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Overview of aerobic bacterial species involved in joint infections in commercial poultry farms

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Abstract

Lameness is a significant issue in poultry industry, resulting in both financial losses and animal welfare issue . This study investigated the bacterial pathogens causing lameness in 50 farms, as well as isolates' antibiotic susceptibility and its virulence characteristics. Bacterial isolation was positive in 23 (46%) of the fifty farms. The most common bacterial species revealed were Staphylococcus spp. (n=12, 24%), followed by *E. coli* (n=9, 18%), were *Pseudomonas aeruginosa* and Enterococcus *spp*. detected in one farm (2%).

Antibiotic susceptibility testing indicated different resistance patterns. *E. coli* isolates showed excellent sensitivity to Amikacin (88.89%), Amoxicillin (77.78%), and Gentamicin (77.78%), but substantial resistance to Ampicillin (77.78%) and Amoxicillin-clavulanic acid (66.67%). *Staphylococcus* isolates were extremely susceptible to enrofloxacin (75%), amoxicillin (75%), but resistant to amikacin, gentamicin, and ceftriaxone (42%). Further molecular screening of three Staphylococcus isolates revealed the presence of important virulence genes. All three isolates tested positive for the regulatory genes *sar*A , *agr*1, as well as the adhesion gene *clf*A. Two strains carried the *fnb*B gene, whereas one carried both *clf*B and *cna*.

Introduction

Poultry general wellness is evaluated by assessing mortality, physiological status, behavior, and typical mobility **(de Alencar Nääs *et al* 2021)**. Lameness increases pain, slows body weight gain, and reduces income **(Granquist *et al* 2019).** The incidence of lameness is typically 1-5%, while it can occasionally exceed 15% (**Wideman *et al* 2013**).

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Lameness is assessed using the Bristol gait grading system, which ranges from 0 (normal) to 5 (unable to walk) (**Kestin *et al*., 1992).**

During the last forty years, broilers have been diagnosed with more lameness issues than laying hens. Lameness in chickens is linked to rapid growth, genetic factors, and variables in the environment (**Williams *et al*., 2000**). The rapid increase in chicken body weight gain, which is not comparable to the development rate of cartilaginous growth plates in the proximal leg bones, is a key factor in Bacterial chondronecrosis with osteomyelitis (BCO) susceptibility on poultry farms (**Wideman *et al.* 2013**). Bacterial infections infecting the tibial and femoral metaphyses produce necrotic degeneration (**Prisby *et al* 2014**). A recent study (**Kittelsen *et al*., 2017**) discovered a link between first-week mortality and poor gait at slaughter age.

Bacterial chondronecrosis with osteomyelitis (BCO) is a significant cause of loss in the chicken industry (**Siegel *et al.* 2019, Ekesi *et al* 2020**). When tissues become diseased or immune compromised, opportunistic microorganisms from the gastrointestinal or respiratory systems can infiltrate and enter the bloodstream. These bacteria, especially *Staphylococcus aureus, Escherichia coli, and Enterococcus cecorum*, which are frequently combined with other bacteria such as Salmonella spp., can then reach the growth plates in long bones (**Assumpcao *et al.* 2024, Choppa *et al.* 2023, Jiang *et al.* (2015**).

Stress damages the growth of the plate's cartilage gap, allowing opportunistic hematogenous bacteria to colonize and cause abscess formation, necrosis, and lameness (**Wideman 2016**). Some bacteria strains can interact with bone collagen, causing osteomyelitis (**Smeltzer and Gillaspy, 2000**). Furthermore, heat stress raises lameness issues in broiler farms through changes in the intestinal microbiota, allowing certain strains of bacteria to reach the bloodstream (**Murugesan *et al.* 2014**).

According to the latest study, effective lameness care in broilers starts in the hatchery, with a focus on suitable environmental conditions, prophylactic Vitamin D3 and day-old probiotic therapy, and, in some cases, the use of enrofloxacin to combat bacterial causes (**Wideman *et al* 2016**). Organic zinc, manganese, and copper (Availa® ZMC) feeding during the early fourth week reduced lameness on broiler farms by 20-25% (**Alharbi *et al*., 2024**).

