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# Biochemical and molecular identification with antimicrobial susceptibility of bacterial species isolated from organs and tissues of *Alectoris chukar* subspecies *Kurdistanica*

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**Abstract**

The current study was conducted on 50 *Alectoris chukar* subspecies *Kurdestanica* that was collected from Sulaymaniyah Province, Kurdistan Region, Northern Iraq, during the period of April to the end of September 2016. Samples of liver, gallbladder, spleen, kidneys, heart, lungs, gizzard, breast, and thigh muscle tissues were tested for bacterial isolates. Preliminary characterization of the isolated bacteria was carried out by morphological and biochemical methods. The VITEK 2® system was used to confirm the isolated species, while the polymerase chain reaction (PCR) was used for the detection of the resistance gene in the bacterial isolates. As a result, tested samples showed the presence of *Staphylococcus sciuri* and *Escherichia coli*. Additionally, antimicrobial susceptibility test was done to determine the bacterial susceptibility to various antibiotics and as a result; *E. coli* showed 100% susceptibility to penicillin, azithromycin, tetracycline, and doxycycline and 75% susceptibility to streptomycin. On the other hand, *S. sciuri* exhibited 75% susceptibility to azithromycin, penicillin, and doxycycline, 50% susceptibility to streptomycin, and 25% susceptibility to tetracycline. Molecular identification showed that only the *S. sciuri* isolates carried the methicillin-resistant *mecA* gene. To our knowledge, this is the first record of isolation of the *S. sciuri* methicillin-resistant *mecA* gene from *A. chukar* subspecies *Kurdestanica*.

## 1. Introduction

*A. chukar* is non-migratory game-bird of the pheasant family Phasianidae that found mainly in the Middle East countries including North Iraq. This bird is a beautiful, round 32–35 cm long partridge, with a light brown back, grey breast, buff belly, and white face framed by a distinct black line (Figure 1) [1, 2]. The bird is also commonly known as chukar partridge, grey chucker, kabk, keklik, chikone, kaukau, kau, chukru, chukor, chickore or nek-pa, as well as it has a unique calling sound [3].

The genus *Alectoris* consists of 7 closely related interfertile species and 24 subspecies, including *A. chukar* [4]. *A. chukar* is a very distinctive species, native to Asia but have introduced to other regions of the world including North America, Canada, Great Britain, and New Zealand by allopatric distribution [5].

*A. chukar* subspecies *Kurdestanica* is found only in the Kurdistan region of Northern Iraq [6]. They tend to avoid agricultural areas and commonly frequent water sources, particularly during Summer. The population of these partridges in the Kurdistan region has tragically declined [7]. The survival of chukar in the wild is limited by poor growth, competition for food, predation, hunting, accidents, habitat degradation, and parasite and bacterial infections [8].

**Figure 1.** *Alectoris chukar* subspecies *Kurdestanica*.

The *Staphylococcus* group of bacteria, including *S. sciuri*, *S. lentus*, *S. vitulinus*, and *S. pulvereri*, principally found in animal species [9], and have been isolated from various human clinical specimens [10, 11]. During outbreaks, more than 50% of hospital patients were shown to be positive for these bacteria [12]. Among sources for these infections are poultry meats and eggs. These infections usually cause life-threatening gastrointestinal tract diseases [13]. Wild birds are among vectors of foodborne and zoonotic diseases and they can serve as useful models for investigations on disease spread among birds and animals and to humans [14].

High-protein and a low-fat ratio of partridge meat foodstuffs are important in terms of nutrition and diet. However, some processes such as the cutting process, the pH value, redox potential, and temperature as well as nutritional composition with partridge meats [15] suitable for food contamination and makes a favorable environment for the growth of microorganisms [16].

There is little information on the prevalence and pathogenicity of *S. sciuri* to animals [17], particularly the wild birds. Thus, the objectives of this study are to determine the occurrence of bacterial pathogens in the organs and tissues of the chukar partridge and susceptibility of isolates to commonly and frequently used antimicrobials.

## 2. Materials and Methods

### *2.1. Area of study*

This study was conducted on *A. chukar* subspecies *Kurdestanica*, during the period of April to the end of September 2016 in the Sulaymaniyah Province, Kurdistan Region of Iraq.

