

Scientific Report

Klebsiella pneumoniae infection in canaries (*Serinus canaria Domestica*): a case report

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Abstract

Background: *Klebsiella pneumoniae* can cause high mortality in birds. In recent years, small farming of canaries has been developed in Iran and infectious diseases are the main obstacle in the progress of these occupations which increases the importance of identification of pathogenic bacterial agents. **Case description:** A flock with 250 one-year-old canaries presented a history of anorexia, lethargy, mild diarrhea, and approximately 30% mortality. Physical examination revealed that the birds were severely debilitated, cachectic, dehydrated with ruffled feathers, and wet discolored stool around the cloaca. Necropsy findings revealed enlarged liver with multiple pale white, irregular foci on the surface of parenchyma, serosal petechial hemorrhages, and enlargement of lungs, liver, and kidneys. The entire intestine was intensely reddened with fibrinonecrotic exudate content. Histopathological findings of the liver elicited multifocal hepatocellular necrosis, hemorrhage, and also basophilic bacterial colonies. The results of biochemical and molecular tests confirmed *K. pneumoniae* as the causative agent. **Findings/treatment and outcome:** Based on antimicrobial susceptibility test, *K. pneumoniae* isolates were susceptible to gentamycin and ciprofloxacin which were administrated for the considered treatment protocol. **Conclusion:** *K. pneumoniae* is one of the most important causes of mortality in canaries with multiple antibiotic resistance, therefore assessments of health conditions can supply suitable information to help decision-making about the sanitary processes, control, prevention, and treatment.

Key words: Canary, Diarrhea, Histopathology, *Klebsiella pneumoniae*, Necropsy

Introduction

Klebsiella pneumoniae is a Gram-negative, non-motile, bacilliform, late lactose fermenting bacterium. It is an encapsulated opportunistic pathogen which is a member of the *Enterobacteriaceae* family. Members of the genus *Klebsiella* are opportunistic pathogens that are mostly identified in various infections of animals and humans. This bacterium is a common saprophyte in the environment and one of the respiratory disease agents in humans and chickens (Podschn and Ullmann, 1998). *Klebsiella* infections can cause sepsis, meningitis, diarrhea, nosocomial pneumonia, arthritis, and urinary tract infections in hospitalized humans. In animals, *Klebsiella* infections are frequently associated with respiratory and urinary tract infections, sepsis, and mastitis (Brisse and Van Duijkeren, 2005). Meanwhile, *Klebsiella* can acquire resistance to multiple antibiotics and is an important cause of nosocomial wound and urinary tract infections in hospitalized humans and animals (Vuotto *et al.*, 2014).

In recent years, non-industrial farming of canaries has

been developed in Iran and infectious diseases, including bacterial infections, are the main obstacle in the progress of these occupations which this subject increases the importance of identification of pathogenic bacterial agents. This report details the identification procedure of *K. pneumoniae* in a canary with respiratory symptoms.

Case description

A total of 250 one-year-old (both sexes) domestic canaries (*Serinus canaria domestica*) with an average weight of 33 ± 0.6 g were presented by an indoor aviculturist to the private veterinary clinic in Northeast Iran due to anorexia, lethargy, diarrhea, and also approximately 30% mortalities during two days. On physical examination, the birds were severely debilitated, cachectic, dehydrated with ruffled feathers and wet discolored stool around the cloaca. According to the history and clinical signs, our differential diagnosis included coccidiosis, secondary necrotizing enteritis, avian poxvirus infection, and other potential bacterial causes.

Based on owner information, an indoor breeding facility was considered, and the birds were kept in pairs in breeding cages (50 × 40 × 40 cm) with artificial lighting. The diet was mostly traditional seed mixture (millets and linseed), soft food (fruits and vegetables), cuttlefish bone, and grit. To have an accurate diagnosis, the fresh canary carcasses were referred to the Avian Diseases Division, Faculty of Veterinary Medicine, the University of Tehran for further examinations. All procedures used in the study are following institutional ethical guidelines which are based on the Directive 2010/63/EU on the protection of animals used for scientific purposes.

