.4±2.44***
Lymphocytes %	57.52±1.03	37.8±2.33***
Monocytes %	4.46±0.18	8.8±3.77**
Basophil %	4.64±0.34	4.2±0.32
Eosinophil %	1.42±0.16	2±0.55

Significant at P < 0.05 **: Significant at P< 0.01 ***: Significant at P< 0.001

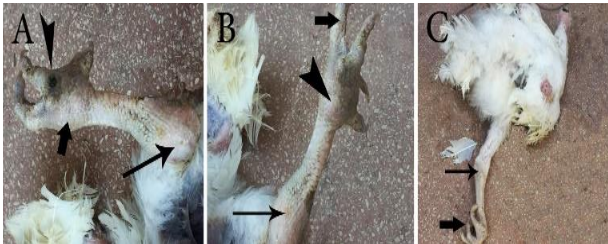

Figure 1: Lame legs of infected turkey (Hybrid Grade Maker , Hybrid Optima, and Hybrid XL breeds)suffering from septic arthritis showing; **(A)** Swollen hock joint **(thin arrow)** and intertarsal joints **(thick arrow)**, necrotic area **(head arrow)**, **(B)** Swollen hock joint **(thin arrow)** and intertarsal joints **(head arrow)**, swollen toes **(thick arrow)**, **(C):** swollen hock joint **(thin arrow)** and intertarsal joints with curved toes **(thick arrow)**.

3.7. Microscopical results

Lame legs in infected arthritic turkeys revealed several forms of tissues injurious. The most commonly observed lesion in the skin was hyperkeratosis with/without focal areas of dermal papillae expansion into the epidermis **(Fig.4a)**. The articular cartilage exhibited variable degrees of chondrocytes necrosis, while the joint capsule of most common cases revealed mononuclear leukocytic cells infiltration of mainly lymphocytes **(Fig.4b)** .Partial fibrosis of the synovium with or without mononuclear cells infiltration also observed **(Fig.4c)** .Another lesion of the articular cartilage exhibited multifocal erosions and/ or ulcerations **(Fig.4d)**, moreover the epiphyseal (articular) bone of the affected joint showed focal decalcification with/without leukocytic cells infiltration**(Fig.5a)**.Some articular joints showed moderate to severe congestion of tendon blood vessels **(Fig.5b)**. In heavily infected cases joint epiphysis of injured joints showed cyst formation in the distorted epiphysis center **(Fig.5c)**. Previously mentioned microscopical lesions were demonstrated in **Figures (4&5).**

Table 8: Some Biochemical parameters of liver and kidney function (mean±SE) in serum of turkeys birds affected by bacterial arthritis compared with control group (N=5)

Parameter	Non-infected	Infected
CRP (ng/ml)	5.6±1.21	24±5.34**
ALT (U/L)	11.54±0.95	23.25±1.98***
AST (U/L)	40.2±2.85	94.5±7.84***
Uric acid (mg/dl)	5.18±0.27	7.18±0.68*
Creatinine (mg/dl)	0.73±0.07	1.62±0.21**
T.Protein (g/dl)	6.36±0.25	4.5±0.12**
Albumin (g/dl)	3.52±0.14	0.85±0.08***
Globulin (g/dl)	2.84±0.21	3.65±0.19**
A/G ratio	1.24±0.07	0.23±0.13**

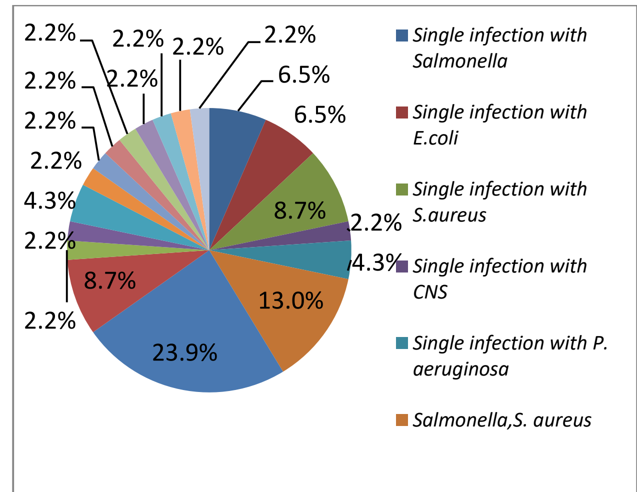

Figure 2: Molecular results of prevalence rate for single and mixed pathogens involved in arthritis by multiplex real-time PCR