### *2.2. Sample collection*

The birds were caught weekly using decoys and traps. Briefly, hunters built a shelter using stone and bushes and then they put a cage with male chukar at a distance of about 10 m from the shelter. The rally

chukar call helped to attract the others in the area to come and fight with the chukar under the observation. Chukars were kept alive in a wood basket and transferred to the Hematology Laboratory in the Department of Clinic and Internal Medicine, College of Veterinary Medicine, University of Sulaimani.

Fifty birds used in this study and they were humanely sacrificed by slaughtering within 2 hours of collection and allowed for complete bleeding. The feathers were removed and the birds were dissected aseptically from the abdominal site. Then, liver, gallbladder, spleen, kidneys, heart, lungs, gizzard, breast, and thigh muscle tissue samples aseptically removed and washed thoroughly with sterile distilled water. Approximately, 1.0 g from each organ was placed in sterile blender bowls containing 5.0 mL of sterile phosphate buffer saline (PBS) and finely ground to form suspensions to be used for further investigation. This study was approved by the ethical committee of the College of Veterinary Medicine, University of Sulaimani.

### *2.3. Bacterial culturing and isolation*

The identification of isolates was done using the procedure described in the Modified Bacteriological Analytical Manual [18]. Briefly, 0.5 g of freshly prepared tissue suspension was first cultured on the buffered peptone water (BPW) (Merck, USA) medium and incubated at 37° C for 24 hours. Later on, 10 µL of this cultured broth was streaked onto blood agar (Difco, Germany) and brain heart infusion (BHI) agar (Merck, USA) plates and incubated at 37° C for 24 hours. After incubation, the agar plates were examined for bacterial colonies. Finally, isolated colonies were picked and transferred to trypticase soy agar slants (BBL) (Sigma Aldrich, USA) and allowed to grow at 37° C for 24 hours [19].

### *2.4. Morphological characteristics using a staining technique*

Smears were prepared from pure colonies on clean microscopic slides before fixing by flaming. Then, the steps of Gram staining were followed properly using a standard procedure [20]. Finally, the smears were washed, air-dried, and examined under light microscope using high power field (HPF; X10) (Leica, Germany).

### *2.5. Bacterial identification using biochemical tests*

Preliminary identification of isolates was done using the triple sugar iron (TSI) test, urease, Simmon's citrate (SC), indole (ID), methyl red (MR), Voges Proskauer, oxidase, and catalase tests.

### *2.6. Bacterial identification using the VITEK 2 system*

For more confirmation of bacterial species, the VITEK 2 system (bioMerieux, Marcy-l´Etoile, France) was used [21, 22] in which the VITEK 2 card was used to confirm the identification of Gram-positive bacteria. Briefly, the bacterial suspensions were adjusted to a McFarland standard of 0.5 in 2.5 mL of 0.45% sodium chloride (NaCl) solution. The cards were filled with isolated bacterial suspension and within 30 minutes placed in the VITEK 2 cassette which then placed in the VITEK 2® system automatic vacuum chamber, sealed, and incubated at 35.5 ± 1.0° C. Colorimetric measurement was done automatically by the machine every 15 minutes for a maximum incubation period of 10 hours. Data were collected at 15 minutes intervals during the incubation period. Finally, data were analyzed using the VITEK 2 database version 4.01, which allows for organism identification in the kinetic mode after 2 hours of incubation [23].

### *2.7. Antimicrobial susceptibility test*

The test was carried out to determine the susceptibility of bacterial isolates using the standard method in which the tetracycline (TE 30), azithromycin (AZM15), streptomycin (S10), penicillin (P10), and doxycycline (DA10) antimicrobial-impregnated discs (6 mm) (HiMedia, India) were used. Briefly, 3 to 4 pure bacterial colonies were picked and placed in tube containing 3 to 5 mL of BHI broth and incubated at 37° C for 3 to 6 hours. Then, the broth was diluted with 0.9% NaCl to obtain turbidity equivalent to 0.5 McFarland standard of $1\times10^8$ cells/mL. A sterile cotton swab soaked in the adjusted bacterial suspension was streaked over the entire surface of Mueller-Hinton (MH) agar (HiMedia, India) and the plate left to stand for 3 to15 minutes at room temperature. The discs were placed on the agar surfaces and the plates incubated at 37° C for 24 hours. Finally, the diameters of inhibition zones were measured and recorded [24].

