

Scientific Report

An outbreak of yolk sac infection and dead-in-shell mortality in common canary (*Serinus canaria*) caused by *Klebsiella pneumoniae*

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(Received 9 Jul 2015; revised version 23 Dec 2015; accepted 30 Jan 2016)

Summary

Yolk sac infection (YSI) and dead-in-shell mortality caused by Enterobacteriaceae in birds are not a rare phenomenon, however there are only a few reports indicating the association between these conditions and *Klebsiella* spp. among canary chicks (*Serinus canaria*). There have been reports of high mortality among 1-3 day old canary chicks in an indoor flock of canaries. In order to study the causative agent, yolk sac samples from dead-in-shell and day-old canary chicks were cultured. *Klebsiella pneumoniae* was isolated and identified based on biochemical tests and using genus and species-specific multiplex PCR and later tested for their susceptibility to 13 antimicrobial agents. The isolates showed susceptibility to Gentamycin, Chloramphenicol, Florfenicol and Streptomycin.

Key words: Antimicrobial susceptibility, Common canary (*Serinus canaria*), *Klebsiella pneumoniae*, Yolk sac infection

Introduction

Yolk retention and yolk sac infection (YSI) are considered as important causes of death in different domesticated birds such as chicken, guinea fowl, duck, turkey, quail and goose. Both YSI and dead-in-shell occur in chicks a few days after hatching, which result in decreased hatchability and increased mortality. Members of the Enterobacteriaceae family, such as *Escherichia coli*, *Salmonella* spp. and *Klebsiella* spp., along with other bacteria such as *Staphylococcus*, *Pseudomonas* and *Clostridia* species, and also *Aspergillus fumigatus* are common causes of YSI and dead-in-shell (Khan *et al.*, 2004). *Klebsiella pneumoniae* is a bacillus, non-motile, oxidase negative, rod shape bacteria with a polysaccharide based capsule, a late lactose fermenting organism and a member of the Enterobacteriaceae family that is a Gram-negative. This bacterium is a common saprophyte in many parts of the environment (Ryan and Ray, 2004) and it can be found everywhere in nature (Bagley, 1985). *Klebsiella* infections can cause a wide range of diseases in humans such as pneumonia, urinary tract infections, septicemia, meningitis, diarrhea, and soft-tissue infections, especially in those with weak immunity (Bagley, 1985; Podschun and Ullmann, 1998). They can also participate in the pathogenesis of the spondylo-arthropathies (Podschun and Ullmann, 1998). This bacterium, however, is an opportunistic pathogen that has been implicated in cases of mastitis (Braman *et al.*, 1973), metritis (Brown *et al.*, 1979), bacteraemia (Fecteau *et al.*, 1997), pneumonia and urinary tract

infections (Roberts *et al.*, 2000), pneumonia and septicemia (Wilson and Madigan, 1989) and poly arthritis in cattle, mares, calves, dogs, foals and in humans, respectively. Additionally, *Klebsiella* can acquire resistance to multiple antibiotics and is an important cause of nosocomial wound and urinary tract infections of hospitalized humans and animals. *Klebsiella* species occasionally cause embryonic mortality and excess losses in young chickens and turkeys (Orajaka and Mohan, 1985).

Case description

An indoor aviculturist (250 canaries) reported dead-in-shell (10 of 24 eggs) and high mortality in his 1-3 day old chicks (11 of 26 chicks). They were depressed, unable to move and eat, showed abdominal distention and were found dead in the first three days after hatching. The breeding facility was located indoors, and the birds were kept in pairs in breeding cages (50 × 40 × 40 cm) with artificial lighting and used newspaper on the bottom of the cages as the substrate. The bird feeds mainly on seeds including: regular and traditional seed mixture (millets and linseed), soft food, cuttlefish bone, grit and conditioning seeds which are changed regularly.

