Journal of Veterinary Diagnostic Investigation
2019, Vol. 31(3) 364–367

DOI: 10.1177/1040638719844297
jvdi.sagepub.com

Surveillance for *Avibacterium paragallinarum* in autopsy cases of birds from small chicken flocks using a real-time PCR assay

Kristin A. Clothier,[1] Andrea Torain, Steve Reinl

Abstract. Infectious coryza is a severe respiratory disease of chickens associated with large economic losses in affected commercial flocks. The fastidious causative pathogen, *Avibacterium paragallinarum*, is difficult to recover and identify, resulting in delayed diagnosis and enhanced spread of the agent. Small poultry flocks are increasingly common in rural and suburban environments. We assessed the frequency of *A. paragallinarum* using real-time PCR and clinical conditions present in samples from such flocks submitted to the California Animal Health and Food Safety Laboratory System (Davis, CA) in 2018. From the 294 samples collected for our study, 86 (30%) were PCR-positive for *A. paragallinarum.* Juvenile birds (≤1 y) were significantly more likely to be PCR-positive ($p = 0.017$), and birds diagnosed with respiratory disease had lower Ct values ($p = 0.001$) than those without. Concurrent infections were also identified, including with *Mycoplasma gallisepticum* (18.6%), *M. synoviae* (18.6%), infectious bronchitis virus (12.8%), and infectious laryngotracheitis virus (7.0%). Only 46.5% of PCR-positive chickens had antemortem respiratory signs, making endemic infections in these flocks highly likely. Our study demonstrates that *A. paragallinarum* is present in small-flock operations including those without respiratory disease and may present a risk for airborne pathogen transmission to commercial poultry operations.

Key words: *Avibacterium paragallinarum*; chickens.

Infectious coryza (IC), caused by *Avibacterium paragallinarum*, is an acute, severe, respiratory disease of chickens associated with large economic losses in affected commercial flocks worldwide.[2,9] Clinical signs include ocular and nasal discharge, rhinitis and sinusitis, and anorexia, with subsequent poor growth in broiler flocks and depressed egg production in layer flocks.[2,6,10] Subclinical carrier birds can be a reservoir for transmission via direct contact, airborne droplets, or fomites.[1,11] Concurrent respiratory agents, including *Mycoplasma synoviae* (MS), *M. gallisepticum* (MG), *Pasteurella* sp., and infectious bronchitis virus (IBV; species *Avian coronavirus*), as well as stress factors, can exacerbate disease.[1,9]

A. paragallinarum is a fastidious gram-negative bacterium in the *Pasteurellaceae* family.[3,5] Detection of *A. paragallinarum* by culture is often hindered both by its fastidious characteristics (slow growth rate, need for specialized media and growth conditions, and minimal reactivity to standard biochemical tests) and the presence of other bacterial organisms present in sample sites, including *Gallibacterium anatis, Pasteurella multocida*, and *Escherichia coli*, among others.[1,5] Delays in sample processing and previous antimicrobial treatment have been shown to prevent recovery.[2]

Vaccination, which is common in affected commercial operations, conveys strong protection against homologous strains of *A. paragallinarum*.[2,4] In our laboratory, several large-scale outbreaks have been identified in well-vaccinated populations with strict biosecurity protocols, prompting questions about the risks of transmission from small and backyard poultry operations in proximity to these affected farms. We used a validated high-throughput real-time PCR (rtPCR) assay to establish the frequency of *A. paragallinarum* in chickens from small flocks and to determine the frequency of clinical disease associated with this organism in these birds.

Samples were collected from small chicken flock cases submitted to the California Animal Health and Food Safety (CAHFS) laboratory system from February to August 2018. Small flocks are defined as containing <1,000 birds in a single operation; birds from larger operations or research facilities were not sampled for our study. Case reports were reviewed for pathology diagnoses and results of ancillary testing, which included evaluation of clinical history; gross and histologic examinations; bacterial cultures of lung, liver, or any diseased tissues identified at autopsy; PCR testing for avian influenza virus; and additional testing based on the autopsy examination including PCR for MG, MS, IBV; infectious laryngotracheitis virus (ILTV; species

California Animal Health and Food Safety Laboratory System, School of Veterinary Medicine, University of California–Davis, Davis, CA.

[1]Corresponding author: Kristin A. Clothier, School of Veterinary Medicine, University of California–Davis, Davis, CA 95616.
kaclothier@ucdavis.edu

Gallid herpesvirus 1); MG and MS serology; fecal flotation and/or mucosal scraping for evidence of intestinal parasites; and electron microscopy of affected tissues. Determination of lesions associated with *A. paragallinarum* was based on the pathologists' diagnosis of cause of death and interpretive comments along with results of ancillary testing.

PCR testing was performed following the laboratory protocol. Swabs from sinus or trachea were placed into 1-mL vials of sterile phosphate-buffered saline (PBS) at pH 7.4, vortexed for 15 s, and discarded. DNA was extracted (MagMax 96 viral RNA isolation kit; Life Technologies, Thermo Fisher Scientific, Waltham, MA) for total nucleic acid extraction in a 96-well plate format. Each 1-mL sample was centrifuged for 10 min at 16,000 × *g*, and the pellet was resuspended in 200 µL of PBS. Extraction was performed on a 50-µL aliquot per the manufacturer's instructions with DNA eluted in the supplied elution buffer. The PCR was designed using a previously published assay that targets the HPG-2 region of *A. paragallinarum*.[6] This target lies within the HPG1 region, a putative protein of unknown function found to be present in *A. paragallinarum* and not in other *Pasteurellaceae*.[5] PCR reactions contained 0.8 µM of each primer, 0.3 µM of probe (Takyon low Rox probe master mix; Eurogentec, Liege, Belgium), TaqMan exogenous internal positive control DNA (Life Technologies) to control for possible PCR inhibition, and 2 µL of template DNA in a total volume of 22 µL. PCR was performed (ABI 7500; Applied Biosystems, Thermo Fisher Scientific) with cycling parameters as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, followed by 60°C for 1 min. Extracted DNA from *A. paragallinarum* 29545 was used as the positive control. Positive and negative extraction and amplification controls were included with each assay. Clinical samples were considered positive at a cycle threshold (Ct) ≤38.0 based on in-house validation testing, which determined that the limit of detection was 10 organisms/mL at Ct = 38.0.