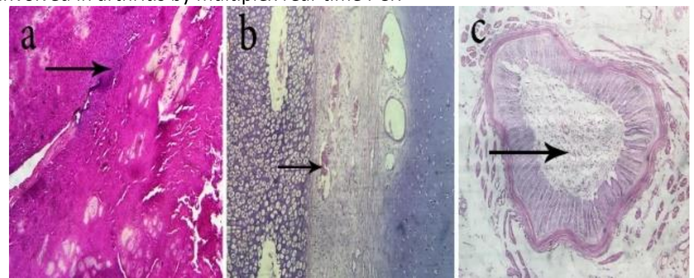

Figure 5. Sacrificed turkey joints suffering arthritis showing; (a) Photomicrograph of the epiphyseal (articular) bone of 8 months old Hybrid Optima turkey showing focal decalcification with leukocytic cells infiltration (arrow) (H&E x100), (b) Photomicrograph of articular surface of 6 months aged Hybrid Grade Maker turkey showing congestion of tendon blood vessels (H&E x100), (c) Photomicrograph of joint epiphysis of 9 months aged Hybrid converter turkey showing cyst formation in the distorted epiphysis center (H&E x200).

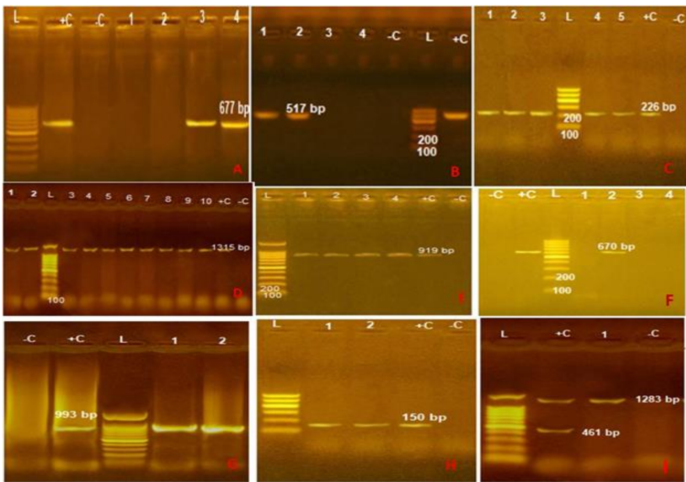

Figure 3. Agarose gel electrophoresis of DNA fragments generated of virulence markers of bacterial pathogens. Lane L: DNA molecular ladder (weight marker, 100 bp); Lanes –C: Negative control; Lanes +C: Positive control. (A, B) PCR amplification products with mgtC and sopB primers targeted 4 Salmonella isolates; (C) PCR amplification products with spa primer targeted 5 S. aureus isolates, (D) PCR amplification products with icaA primer targeted 10 Staphylococci isolates (5 S. aureus, 5 CNS) , (E, F) PCR amplification products with ompA and neuC targeted 4 E.coli isolates, (G, H) PCR amplification products with aprA and tox A targeted 2 P. aeruginosa isolates, (I) Multiplex PCR amplification products with magA and rmpA generating the specific amplicons at 1283 and 461bp, respectively targeted 1 Klebsiella isolate