### 2.8. Polymerase Chain Reaction (PCR)

#### 2.8.1. DNA extraction

The individual bacterial colony was used for DNA extraction using a genomic DNA extraction kit (GeneNet, South Korea) according to the manufacturer's instructions. The purified DNA was then quantified and stored at - 20° C for subsequent PCR assay.

#### 2.8.2. PCR amplification

The PCR amplification reaction was conducted based on the manufacturer instructions using GeneNet PCR Premix. The PCR primers were designed for the *mecA* gene-specific to chromosomal DNA of *S. sciuri* with the following sequences: SAMECA358 (ATCCATCAATATTGAACCA) and SAMECA1482 (TATATCTTCACCAACACC) to amplify 1120 bp ***[25]***. Bacterial 16S rRNA gene was used as a positive (internal) control for the bacterial genome. Bacterial specific primers, 16SF (AATACGTTCCCGGGCCTTG) and DG74 (AGGAGGTGATCCAACCGCA) ***[26]*** were constructed by Macrogen, South Korea. The PCR reaction was performed with 5 µL of DNA template and 1.0 µL of 10 pmol forward and reverse primers, the reaction mixture then made up to a final volume of 20 µL with DEPC-$H_2O$. The thermal cycler parameter was initial denaturation at 94° C for 5 minutes, 35 cycles including denaturation at 94° C for 30 seconds, annealing at 57° C for 30 seconds, extension at 72° C for 30 seconds, and a final extension phase run at 72° C for 5 minutes.

#### 2.8.3. Electrophoresis and gel analysis

Electrophoresis of PCR products was performed on 1% agarose gel pre-stained with 5 µL of DNA safe dye (GeneNet, South Korea). Approximately, 7.0 µL of PCR products were loaded on the gel and visualized by the gel documentation system (BioRad, USA). The amplicon size was estimated according to the migration pattern of the 100 bp DNA ladder.

#### 2.8.4. Direct sequencing

The sequencing of PCR products was done by Macrogen, South Korea. The identity of each nucleotide was verified twice. The coding sequences were submitted to Genbank database with the accession number of MG706986.

## 3. Results

### 3.1. The incidence of bacterial isolates

Only 2 species of bacteria were isolated from the tissue samples in which *E. coli* was the most prevalent bacteria in 11 (2.44 %) samples of the liver, kidney, gizzard, and breast muscle tissues while *S. sciuri* was recovered from 5 (1.11 %) cultured liver, kidneys, heart, and thigh muscle specimens (Table 1).

**Table 1.** Percentage of bacterial isolates from the organ and tissues of *Alectoris chukar* subspecies *Kurdestanica*.

| Bacterial Species | Number of isolates | Positive samples |
|---|---|---|
| *E. coli* | 11 (2.44 %) | Liver, kidney, gizzard and breast muscle |
| *S. sciuri* | 5 (1.11 %) | Liver, kidneys, heart and thigh muscle |

### 3.2. Bacterial cultures and morphological features

The *E. coli* and *S. sciuri* (Figure 2) colonies were morphologically characterized by round, convex, smooth surface, and entire margin. *E. coli* showed characteristic Gram-negative staining with medium-size, and rod-shaped morphology. In addition, *E. coli* showed a clear green metallic sheen on eosin methylene blue (EMB) agar medium. Whereas, *S. sciuri* isolates were Gram-positive, and appeared as small size, cocci-shaped bacteria.