Microbiology and antimicrobial susceptibility testing

To check out necrotizing enteritis, a sample of 50 g liver sample was added into 450 ml of distilled water and mixed in a vortex mixer (TARSON) for 15 min. A 50-ml aliquot from the mixture was transferred to a beaker and placed in the water bath at 80°C for 10 min. After cooling the mixture, 1 ml of it was inoculated on blood agar containing 7% defibrinated sheep blood and incubated anaerobically at 37°C for 48 h. Suspected colonies were sub-cultured on selective culture media of *C. perfringens* including tryptose sulfite cycloserine agar (TSC).

For detection of *Salmonella* spp., three debilitated canaries were humanly killed by CO₂ asphyxiation. One gram of cecal contents was aseptically removed and placed into sterile tubes containing 9 ml of peptone water buffer and incubated overnight at 37°C. To be enriched in a selective media 0.1 ml of each tube was transferred to 10 ml of rappaport vassiliadis (RV) broth (Merck, Germany) and incubated overnight at 37°C. Following enrichment, each sample of cecal contents was streaked for isolation on xylose lysine deoxycholate (XLD) agar plates (Merck, Germany). The plates were incubated at 37°C for 24 h and then observed for either the presence or absence of characteristic *Salmonella* colonies.

The presence of the coccidian organism (Isospora) was evaluated by wet smears of intestinal and fecal samples taken from live and dead canaries. In terms of determining the distribution of other potential bacterial agents especially Enterobacteriaceae, *Klebsiella*, *Pseudomonas*, *Enterobacter*, *Proteus*, and some Gram-positive bacteria such as *Staphylococcus* spp., *Streptococcus* spp., and *Enterococcus* spp. were assessed by Gram staining of fecal specimens and mucosal intestinal scraping, agar culture, colonial and microscopical morphology, hemolytic pattern and biochemical experiments as described formerly (Chanos and Mygind, 2016).

In the next step, biochemical tests and bacterial culture were performed using sterilized swabs from seven liver samples of birds. The specimens were enriched using a nutrient broth for 24-48 h at 37°C anaerobically/aerobically, then cultured on Blood agar aerobically at 37°C for 24-48 h which represented mucoid non-hemolytic colonies. To have definite

colonies of bacteria, suspected colonies were re-cultured on Mac Conkey's agar for 24 h.

To treat the canaries, the antimicrobial susceptibility of *K. pneumoniae* was tested by Kirby-Bauer disk diffusion method on Mueller-Hinton agar based on the recommendations provided by the National Committee for Clinical Laboratory Standards Guidelines (CLSI, 2019). *K. pneumoniae* isolate was tested against the following 13 antimicrobial agents (µg/disk): ciprofloxacin (5 µg), enrofloxacin (5 µg), chloramphenicol (30 µg), cefazolin (30 µg), cefixime (5 µg), ampicillin (10 µg), erythromycin (15 µg), kanamycin (30 µg), streptomycin (30 µg), amikacin (30 µg), gentamicin (10 µg), florfenicol (30 µg), and oxytetracycline (30 µg). All anti-bacterial disks were purchased from Padtan Teb Co. (Tehran, Iran).

Histopathology

Samples of livers were collected in 10% neutral buffered formalin for histopathological assessment. Tissue samples were routinely processed, dehydrated, and embedded in paraffin wax, sectioned at 5 µm thickness (Rotary Microtome RM2 145, Leica), and stained with hematoxylin and eosin (H&E). Sections were examined using a light microscope (Nikon, Eclipse E600), and representative images were taken.

Polymerase chain reaction (PCR)

Tissue samples of pulmonary lesions were kept at 20°C until use. The specimens were minced and ground with sterile quartz sand by a mortar and pestle, suspended in a balanced salt solution containing 50 IU/ml penicillin and 50 mg/ml streptomycin. After centrifugation at 100 g for 10 min with low-speed, 0.1 ml of the supernatant of the 15 suspensions was inoculated on the CAMs of 11-day-old chicken embryos from commercial and specific-pathogen-free (SPF) as well. The inoculated eggs were incubated at 37.5°C for 7 days and then evaluated for pocks on the CAM. No more passages were performed. Extraction of DNA was performed by 25 mg of the pulmonary lesions of seven clinical cases and 25 mg of the two lyophilized vaccines by QIAamp DNA Mini Kit (Qiagen, Italy, Milan) according to the manufacturer's instructions. Proteinase K was used for tissue digestion at 56°C for 24 h. Obtained DNA specimens were kept at 80°C up to analysis. PCR reactions for APV-specific PCR and fpv140 were performed in a volume of 25 µl PCR buffer containing 1.5 mM of MgCl₂, 6 pmol of each primer, 200 mM of each dNTP, and 1.25 U Taq polymerase (Invitrogen, Milan, Italy). The APV specific PCR was carried out using a primer pair explained in a study (Lee and Lee, 1997) according to the sequence of P4b gene of Fowlpox virus strain HP444. After the initial denaturation, the amplification was performed for 2 min at 94°C for 35 cycles including 1 min denaturation at 94°C, 1 min annealing at 60°C, and 1 min extension at 72°C. The final extension step was performed for 2 min at 72°C. Afterwards, five microliters of the PCR products were detached by 2% (for P4b) and 1.2% (for