Dead-in-shell and 1-3 day old canary chicks were brought to the Avian Medicine Division of the Veterinary Faculty of Ferdowsi University of Mashhad for further investigation. Necropsy revealed a distended yolk sac in chicks, that was hyperemic and yellow-greenish watery. There were signs of septicemia,

Table 1: List of primers used in this study

Primer name	Sequence (5'-3')	Target gene	Product size (bp)
KP (F) (R)	CAA CGG TGT GGT TAC TGA CG TCT ACG AAG TGG CCG TTT TC	<i>rpoB</i>	108
KO (F) (R)	GAT ACG GAG TAT GCC TTT ACG GTG TAG CCT TTA TCA AGC GGA TAC TGG	<i>pehX</i>	343
Kleb. (F) (R)	CGC GTA CTA TAC GCC ATG AAC GTA ACC GTT GATCAC TTC GGT CAG G	<i>gyrA</i>	441

including petechial hemorrhages on the serosal surface of visceral organs, enlargement of subcutaneous blood vessels and congestion and enlargement of the lungs, liver and kidneys.

Swabs were taken from yolk sac and liver of the two canaries for bacterial isolation. Samples were enriched in nutrient broth anaerobically/aerobically for 24-48 h at 37°C then cultured on Blood/MacConkey's agar aerobically at 37°C for 24-48 h. Another 24 h of incubation at 37°C on MacConkey's agar was done in order to purify the suspected colonies. Bacterial isolates were identified as *Klebsiella* spp. by non automated biochemical tests (Table 1).

Klebsiella isolates were recovered from dead-in-shell embryo and YSI and their susceptibility to a panel of 13 antimicrobial agents, determined by the agar disk-diffusion method, and also the interpretations of the results, were carried out according to the National Committee for Clinical Laboratory Standards Guidelines (CLSI)(1). The tested antimicrobial agents and their concentrations (µg) were as follows: ciprofloxacin (5), enrofloxacin (5), Chloramphenicol (30), cefazolin (30), cefixime (5), ampicillin (10), erythromycin (15), kanamycin (30), streptomycin (30), amikacin (30), gentamicin (10), florfenicol (30), and oxytetracycline (30). All anti-bacterial disks were purchased from Padtan Teb Co. (Tehran, Iran). The resistance of the isolates against antimicrobial agents was classified as susceptible, intermediate and resistant, based on the standard interpretation chart updated according to the current CLSI standard (CLSI, 2014).

The isolates were subjected to multiplex PCR to confirm biochemical test results by using Chander *et al.* (2011) method with some modification. The DNA was extracted from single colony using an extraction kit (Bioneer, South Korea) according to the manufacturer's instructions. Briefly, 200 µL of the sample suspension was incubated with 200 µL of lysis buffer and 10 µL proteinase K at 65°C for 30 min. After incubation, 250 µL of binding buffer and 250 µL of ethanol, 80% were added to the lysate. The sample was then washed following the manufacturer's recommendations. Nucleic acid was eluted with 100 µL of elution buffer provided in the kit (Chander *et al.*, 2011).

Briefly, the PCR reaction mixture consisted of 5 µL template DNA, 12.5 µL 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/ul), 1 µL (10 pmol/µL) of each forward and reverse of primers (Table 1) and distilled water to the final volume of 25 µL. 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 (Chander *et al.*, 2011). The PCR products were analyzed by 1% agarose gel and ethidium bromide staining followed by visualization with ultraviolet transillumination 5 µL PCR products. All reactions contain positive control's *K. pneumoniae* and *Klebsiella oxytoca*, kindly provided by Dr. M. Rad and Distilled water as negative control.

All isolates were identified as *K. pneumonia* by the biochemical tests and were also confirmed by using the PCR test (Fig. 1). Tested isolates were susceptible to Gentamycin, Chloramphenicol, Florfenicol and Streptomycin, intermediate susceptible to kanamycin, amikacin and oxytetracycline and resistant to ciprofloxacin, enrofloxacin, cefazolin, cefixime, ampicillin and erythromycin.