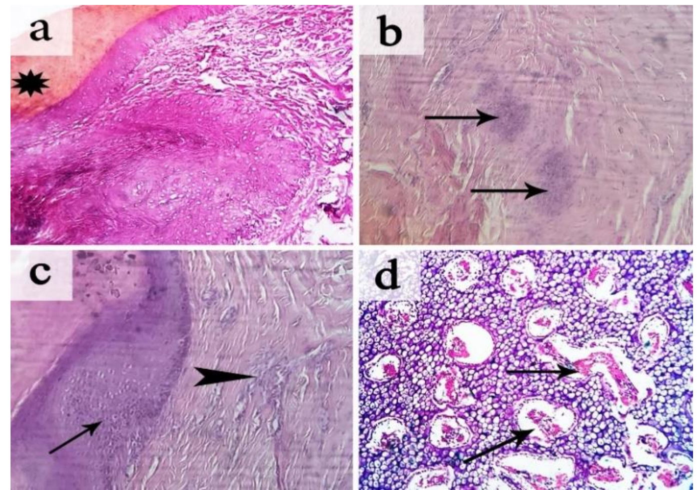

Figure 4. Sacrificed turkey joints suffering septic arthritis showing; (a) Hyperkeratosis (star) of 4 months aged Hybrid Grade Maker turkey with focal area of dermal papillae expansion into the epidermis (H&Ex200), (b) Photomicrograph of the joint capsule 7 months aged Hybride Converter turkey showing mononuclear cells infiltration (arrows) (H&Ex100), (c) Photomicrograph of the joint capsule 10 months aged Hybride Grade Maker turkey showing fibrosis of the synovium (head arrow) with mononuclear cells infiltration (arrow) (H&E x100), (d) Photomicrograph of the articular cartilage of 13months aged HybrideXL turkey showing multifocal erosions and ulcers (arrow) (H&E x100).

4. DISCUSSION

Purulent arthritis, bacterial chondronecrosis with osteomyelitis were considered the most common causes of lameness in turkey breeds. In the present study, the isolation and identification results of bacterial agents using conventional methods depending on their phenotypic characteristics on the specific media for each microbe revealed that bacterial infection detected within the range of 40%, and the most predominated organisms were *Staphylococcus* spp., *Salmonella* spp. in addition to *E. coli*. These data were fairly following Manohar et al. [19] and Kierończyk et al. [20] who demonstrated that multiple organisms can cause septic arthritis in poultry.

The PCR assays were optimized successfully and showed a significantly higher rat e of detection of arthritis bacterial pathogens singly and mixed in turkey than that was detected by the conventional culture methods and these findings were commonly reported by other investigators [27, 28].

The most frequent samples of mixed infections were *S. aureus* and CNS with a percentage 23.9% followed by *S. aureus* and *Salmonella* with a percentage 13%. The obtained results were similar to those reported in South Africa, [3], Brazil [1], and the USA [23].

The presence of mixed infection in arthritic joint samples was proved in previous studies as Tawfik et al. [24] who mentioned that *Salmonella* spp. associated with arthritis in poultry is found either as the sole bacterium genus or as part of a cocktail of bacteria as *S. aureus and E. coli.* These data support the importance of joints as the site for isolation in screening livestock to identify emerging pathogens.

Concerning the antimicrobial susceptibility pattern of the obtained isolates, higher rates of sensitivity were observed to amikacin, florfenicol, and neomycin with percentages comparable to those found in many developing countries, especially Ghana [25] and Iran [26].

Also, relatively high level of antibiotic resistance among the examined isolates was detected especially to novobiocin, amoxicillin-clavulanic acid, streptomycin and colistin which mostly resulted from the long-term and widespread abuse of these antimicrobials in animal farms representing a significant disease burden in Egypt. Furthermore, 29.1% (21/72) of tested isolates exhibited multidrug resistance, which was lower than that recorded in Japan and Nigeria by percentage 40.6% and 32.1%, respectively. The consequence of this behavioral change over the years and countries is the gradual emergence of

resistant bacterial strains refractory to multiple antimicrobial agents [27].

The large genome and its genetic complexity allow these pathogens to thrive in diverse ecological conditions. Multiple virulence factors impact their pathogenesis.

Regarding Salmonella, we examined the isolates for the *sopB* and *mgtC* genes, and the results revealed that each of them was detected in 50% of the isolates as compared with 100% of the isolates tested in India [28]. The *mgtC* magnesium transport protein is a putative P-type ATPases which encodes a membrane protein that is indispensable for Salmonella survival in macrophages [29]. *SopB* gene implicated in the translocated effector protein of T3SS for SPI-5 translocated into the host cytosol, where it mediates inflammation and fluid secretion in the intestinal mucosa [30].

Molecular profile of examined staphylococci isolates (5 *S. aureus* and 5 CNS) revealed that *icaA* detected in all of them and *spa* gene was detected in all examined *S. aureus* isolates (100%) indicating high pathogenicity of these isolates represented in biofilm formation and spread in the host [31].