Lameness (Arthritis) is a serious economic concern in Egyptian breeder chicken flocks, as evidenced by a research in Dakahlia that linked it to a 5-7% decrease in egg output. Mycoplasma synoviae (MS) is the most common cause of concern, with antibodies identified in 87.5% of examined birds, as well as high seropositivity for *Mycoplasma gallisepticum* (MG) and *Salmonella gallinarum-pullorum*. In addition, bacteriological examination of the joints yielded several other causes, primarily Staphylococcus spp. and *E. coli* serotypes **(Amer *et al* 2019**). *Staphylococcus aureus (S. aureus)* was isolated from 55% (110/200) of broiler chicks with arthritis at the finishing age (more than 30 days) in Ismailia, Egypt **Hamed, (2012).** Furthermore, Avian Reovirus (ARV) has also been isolated and detected in connection with arthritis in Egyptian broiler breeder farms (e.g., strain Egypt/arv/giza 2011, as reported by **Bekheet *et al.* 2024**). The current study was to identify and characterize aerobic bacterial infections that cause lameness in broiler chickens. It involves determining the main bacteria, studying their virulence genes using PCR, and understanding their role in lameness and evaluating Amoxicillin's treatment effectiveness at 15 mg/kg body weight.

Materials and Methods

1- Sample collection

The investigation was carried out at the Reference Laboratory for Veterinary Quality Control in Poultry Production.

Table (1). Broiler Farms Sampled using Clinical and Post-mortem Examination Procedures.

No of farms	Location of farms	Source of samples	Signs	Necropsy procedures	Grading systems
50 broiler farms ranging From 1 to 45 days age	Cairo and Qena governates	Aseptically collected swabs from hip and hock joints of lame chickens	Goblet gait, wing-tip dipping, footpad dermatitis, decreased walking/standing, kinky back, increased hock burns	Lame birds were humanely killed for necropsy and examination of tibial and femoral lesions .	**Wideman's (2016) grading system**

2- Isolation and identification of Staphylococcus, Salmonella, *E. coli*, Pseudomonas, and Enterococcus species.

During the postmortem examination, sterile swabs were taken under complete aseptic condition to identify and isolate aerobic bacteria that cause chondronecrosis and osteomyelitis. The target bacterial species included Staphylococcus, Salmonella, *E. coli,* Pseudomonas, and Enterococcus species.

2.1 Bacterial isolation of *Staphylococcus*

Staphylococcus aureus was isolated and identified using standard procedures such as BAM: 2001 and ISO 6888-1: 2003. Isolated colonies were detected using morphological, microscopical, and biochemical methods. **Sneath *et al*. (1986) and Quinn *et al*. (2002)** reported catalase +ve coagulase +ve oxidase -ve.

2.2 Bacterial isolation of *E. coli*

E. coli was isolated and identified using the procedures described (**Nolan *et al*., 2013**). A loopful of each incubated sample was streaked onto MacConkey's agar (Oxoid, Manchester, UK) and Eosin Methylene Blue agar (Lioflichem, Roseto degli Abruzzi, Italy) plates and incubated aerobically at 37°C for 24 hours. Additional biochemical testing was performed on suspected *E. coli* colonies (indole test, methyl red, Voges Proskauer "VP", citrate utilization, oxidase test, and Triple Sugar Iron "TSI").

2.3 Bacterial isolation of *Salmonella spp.*

Salmonella spp. was isolated and identified according to **ISO 6579-1:2017/Amd1:2020** criteria. The samples were pre-enriched in buffered peptone water (Oxoid®) and incubated at 37°C for 16-18 hours. A 0.1 mL portion of pre-enrichment broth was added to Modified Semisolid Rappaport-Vassiliadis (MSRV) medium (Oxoid®) and incubated at 41.5° C for 24 hours. Simultaneously, 1 mL of the pre-enrichment broth was added to Muller-Kauffmann Tetrathionate (MKTTn) broth (Oxoid®) and incubated aerobically at 37°C for 24 hours. Selective plating was next performed, with enriched samples streaked onto Xylose Lysine Deoxycholate (XLD, Oxoid®) and Hektoen Enteric (HE, Liofilchem®) agars. Plates were incubated aerobically at 37 degrees Celsius for 24 hours. Colonies were confirmed using biochemical tests, including Urea agar, Triple Sugar Iron (TSI) agar, and Lysine Iron agar (LabM, Oxoid®, and Liofilchem®).