fpv140) agarose gel electrophoresis and subsequently stained with ethidium bromide. PCR products obtained from amplifying P4b gene with the characteristic size were purified by QIAquick PCR purification kit (Qiagen, Milan, Italy). One commercially available vaccine strain against the fowlpox virus (Razi Institute, Iran) was also included.

To detect *K. pneumoniae*, the isolate was subjected to multiplex PCR to confirm results obtained from biochemical tests by using the method adopted from a study with some modification (Chander *et al.*, 2011). The DNA extraction from the individual colonies was performed by an extraction kit (Bioneer, South Korea) in accordance with the manufacturer's guidelines. In brief, 200 μ L of the specimen suspension was incubated with 200 μ L of lysis buffer and 10 μ L proteinase K at 65°C for 30 min. Following incubation, 250 μ L of binding buffer and 250 μ L of ethanol 80% were combined with the lysate. After that, washing the sample was performed according to the producer's guidelines. Elution of nucleic acid was carried out with 100 μ L of elution buffer. The PCR reaction mixture composed of 5 μ L template DNA, 12.5 μ L Taq DNA Polymerase Master Mix RED kit (Ampliqon, Denmark) which contained Tris-HCl 150 mM pH = 8.5, (NH₄)₂SO₄ 40 mM, MgCl₂ 1.5 mM, 0.2% Tween 20, dNTPs 0.4 mM, Ampliqon Taq DNA Polymerase (0.05 Unit/ μ L), 1 μ L (10 pmol/ μ L) of each forward and reverse of primers and distilled water to the final volume of 25 μ L. The list of primers used in this study is presented in Table 1. Thermal cycling (Techne TC, 3000, England) was carried out at 95°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min which terminated the PCR reaction. The PCR products (5 μ L) were visualized on 1% agarose gel in Tris-acetate-EDTA buffer by electrophoresis.

Table 1: List of primers used in multiplex PCR assay for confirmation of *K. pneumoniae*

Primer name	Sequence (5'-3')	Target gene	Product size (bp)
<i>Klebsiella</i> spp.	F: CGCGTACTATACGCCATGAACGTA R: ACCGTTGATCACTTCGGTCAGG	<i>gyrA</i>	441
<i>K. pneumoniae</i>	F: CAACGGTGTGGTTACTGACG R: TCTACGAAGTGGCCGTTTTC	<i>rpoB</i>	108

Results

Necropsy, microbiological, and histopathological findings

Postmortem examination revealed enlarged liver with multiple pale white, irregular, and variably sized foci on the surface, and the parenchyma and many foci were confluent (Fig. 1A). There were petechial hemorrhages on the serosal surface of visceral organs, enlargement of subcutaneous blood vessels, and congestion and enlargement of the lungs, liver, and kidneys. In addition, the entire intestine was intensely reddened, and the mucosal surfaces were covered by fibrinonecrotic exudate. Meanwhile, airsacculitis especially cranial and caudal thoracic air sacs were involved but

pneumonia/bronchopneumonia was not observed.