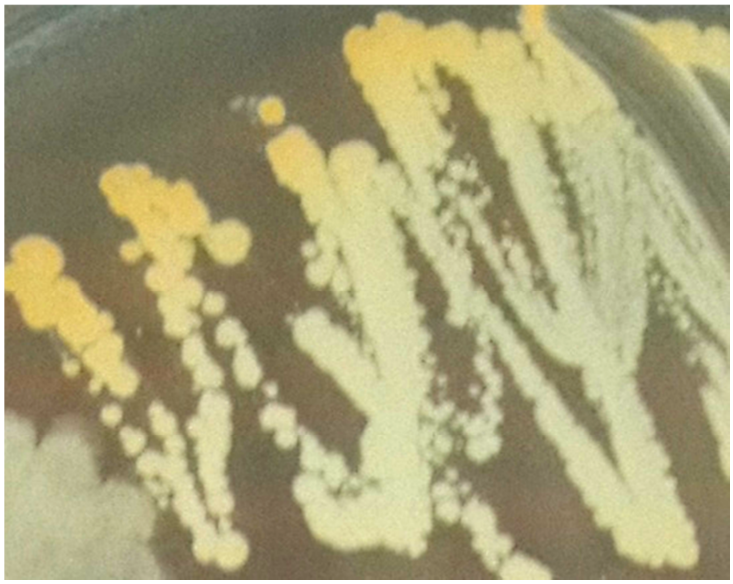

**Figure 2.** *Staphylococcus sciuri* colonies isolated from organs of *Alectoris chukar* subspecies *Kurdestanica*.

### *3.3. Biochemical tests*

The results of biochemical tests are shown in Table 2. Both, *E. coli* and *S. sciuri* were negative for urease and citrate tests and positive for catalase and methyl red tests. Only *E. coli* fermented glucose and sucrose and were negative for each oxidase, Voges Proskauer, and indole tests.

**Table 2.** Biochemical identification of bacterial isolates from organs of *Alectoris chukar* subspecies *Kurdestanica*.

| **Bacterial Species** | **Biochemical Tests** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **Triple Sugar Iron Test** | **Urease** | **Simmon Citrate** | **Indole** | **Methyl Red** | **Voges Proskauer** | **Oxidase** | **Catalase** |
| ***E. coli*** | -/-/gas | -ve | -ve | +ve | +ve | -ve | -ve | +ve |
| ***S. sciuri*** | +/+ | -ve | -ve | -ve | +ve | +ve | +ve | +ve |

### *3.4. VITEK 2 system*

The new VITEK 2 card for Gram-positive bacteria seems to be a promising new tool for routine, rapid identification of a wide variety of bacterial contaminants from different samples. In this respect, 43 tests were conducted for further confirmation of *S. sciuri* isolates using the VITEK 2 system and a probability of about 99% were obtained to identify this species.

### *3.5. Antimicrobial susceptibility test*

All *E. coli* showed 100% susceptibility to penicillin, azithromycin, tetracycline, and doxycycline and 75% to streptomycin. On the other hand, the *S. sciuri* showed 75% susceptibility to azithromycin, penicillin, and doxycycline, 50% and 25% to streptomycin and tetracycline, respectively (Figure 3 and Table 3).