2.4 Bacterial isolation of *Enterococcus spp*

Two complex culture media are commonly utilized for the identification of Enterococcus: Kanamycin Aesculin Azide (KAA) agar and Slantez and Bartley (SB) agar. On KAA agar, Enterococcus manifests as a black colony due to the hydrolysis of Esculin , while on SB agar, the colonies appear red or maroon as a result of decreased triphenyltetrazolium chloride (**Slanetz and Bartley, 1957**). The biochemical identification process is referenced from the study by **Manero and Blanch (1999).**

2.5 Bacterial examination of *Pseudomonas aeruginosa*

The samples were first enriched with nutrient broth and then incubated at 37°C for 24 hours (**Abbas *et al*., 2022**). The turbid samples were then inoculated on tryptose soya agar and Pseudomonas agar to form typical colonies of *P. aeruginosa* (large, irregular, transparent, and producing a greenish diffusible pigment). *P.aeruginosa* was identified by microscopic morphology, Gram-staining, oxidase, urease, catalase test, Triple Sugar Iron test, indole, and citrate utilization assays (**Poursina *et al*., 2023, Badr *et al*. 2016**).

3- Antibiotic sensitivity test

Antibiotic susceptibility of bacterial isolates from lameness cases was performed according to The **CLSI 2021** . isolates were tested against 14Oxoid® (UK) antibiotics, including: Amoxicillin (20 µg), Amoxicillin-clavulanic acid (20/10 µg), Ampicillin (10 µg), Ceftriaxone (30 µg), Cefotaxime (30 µg), Amikacin (30 µg), Enrofloxacin (5 µg), Ciprofloxacin (5 µg), Doxycycline (30 µg), Gentamicin (10 µg), Sulfamethoxazole-trimethoprim (23.75 µg/1.25 µg), Streptomycin (10 µg), Spectinomycin (100 µg), Chloramphenicol (30 µg). Zone diameters were assessed using **CLSI 2021** standards to identify sensitivity, intermediate, and resistance patterns.

4- Molecular detection of virulence genes bind with collagen

DNA extraction

A PCR (polymerase chain reaction) test was

run on three Staphylococcus samples at RLQP biotechnology unit at the Animal Health Research Institute (AHRI) to detect virulence genes. DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH), with particular modifications to the instruction manual. Two hundred µl of sample suspension were incubated with 10 µl of proteinase K enzyme and 200 µl of lysis buffer at 56°C for ten minutes. After incubation, 200 µl of 100% ethanol were added to the lysate. The sample was subsequently washed and centrifuged according to the manufacturer's guidelines. Nucleic acid was eluted by 100 µl of the kit's elution buffer.

Oligonucleotide primers: The primers used were supplied by Metabion (Germany) and are mentioned in table (1).

PCR amplification Primers were used in a 25-µl reaction using 12.5 µl of EmeraldAmp Max PCR Master Mix (Takara, Japan), 1 µl of each primer at 20 pmol concentration, 5.5 µl of water, and 5 µl of DNA template. The reactions were carried out in an Applied Biosystems 2720 thermal cycler.

Analysis of PCR products. The PCR products were separated by electrophoresis on a 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature with 5V/cm gradient. Gel analysis involved loading 15 µl of products into each gel slot. Fragment sizes were determined using a Generuler 100 bp ladder (Fermentas, Germany).The agarose gel was documented using a Gel Documentation System (e.g., Alpha Innotech, Bio-Rad, or equivalent equipment). The image was captured automatically under UV illumination after ethidium bromide and data was analyzed through computer software (automatic image capture protein simple formerly cell bioscience , USA).