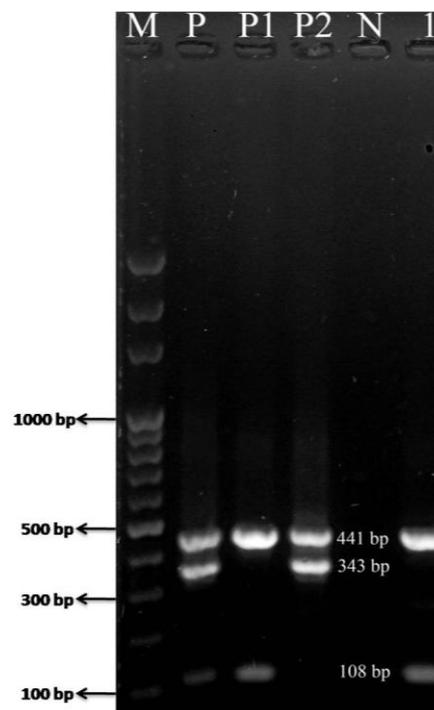


Fig. 1: The presence of *Klebsiella pneumonia* was confirmed with mPCR assay. Lane M: 100 bp marker, Lane P: Positive control including *K. pneumonia* (108 bp) and *Klebsiella oxytoca* (343 bp) and 441 bp PCR fragment of the *gyrA* gene (*Klebsiella* genus-specific gene), Lane P1: Positive control *K. pneumonia*, Lane P2: Positive control *K. oxytoca*, Lane N: negative control, and Lane 1: *K. pneumonia* isolated from the yolk sac sample

Discussion

Most reports present YSI and dead-in-shell or day-old mortality in poultry. However, few reports exist in the literature on companion birds. In this report, we present an outbreak of YSI caused by *K. pneumoniae* in a domestic flock of canaries.

Jahantigh (2010) reported *Klebsiella* spp. as 15% of bacterial flora of dead-in-shell embryos of ostrich along with *Bacillus* spp. (45%), *Staphylococcus* spp. (25%), *E. coli* (10%) and *Proteus* spp. (5%). Of 79 pooled samples containing 632 dead-in-shell chicken embryos, cultured from two hatcheries in Nigeria, 13 isolates were *Klebsiella* spp. (Orajaka and Mohan, 1985). Giacopello *et al.* (2014) reported the prevalence of Gram-negative bacteria among canaries with clinical disease, and the antimicrobial sensitivity patterns of the isolates. 6 of 88 isolates belonged to *Klebsiella* spp. (five *K. oxytoca* and one *Klebsiella* GR.47). The isolates in their study showed most susceptibility to Norfloxacin, Oxytetracycline, and Ciprofloxacin and most resistance to Amoxicillin, Cefadroxil, Erythromycin and Penicillin G (Giacopello *et al.*, 2014); while in our study, the isolates were susceptible to Gentamycin, Chloramphenicol and Streptomycin, and resistant to ciprofloxacin, enrofloxacin, cefazolin, cefixime, ampicillin and erythromycin.

Husseina *et al.* (2008) studied on bacteriology, pathology and antimicrobial resistance of YSI in broiler chicks in Suleimani district and reported *K. pneumoniae* as 12% of bacterial isolates from yolk sac samples and suggested Enrofloxacin, Florfenicol or Co-trimoxazole as being useful for the treatment of YSI. Ajayi and Egbeki (2011) recovered 90 strains of *K. pneumoniae* from 150 faecal samples of local birds in Nigeria. The frequency of antibiotic resistance among the isolates from local birds ranged between 28% and 88% among *K. pneumoniae*.

In conclusion, the results of the current study in a canary aviary focused on the importance of *K. pneumoniae* as a primary pathogen to induce YSI and dead-in-shell in causing the high mortality of canary chicks during their first week of their post-hatching life and thus, posing a great threat to this small to large colonies for aviculturists in Iran. Identification of the causative agent of YSI and dead-in-shell is important in order to choose the effective treatment protocol. Blind antibiotic therapy could lead to the development of antibiotic-resistant strains, and this highlights the importance of considering antimicrobial sensitivity pattern results in the formulation of treatment protocols. Moreover, a further study has been carried out throughout the country on epidemiological investigation of YSI and dead-in-shell in canary aviaries and its economic impact. The study also tries to assess the origin tracking by molecular methods in *K. pneumoniae* isolates obtained from human and bird's sources, and finally, solutions to prevent and control the disease are encouraged.

Acknowledgements

The authors would like to thank Dr. M. Rad for kindly providing positive controls (*Klebsiella oxytoca* and *Klebsiella pneumoniae*) and Mr. Ali Kargar for technical assistance.

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