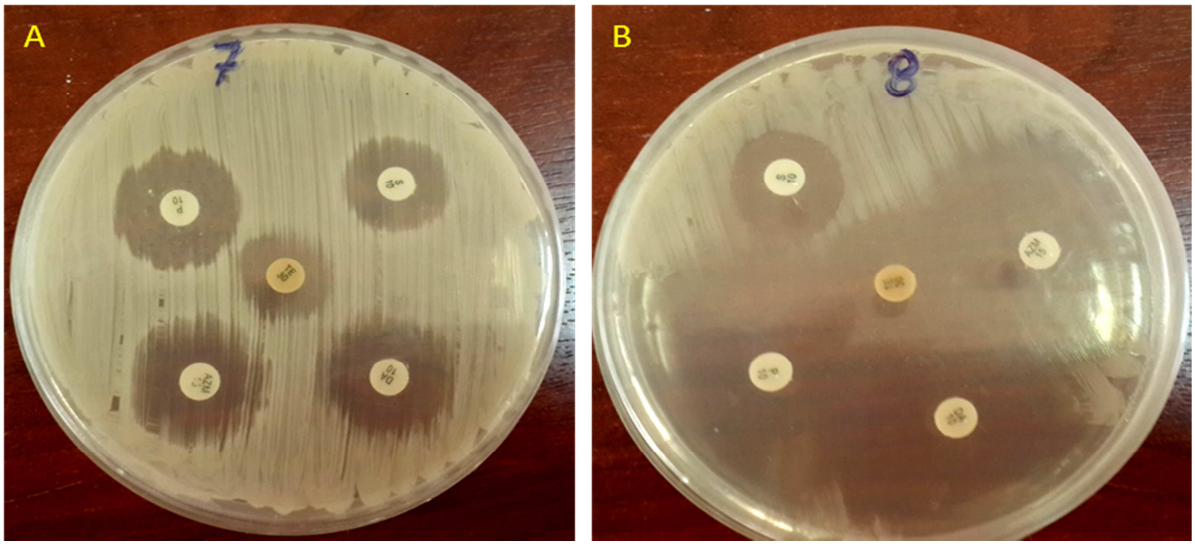


**Figure 3.** Antimicrobial susceptibility test for (A) *S. sciuri* and (B) *E. coli* isolates from organs of *Alectoris chukar* subspecies *Kurdestanica*.

**Table 3.** The percentage of antimicrobial susceptibility tests for (A) *S. sciuri* and (B) *E. coli* isolates from *Alectoris chukar* subspecies *Kurdestanica*.

| Bacterial Species | Susceptibility (%) | | | | |
|---|---|---|---|---|---|
| | Penicillin | Tetracycline | Azithromycin | Doxycycline | Streptomycin |
| ***S. sciuri*** | 75 | 25 | 75 | 75 | 50 |
| ***E. coli*** | 100 | 100 | 100 | 100 | 75 |

### *3.6. Molecular detection*

Molecular analysis was conducted only for *S. sciuri* to determine the common resistance gene in this genus. All *S. sciuri* isolates were showed positive for the methicillin-resistant *mecA* gene (Figure 4). The results were further confirmed by the sequencing of the PCR product and the sequences were submitted into NCBI Genbank.

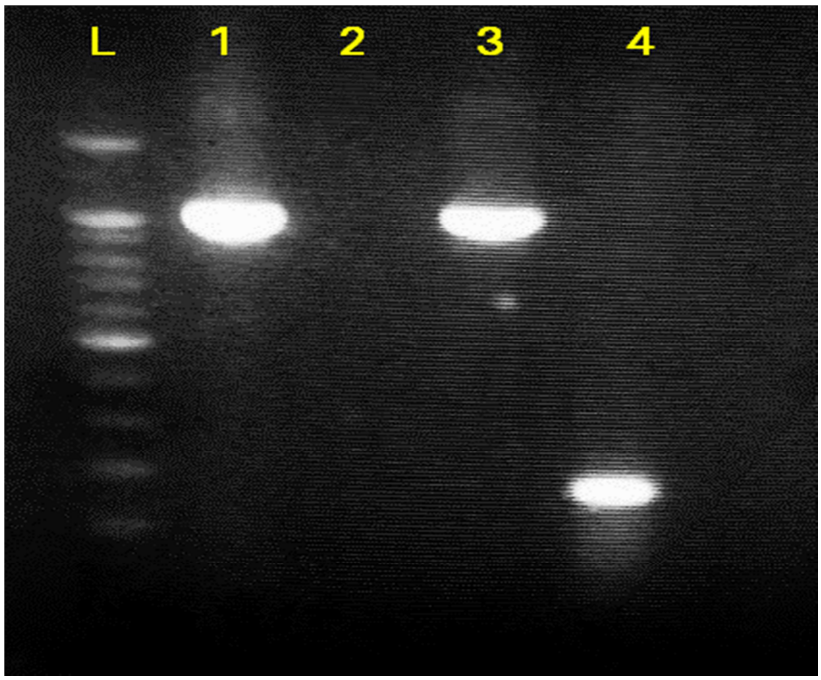


**Figure 4.** Agarose gel electrophoresis showing PCR amplification of the 1120 bp *S. sciuri meA* gene isolated from *Alectoris chukar* subspecies *kurdestanica*. Lane L: 100 bp DNA ladder; Lane 1: *S. sciuri* isolate; Lane 2: Negative control; Lane 3: Positive control; and Lane 4: 167 bp positive control for bacterial 16S rRNA gene.

## 4. Discussion

Poultry diseases and poultry meat contaminates are among the most important public health concerns because they can cause morbidity and even death to humans. Thus, poultry processing plants require the implementation of the Hazard Analysis Critical Control Point (HACCP) system to ensure that food products is free from contamination by spoilage organisms [27].