Table (2). Primers, Target Genes, Amplicon and Thermal Cycling Conditions for Virulence Genes in *Staphylococcus aureus*.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
clfA	GCAAAATCCAG-CACAACAGGAAACGA CTTGATCTCCAGCCA-TAATTGGTGG	638	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Mason *et al.*, (2001)**
clfB	ACA TCA GTA ATA GTA GGG GGC AAC TTC GCA CTG TTT GTG TTT GCA C	205	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Tristan *et al.*, (2003)**
fnbB	GTAACAGCTAATGGTCG AATTGATACT CAAGTTCGATAGGAG-TACTATGTTC	524	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
cna	GTCAAGCAG-TTATTAACACCAGAC AATCAGTAATTGCAC-TTTGTCCACTG	423	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	
sarA	CAGCGAAAACAAAGA-GAAAG TCGTTGTTTGCTTCAG-TGAT	223	94˚C 5 min.	94˚C 30 sec.	55˚C 30 sec.	72˚C 30 sec.	72˚C 7 min.	**Wood *et al.*, (2009)**
agrI	**Pan:** ATG CAC ATG GTG CAC ATG C GTC ACA AGT ACT ATA AGC TGC GAT	441	94˚C 5 min.	94˚C 30 sec.	55˚C 40 sec.	72˚C 45 sec.	72˚C 10 min.	**Abd El-Hamid and Bendary, (2013)**

5- Experimental study

Thirty Cobb chickens were divided into three equal groups: a positive control, a negative control, and a treatment group consisting of ten chickens. The treatment group was inoculated via oral administration with a Staphylococcus strain. Following inoculation four days before treatment, chickens were given oral amoxicillin at 15 mg/kg twice daily for five days, starting with the appearance of clinical signs.

Table (3). Experimental Design and Treatment Regimens for Broiler Chicken Groups

Group	Number of Chicks	Treatment
Negative Control	10	Oral administration of saline solution.
Positive Control	10	Oral administration of Staphylococcus strain at a dose of 200 µL of 10^8 CFU/mL.
Treated Group	10	Oral administration of Staphylococcus strain at a dose of 200 µL of 10^8 CFU/mL, followed by oral administration of amoxicillin (15 mg/kg) twice daily at 6 hour period for 5 days, starting after the appearance of clinical signs (4 day).
Reference	**AlRubaye *et al*. 2020; AlRubaye *et al*. 2017**). **Ledesma *et al*. (2018).**	

The administration of about 10^8 CFU/mL of this Staphylococcus strain orally to 20-day-old broilers resulted in nearly equal occurrences of lameness. separated into three groups, each comprising ten chicks as a negative control and positive control. After the lesion appeared, we observed the experiment for 10 days (the treated group received amoxicillin 15 mg/kg orally twice daily for five consecutive days.

Results

From 50 lameness-affected farms, isolated bacteria were as showed in table (4).

Table (4). Prevalence and Distribution of Aerobic Bacterial Isolates Collected from Joints of Lameness-Associated Broiler Flocks

Bacterial isolates	Number of farms
Farms with positive isolations	23 of 50 (46%)
Staphylococcus spp..	12 (24 %)
E.coli	9 (18%)
Pseudomonas aeruginosa	1 (2%)
Enterococcus spp	1 (2%)
Salmonella spp.	Not detected

Antibiotic sensitivity studies on *E. coli* isolates showed in table (5) .

Table (5). Antimicrobial Susceptibility Profile of nine *Escherichia coli* Isolates Recovered from Lameness-Affected Broiler Joints