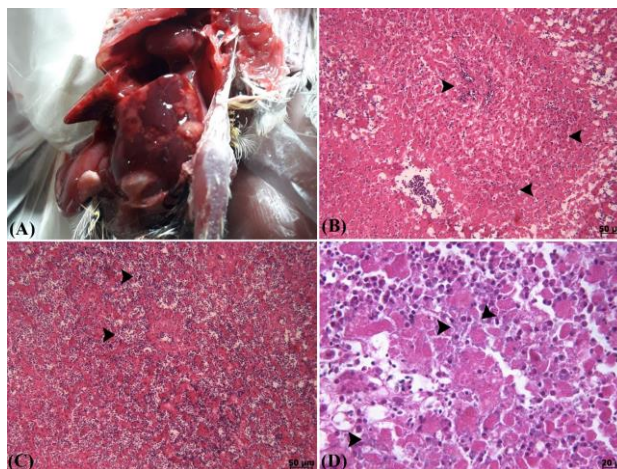


Fig. 1: (A) An enlarged liver with multiple pale white and irregular foci on the surface, (B) Hepatocellular necrosis (arrowheads) (H&E staining, $\times 20$, scale bar: 50 μ m), (C) Hemorrhage (arrowheads) (H&E staining, $\times 20$, scale bar: 50 μ m), and (D) Basophilic bacterial colonies in the liver (arrowheads) (H&E staining, $\times 60$, scale bar: 20 μ m)

After the procedure of culture on blood agar and MacConkey's agar and obtaining non-hemolytic and lactose-fermenting colonies, the bacterial isolates were identified as *K. pneumoniae* by non-automated biochemical tests (Table 2). Histopathological findings revealed multifocal hepatocellular necrosis, hemorrhage, and inflammatory cells infiltration (Figs. 1B and C). In addition, basophilic bacterial colonies were also seen in the liver (Fig. 1D). All samples selected and sub-cultured on selective culture media of *C. perfringens* were negative for *C. perfringens*. In addition, black colonies characterizing *Salmonella* spp. were not observed on XLD agar.

Table 2: Biochemical properties of *K. pneumoniae* strains isolates in the present study

Test name	Results
Indole production test	Negative
Methyl-red test	Negative
Voges-proskauer test	Positive
Citrate utilization test	Positive
Hydrogen sulfide production	Negative
Urea hydrolysis test	Positive
Ornithine decarboxylase	Negative
Motility at 36°C	Non-motile
D-glucose (acid/gas)	Positive/Positive
D-mannitol fermentation	Positive
Sucrose fermentation	Positive
Lactose fermentation	Positive
D-sorbitol fermentation	Positive
Cellobiose	Positive
Esculin hydrolysis	Positive
Acetate utilization test	Positive

Antimicrobial susceptibility findings

The results of the antibiotic susceptibility tests were interpreted according to the guidelines provided by CLSI (2019). The isolates were assigned into resistant,

intermediate, and susceptible groups. *Klebsiella pneumoniae* isolates were susceptible to ciprofloxacin and gentamycin. Ciprofloxacin was then administrated for the treatment of the flock. Gentamicin was not prescribed because it is not absorbed orally and must be given parentally, which would be a challenge in a flock of 250 small, fragile birds. Intermediate susceptibility was observed to streptomycin, kanamycin, amikacin, enrofloxacin, and oxytetracycline. Antibiotic resistance was shown for cefazolin, cefixime, ampicillin, erythromycin, chloramphenicol, and florfenicol.

PCR analysis

The results for detection of canarypox virus in PCR technique were negative in all isolates (data are not shown). In all reactions, positive controls were *K. pneumoniae* and *Klebsiella oxytoca*, and the negative control was distilled water. Every seven samples in the current study were positive for *Klebsiella* species and *K. pneumoniae*. In gel electrophoresis, both products of 441 bp and 108 bp were observed (data are not shown).

Discussion

Klebsiella pneumoniae in the *Enterobacteriaceae* family is a common saprophyte in the environment and one of the respiratory disease agents in humans and chickens. Enterobacteria do not belong to the intestinal flora of granivorous pet birds which are able to spread in the environment and become ubiquitous under suitable situations (Gerlach, 1994; Glünder, 2002). *K. pneumoniae* can be detected in fecal samples and oropharynx of many species of healthy psittacines and passerines (Gibbs *et al.*, 2007), however, these enterobacteria mostly act as respiratory pathogens, especially among immunosuppressed and stressed birds (El Fertas-Aissani *et al.*, 2013). It seems that keeping captive birds can impact on normal flora of the intestinal and respiratory tract and subsequent colonization by Gram-negative bacteria. In this case report, it seems that lack of hygiene and biosecurity, introducing new canaries to the flock without quarantine and health status monitoring, induced stress and immunosuppression especially during mating and feeding the chicks, or presence of concurrent diseases may play important roles in the occurrence of *K. pneumoniae* infection. In addition, arbitrary usage of different antibiotics by aviculturists without consultant of vets and antimicrobial susceptibility testing could modify the intestinal and respiratory tract microbial flora, favoring colonization by Gram-negative bacteria. Based on the history and owner descriptions, the disease started suddenly and the rate of mortality was relatively severe during utilization of antibiotics arbitrarily by the aviculturist which shows the multiple antibiotic resistance against *K. pneumoniae* as an aggregating factor in the infectious process of disease.