The *A. chukar* subspecies *Kurdestanica* is widely distributed in Kurdistan, Iraq, and it is one of the most important game birds. The meat of this bird is popular but expensive and is the choice of meat in many restaurants. However, the safety of chukar meat for consumption has not been ascertained. The meat may be a source of food-borne and zoonotic diseases. Among species of bacteria that were shown to contaminate chukar meat are *E. coli* and *S. sciuri.*

*S. sciuri* usually present in a wide range of habitats including healthy farm and wild animals [28]. In humans, these bacteria have been isolated in hospital environments and hospitalized patients [29]. The bacteria have also been isolated from wound swabs, surgical wound and soft tissue infections [30], the blood of patients with septic shock, probably from indwelling catheters [31], and in patients with continuous ambulatory peritoneal dialysis (CAPD) [32, 33]. To date, there is no information on the prevalence of these bacteria in the *A. chukar* subspecies *Kurdestanica*.

In the current study, *S. sciuri* was isolated from liver, kidneys, heart and thigh muscle of healthy chukar partridges. The bacteria were investigated to determine their antimicrobial-resistant properties or whether they possess the methicillin-resistant genes. An earlier report showed that *S. sciuri mecA* did not confer resistance to beta-lactam antibiotics [34]. This finding was confirmed by our study, where *S. sciuri* was shown to be 75% susceptible to both azithromycin and penicillin. On the contrary, isolated *S. sciuri mecA gene* in healthy chicken [35] and in the hospital environment [36] was showed 100% and 65.2% resistant to penicillin, respectively.

On the other hand, it was found that the isolated *S. sciuri* strain from surgical wound infection was susceptible to trimethoprim-sulfamethoxazole, erythromycin, chloramphenicol, ciprofloxacin and vancomycin and resistant to gentamicin, clindamycin, rifampicin, methicillin, ampicillin, and ceftriaxone. The multiresistant of the strain had a serious impact on the prolonged course of the infection [37, 38].

Simultaneously, *E. coli* was isolated from liver, kidney, gizzard and breast muscle of healthy chukar partridges and were found to be totally susceptible to each penicillin, tetracycline, azithromycin, and doxycycline, whereas 25% resistant to streptomycin. Instantly, the antibiotic resistance of *E. coli* to the common commercial antibiotic is a potential threat to food safety and public health which is found by other researchers that isolated this bacterium from raw poultry meat to be about 92% resistance to ampicillin and tetracycline, 15.8% to kanamycin, 23.7% to streptomycin [39]. On the other hand, Hossain et al. (2008) reported that the *E. coli* isolates from chicken meat from Bangladesh were found 100% resistant to nalidixic acid and 63% to ampicillin [40]. In contrast, our study showed that *E. coli* isolates of poultry meat and visceral organs are not resistant to antimicrobial drugs, commonly in use against Gram-negative bacteria.

Generally, a Gram-negative bacterium possesses an external lipopolysaccharide membrane that protects the peptidoglycan layer and allows the bacteria to survive in harsh environments. Presumably, this protective layer is one of the contributing factors toward the greater ability of *E. coli* than *S. sciuri* to survive the toxic effects of some frequently used and most popular antimicrobials *[36].* But in opposite to that theory, in this study, *S. sciuri* isolates were showed more resistance to the used antibiotics that may be due to the effect of the *mecA* methicillin resistance gene detected in the *S. sciuri*.

## 5. Conclusion

This is the first report of isolation of *S. sciuri* with methicillin-resistant *mecA* gene isolation from the *A. chukar* subspecies *Kurdestanica*. In light of the emergence of new infectious diseases in wildlife, it is currently of great interest to address the role of wild birds as potential vectors of pathogenic bacteria for human while they only act as a carrier. In the *A. chukar* subspecies *Kurdestanica*, the predominant bacteria contaminant in meats and organs were *E. coli* and *S. sciuri*. The birds used in this study were wild-caught,

thus, the prevalence of these bacteria may indicate that they are carriers of the bacteria that could potentially cause food-borne disease epidemics in humans consuming chukar partridge meats. For this purpose, hygienic conditions have to be proven on breeding and slaughtering enterprises, prevent cross-contamination and kept the cold chain until consumption.

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