Antimicrobial drugs	Sensitivity	Intermediate	Resistant
Amoxicillin-clavulanic acid (20/10 µg),	1(11%)	2 (22%)	6(67%)
Ampicillin (10 µg)	1(11%)	1(11%)	7(78%)
Ceftriaxone (30 µg)	5(56 %)	2(22%)	2(22%)
Cefotaxime (30 µg)	6 (67%)	2(22%)	1(11%)
Amikacin (30 µg)	8(89%)	1(11%)	0
Enrofloxacin (5 µg)	6 (67%)	2(22%)	1(11%)
Ciprofloxacin (5 µg)	6(67%)	2(22%)	1(11%)
Doxycycline (30 µg)	3(33%)	2(22%)	4(45%)
Gentamicin (10 µg)	7(78%)	1(11%)	1(11%)
Sulfamethoxazole-Trimethoprim (25 µg)	3(33%)	2(22%)	4(45%)
Streptomycin (10 µg)	4(45%)	2(22%)	3(33%)
Chloramphenicol (30 µg)	5(56%)	1(11%)	3(33%)
Amoxicillin (20 µg)	7(78%)	0	2(22%)
Spectinomycin (100 µg)	4 (44.5%)	(44.5%)	1(11%)

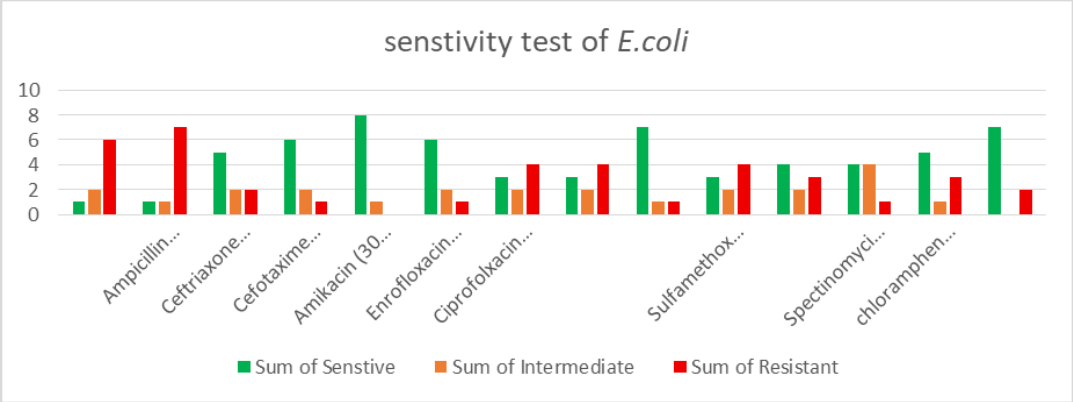

Figure (1). Antibiotic susceptibility test of *E.coli* against 14 antibiotics.

Table (6). Antimicrobial Susceptibility Profile of Staphylococcus Isolates Recovered from Lameness-Affected Broiler Joints

Antimicrobial drugs	Sensitivity	Intermediate	Resistant
Amoxicillin-clavulanic acid (20/10 µg),	6(50 %)	2 (16.7 %)	4(33.3%)
Ampicillin (10 µg)	7(58.3%)	3(25%)	2(16.7%)
Ceftriaxone (30 µg)	5(41.6 %)	2(16.7%)	5(41.6%)
Cefotaxime (30 µg)	8 (66.6%)	2(16.7%)	2(16.7%)
Amikacin (30 µg)	4(33.3%)	3(25%)	5(41.6%)
Enrofloxacin (5 µg)	9 (75%)	2(16.7%)	1(8.3%)
Ciprofloxacin (5 µg)	8(66.6%)	3(25%)	1(8.3%)
Doxycycline (30 µg)	7(58.3%)	3(25%)	2(16.7%)
Gentamicin (10 µg)	3(25%)	4(33.3%)	5(41.6%)
Sulfamethoxazole-Trimethoprim (25 µg)	4(33%)	5(41.6%)	3(25%)
Streptomycin (10 µg)	9(75%)	1(8.3%)	2(16.7%)
Chloramphenicol (30 µg)	8(66.6%)	2(16.7%)	2(16.7%)
Amoxicillin (20 µg)	9(75%)	2(16.7%)	1(8.3%)
Spectinomycin (100 µg)	6 (50 %)	4(33.3%)	2(16.7%)