An outbreak of *K. pneumoniae* infection had been reported in many animals (Fecteau *et al.*, 1997; Davies *et al.*, 2016; Yang *et al.*, 2019; Roberts *et al.*, 2000). Similar recent reports about the isolation of this

microorganism from canaries suffered from cutaneous xanthogranuloma and yolk sac infection (Razmyar and Zamani, 2016; Shokrpour *et al.*, 2019). Consistent with the current study, the clinical signs and necropsy findings were similar in dogs with *K. pneumoniae* infection in which diarrhea, dehydration, and enlarged liver with multiple focal necrosis were the dominant reported symptoms (Roberts *et al.*, 2000). This bacterium had been recognized as a causative factor of genital system infection of the mare, bovine mastitis, critically sick neonatal calves, severe enteritis, and diarrhea in dogs (Braman *et al.*, 1973; Atherton, 1975; Fecteau *et al.*, 1997; Saidenberg *et al.*, 2012; Roberts *et al.*, 2000). In addition to bacterial infection caused by *Klebsiella* spp. in animals with different clinical manifestations, there are other concerns about the probability of transmission of this microorganism to animals and humans (Saidenberg *et al.*, 2012).

K. pneumoniae is known to be an opportunistic causative agent of nosocomial infections in humans with various presentations of disease and the emergence of multidrug-resistant of this bacterium has made it difficult to control, worldwide (Ripabelli *et al.*, 2018). In a similar study, the researchers reported the prevalence of Gram-negative bacteria between breeder canaries with clinical disease and the antimicrobial susceptibility patterns of the isolates in which 6 of 88 isolates classified in *Klebsiella* spp and most susceptibility was observed to norfloxacin, oxytetracycline, and ciprofloxacin. The most resistance was found for amoxicillin, cefadroxil, erythromycin, and penicillin G (Giacopello *et al.*, 2015); while in our study, the isolates were susceptible to gentamycin and ciprofloxacin and resistant to cefazolin, cefixime, ampicillin, erythromycin, chloramphenicol, and florfenicol. As well, we discovered a relatively large similarity between the antimicrobial susceptibility of the *K. pneumoniae* isolate of the current study and the recent case report (Razmyar and Zamani, 2016). In another study, gentamicin efficacy against *K. pneumoniae* isolated from Passeriformes and Psittaciformes was demonstrated. In the current study, ciprofloxacin showed proper efficiency for treatment protocol, but in another study, *K. pneumoniae* strains isolated from chickens were resistant to this antimicrobial agent, therefore, these similar and contradictory findings of antimicrobial susceptibility of *K. pneumoniae* would be due to different species, age, route of infection, environmental condition, and bacterial resistant profiles.

Based on the results of this study, *K. pneumoniae* is one of the most important causes of mortality in canaries with multiple antibiotic resistance, and its control, prevention, and treatment are of great importance. Isolation of this bacterium had been reported in extra-enteric disease in canaries and this study demonstrated pathogenic *K. pneumoniae* with gastrointestinal origin and systemic lesions which should be considered as a differential diagnosis for high mortalities in canaries. In addition, the precision mechanism of horizontal transmission of *K. pneumoniae* has not been identified to date. It appears that the bacterium has entered the host

via the gastrointestinal tract and vertical transmission has not been definitively confirmed yet and needs further investigations. Also, due to the wide range of this bacterium hosts, wild and domesticated birds and mammals may be raised as the reservoir of infection. According to the results, *K. pneumoniae* is one of the most important causes of mortality in canaries with multiple antibiotic resistance, therefore assessments of health conditions can supply suitable information to help decision-making about the sanitary processes, control, prevention, and treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

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