Concerning *E.coli*, *ompA* and *neuC* genes were detected with percentage 100% and 25%, respectively indicating strong virulence biomarkers of the examined isolates. The perfect beta-barrel structure of *ompA* is important for outer membrane stability and the presence of K1 capsule is encoded by *neuC* [32].

Furthermore, 100% prevalence of *toxA and aprA* genes in all analyzed *P. aeruginosa* strains promote the development of bacteria within the infected host and interfere with the host immune system [33].

Regarding *K. pneumoniae* isolate, *magA* gene was only detected which an important virulence gene in the host invasion, with a length of 1.2 kb and encoding an enzyme protein known as wzy, which acts as a polymerase in capsule synthesis. Therefore, the presence of *magA* in *K. pneumoniae* strain confers resistance to serum and phagocytosis [34]

The obtained findings were following Lucke et al. [35]and Youssef et al.[22], as they found a significant increase in total leukocytic counts, lymphocyte, and monocyte between infected and control groups especially in infected birds with *S. aureus*.

Biochemical analysis showed an increase in CRP in the infected group compared to the non-infected one. Furthermore, the increase in serum AST and ALT levels could be attributed to liver cells damage which may be produced by isolated bacteria [36] and [37] circulating in blood in both ascending and descending infection.

Also, the significant change in total protein, albumin may be due to bacterial toxins increase the capillary permeability and permitted the escape of plasma proteins into tissue resulting in hypoproteinemia [38]. Uric acid and creatinine in the present work could be increased due to liver and kidney damage which could be associated with a bacterial infection [22**,** 39].

The elevation of total globulin indicates an activation of the immune system which is due to infection or inflammatory diseases [40]. This increase might be attributed to the increased protein catabolism, febrile respiratory diseases, impaired cardiac function and decreased renal blood flow [22]**.** Zaki et al. [39] and Youssef et al. [22] mentioned that *S. aureus* is responsible for Bumble foot and septic arthritis in broilers and layers which cause a significant increase in AST, ALT, uric acid, creatinine, gamma, alpha globulin. Total protein and albumin levels were significantly decreased. These data were completely in accordance with our results.

Colored exudate in bacterial infection cases is typically detected by Echols [41] who proved that turbidity of synovial fluid can be another indicator of bacterial infection. Synovium fibrosis is a characteristic lesion of chronic cases as that reported by Davis et al. [41] and Eldin & Ibrahim [43]. Some forms of grossly observed injuries are demonstrated in **Figure1** are almost parallel to those obtained by Degernes et al. [5] and Hassan et al. [4]. Meanwhile, our microscopical results are in harmony with those recorded by some authors [49, 5, 4, 47, 48]**.** The obtained results of heterophilic and /or lymphocytic infiltration of the synovial membrane, synovial fold, and tendon collagen in most of the arthritic cases depends on the type of infection whatever bacterial or viral one consequently which supported by the results of previously mentioned authors. The infection degree and duration from mild to severe and from acute to chronic determine the existence and forms of obtained lesions**;** cyst formation in the distorted epiphysis center is noticed only in severe cases while leukocytic cells infiltration is noticed in all forms. Associated microscopical lesions with different isolated microbes were demonstrated in detail in lesions score **(Table 7).**

CONCLUSION

Multiplex real-time PCR holds the potential to be a more reliable, economical, and rapid gold diagnostic tool for simultaneous detection of arthritis pathogens in turkey and provides an efficient way to ask several related epidemiological questions and also provides explanations for grossly and pathologically observed lesions accompanied with hematological and biochemical investigations.

Conflict of interest

The authors declare that they have no competing interests.

Research ethics committee permission

The current research work is permitted to be executed according to standards of Animal Research Ethics committee, Animal Health Research Institute (AHRI), Dokki, Egypt

Authors' contribution

Azza S. El-Demerdash and Rehab E. Mowafy participated in the conception and design of the study, acquisition of data, and writing the paper and revising it critically for important intellectual contents. Rehab E. Mowafy and Sanaa M.Salem contributed in collection of samples and histopathological part. Sahar N.Mohamady contributed in measuring hematological and biochemical parameters. Azza S. El-Demerdash and Samah F.Ali performed microbiology part . Azza S. El-Demerdash and El-Toukhy E.I contributed in molecular part. All the authors have approved the final article version to be submitted.

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