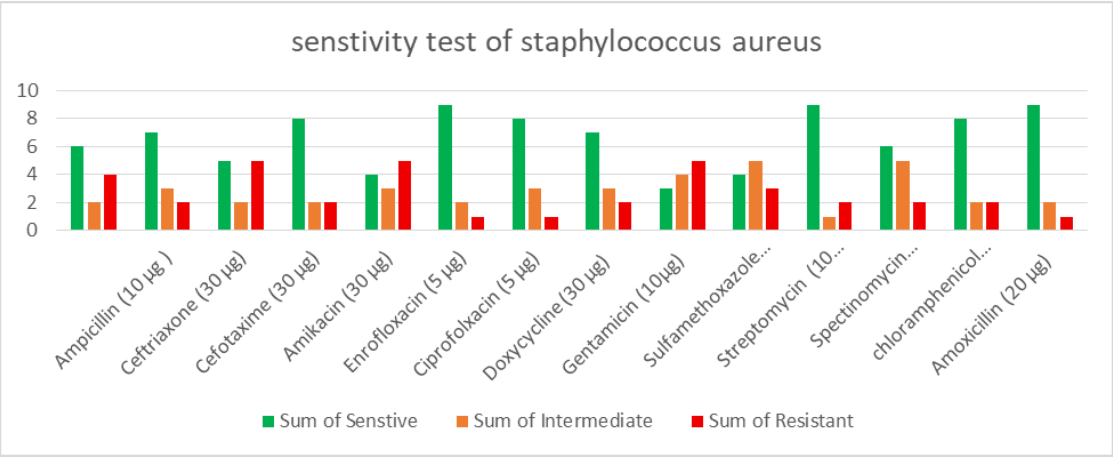

Figure (2). Antibiotic susceptibility test of *Staphylococcus aureus*

An Enterococcus isolate was susceptible to ampicillin, amoxicillin-clavulanic acid, ceftriaxone, and cefotaxime, but resistant to the other antibiotics tested in this study. *Pseudomonas aeruginosa* was susceptible to ciprofloxacin, enrofloxacin, amikacin, and gentamycin, but resistant to the other antibiotics tested.

Molecular findings were All of Staphylococcus strains tested positive for the *sar*A, *agr*1, and *clf*A genes. The *fnb*B gene was detected in two strains, whereas *cna* and *clf*B were found in one. The findings are shown in table (7) and figures (3, 4).

Table (7). Molecular Detection of Virulence-Associated Genes in Isolated Staphylococcus Strains

***Staphylococcus* sample**	***clfA***	***clfB***	***fnbB***	***cna***	***agrI***	***sarA***
1	+	+	+	+	+	+
2	+	-	-	-	+	+
3	+	-	+	-	+	+

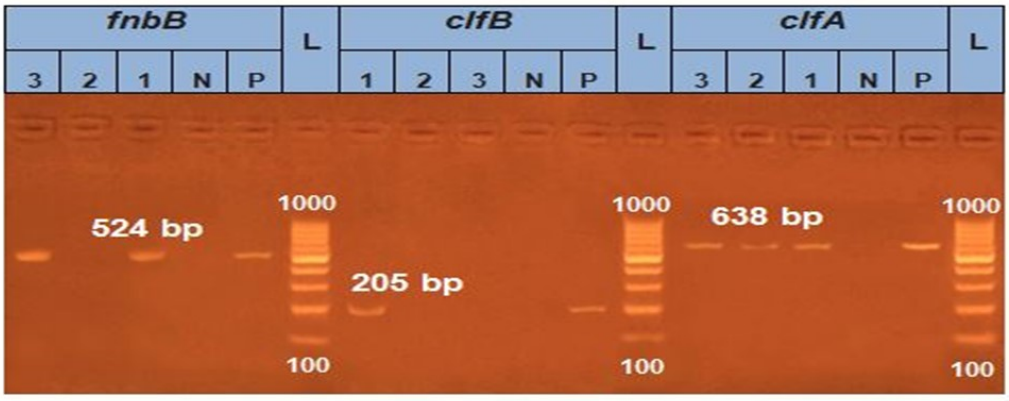

Figure (3). Agarose Gel Electrophoresis of PCR Amplicons Demonstrating the Presence of *fnbB*, *clfB*, and *clfA* Genes in *Staphylococcus aureus* Isolates.
N (Negative Control), P (Positive Control), L (ladder) and bp (base pair)

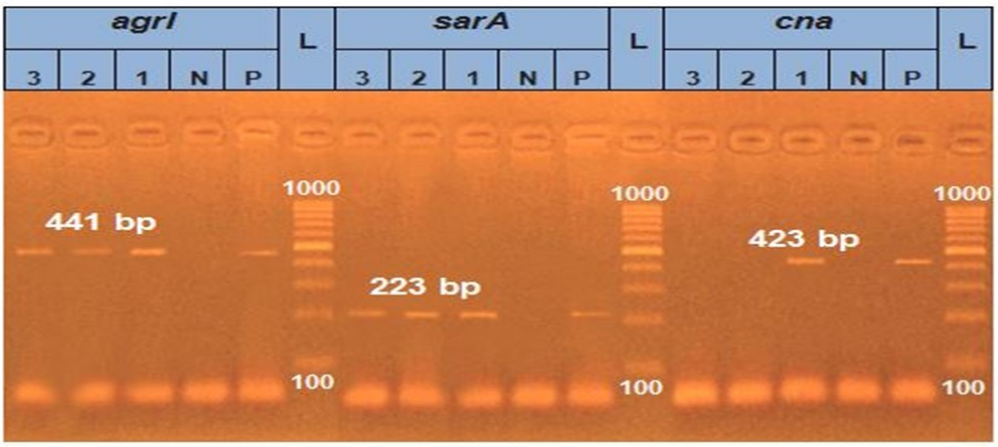

Figure (4). Agarose Gel Electrophoresis of PCR Amplicons Demonstrating the Prevalence of *agr1*, *sarA*, and *cna* Genes in *Staphylococcus aureus* Isolates.
N (Negative Control, P (Positive Control), L (ladder) and bp (base pair)

Table (8). Clinical Signs and Mortality Rates Observed in Experimental Broiler Groups inoculated Staphylococcus Challenge

Groups	Signs				Mortality
	Day 3	Day 4	Day 5	Day from 6-10	
Negative Control	Normal	Normal	Normal	Normal	No mortality
Positive Control	Depression In all birds	5 birds showed redness in hock joint	5 chicken redness and edema in hock joint and 2 unable to stand	10 walk with stiff gait Redness swelling ,edema, and 3 chicken unable to stand	No mortality
Treated Group	Depression In all birds	4 birds showed redness	Treated with amoxicillin 15 mg/kg	Only 2 chicken showed redness, swelling	No mortality

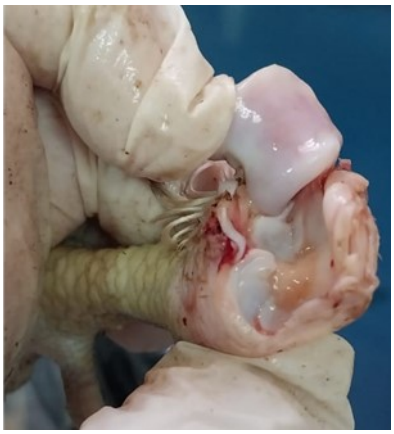

Figure (5). Synovial fluid

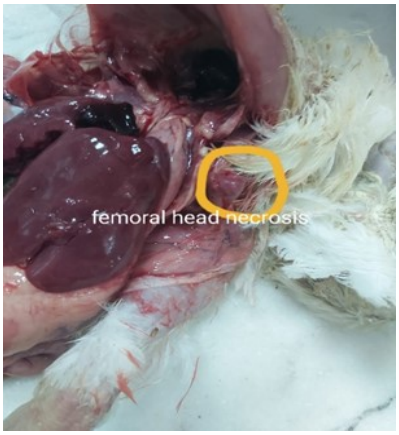

Figure (6). Femoral head necrosis

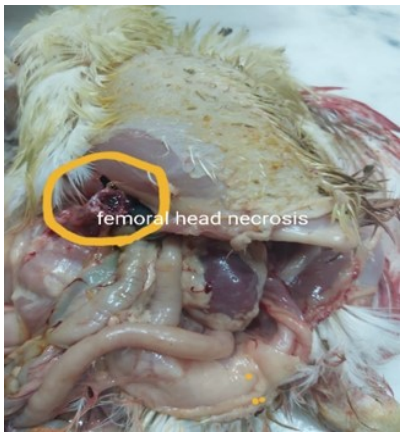

Figure (7). Femoral head necrosis

Discussion

The study revealed that , 46% of lameness cases were caused by aerobic bacteria on fifty farms. *Staphylococcus aureus* was detected in 24 % of bacterial infections, *E. coli* in 18 %, and *pseudomonas aeurogenosa* and Enterococcus in 2 % each. These data were consistent with (**Rasheed, B. Y. (2011**), who observed Staphylococcus isolation in arthritic chickens at around 50%. Furthermore, **Amer *et al.* (2019)** concluded that *E.coli* isolated from joint samples were 47%. **Dinev *et al.* (2013)** shown that *P. aeruginosa* is not a common cause of lameness, but rather happens as a result of a contaminated vaccination injection or aerosol. **Zeshan *et al* 2015** found that Enterococcus was responsible for around 5% of lameness. Antibiotic sensitivity tests against *E. coli* revealed that it was sensitive to amikacin but highly resistant to ciprofloxacin and doxycycline . The results are on the same page as **Rizal *et al* (2024)**. Antibiotic susceptibility testing for *Staphylococcus aureus* indicated that it is very susceptible to enrofloxacin and amoxicillin but resistant to gentamycin, ceftriaxone, ampicillin, and doxycycline, as shown by **Hamed *et al* (2021)**. Enterococcus resistant to tetracycline, as reported by **Ribeiro *et al.* (2023).** *Pseudomonas aeruginosa* was susceptible to ciprofloxacin, enrofloxacin, and amikacin but resists ampicillin, as determined by **Ramatla *et al* (2024)**, who reported *Pseudomonas aerogenous* resist ampicillin and colistin. Some isolates have ESBL and colistin resistance genes, which raises public health concerns.

The examined three *Staphylococcus aureus* strains for the presence of six particular genes, which were classified into two categories: adhesion genes and regulatory genes. The adhesion genes were *clf*B, which creates a protein that binds to fibrinogen to promote bacterial clumping; *fnb*B, which produces a protein that attaches to fibronectin on host cells for adher-

ence; and *cna*, which produces a protein that plays a crucial role in septic arthritis **Wright and Nair (2010).** *Agr*I and *Sar*A were the regulatory genes studied. These genes are critical for managing the production of several virulence factors, such as toxins and adhesion proteins, which work together to regulate the bacteria's ability to cause disease (**Cheung *et al.* 2004; Cassat *et al.* 2013**). One of three S. aureus strains tested positive for all six of these genes. *Clf*A. *Clf*B, *fnb*B, *Cna, Agr*I, and *Sar*A. An experiment was conducted to assess the effects of a Staphylococcus spp infection and amoxicillin treatment in 20-day-old chickens. While the bacterial strain wasn't lethal, it caused clinical signs of disease, including depression and staphylococcal arthritis in the joints, which impaired the birds' movement. The study confirmed that the infection could be effectively treated with a five-day course of amoxicillin, as the birds' condition significantly improved . the result was on the same page with (**Mutalib *et al* 1982).**

Conclusion

The aerobic bacterial infections that cause lameness in broiler chickens were investigated in this study. In fifty farms under investigation, lameness were caused by Staphylococcus species, *E. Coli*, *Pseudomonas aeruginosa*, and Enterococcus. Staphylococcus had a high prevalence incidence of 46%. Five virulence genes were investigated by PCR in three isolates. selected a strain that tested positive for each of the five virulence genes to inoculate, validate the results, and administer amoxicillin in drinking water for five days at a dose of 15 mg/kg according to the manufacturer's instructions

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