Statistical analyses were performed (SAS v.9.4; SAS Institute, Cary, NC). Observed Ct values were assessed using a least squares means procedure for PCR-positive birds with and without respiratory signs and between juvenile (<1 y old), young adult (1–3 y old), and adult (>3 y old) birds. Comparisons of disease diagnoses between *A. paragallinarum*–positive and –negative chickens were assessed with the Fisher exact test.

A total of 292 samples (252 sinus and 40 tracheal swabs collected at the pathologist's discretion; these sites are routinely sampled for *A. paragallinarum* culture to minimize overgrowth from other bacterial organisms) from 264 accessions were included (Table 1). Cases were submitted from 41 of the 58 California counties with birds from a broad age range (34.9%: <1 y old; 29.8%: 1–3 y old; 35.3%: >3 y old.)

Overall, 29.5% (86 of 292) of samples were PCR-positive for *A. paragallinarum*, including 27.0% (68 of 252) of the sinus swabs and 45.0% (18 of 40) of the tracheal swabs. Although tracheal swabs yielded a higher percent positive for this assay, side-by-side comparison testing was not possible from our convenience sample. Seven small flocks had 2 PCR-positive birds, and 1 small flock had 3 PCR-positive birds identified; the remaining 69 positive birds were each from an individual flock. Thirty-four (39.5%) PCR-positive birds had a history of respiratory disease described as sneezing, ocular and nasal discharge, swollen eyelids, swollen heads, or open-mouth breathing. Two birds with reported dyspnea had non-respiratory conditions contributing to death (ovarian adenocarcinoma, hemorrhagic liver syndrome). Juvenile birds were significantly more likely (p = 0.017) to be positive for IC than young adults or adults. Chickens for which respiratory disease was diagnosed as the cause of death (32 of 86, 37.2%) had significantly lower *A. paragallinarum* Ct values (p = 0.001) than birds with other disease processes. Concurrent infections detected by PCR in these birds included MG (18.6%), MS (18.6%), IBV (12.8%), and ILTV (7.0%); 4.7% had no other respiratory agents identified.

Less than 40% (34 of 86) of *A. paragallinarum*–positive chickens had a history of respiratory disease; these birds had statistically significantly lower Ct values (p = 0.03) than birds without respiratory signs. No significant difference in Ct value was detected between age groups (p = 0.18). Common clinical histories from PCR-positive chickens without respiratory signs included acute death (23 of 86, 26.7%) and lethargy (20 of 86, 23.3%).

Cause of death diagnoses demonstrated that neoplasia was more common in PCR-negative birds (27.6%) than PCR-positive (12.8%) ones. Other conditions were similar among the groups, with coelomitis (12.8% and 13.6%), hepatic lesions (2.3% and 5.3%), and enteric disease (5.8% and 3.9%) being identified from positive and negative birds, respectively. The presence of Marek's disease was not significantly different (p = 0.20) between PCR-positive (17.5%) and PCR-negative (13.2%) chickens.

A program providing low-cost autopsy and ancillary testing for small poultry operations is provided at all 4 CAHFS locations. Although our study was performed on a convenience sample of animals submitted to the diagnostic laboratory and cannot provide prevalence data, it demonstrates that *A. paragallinarum* is common in these California flocks. Over 50% of the PCR-positive chickens from small flocks had no signs or lesions of respiratory disease, and nearly one-quarter of these samples had Ct values <30, indicating the potential for shedding large numbers of bacteria (≥10^4 CFU/mL based on limit of detection testing for the assay).

Coinfection with other respiratory pathogens was also a common finding in the small-flock birds, which are less likely to have well-developed vaccination or biosecurity flock plans. More than 30% of birds with IC were concurrently infected with MG, MS, and IBV. Interestingly, the frequency of Marek's disease that would be expected to result in immu-

Table 1. Results of PCR testing for *Avibacterium paragallinarum* and clinical information for small-flock samples submitted to the California Animal Health and Food Safety laboratory system from February to August 2018.

	PCR-positive (n = 86)	PCR-negative (n = 206)
Age group		
Juvenile (≤1 y)	39 (45.3)*	62 (30.1)
Young adult (1–3 y)	17 (19.7)	70 (34.0)
Adult (>3 y)	31 (36.0)	74 (35.9)
Clinical conditions		
Respiratory cause of death at autopsy	32 (37.2)†	7 (3.4)
Non-respiratory cause of death at autopsy	54 (62.8)	199 (96.6)

	PCR-positive (n = 86)		PCR-negative (n = 206)	
Concurrent respiratory pathogens	With respiratory disease	Without respiratory disease	With respiratory disease	Without respiratory disease
Mycoplasma gallisepticum (MG)	2 (6.3)	0	0	0
Mycoplasma synoviae (MS)	0	1 (3.1)	0	1 (0.5)
Infectious bronchitis virus (IBV)	1 (3.1)	1 (3.1)	0	1 (0.5)
Infectious laryngotracheitis virus (ILTV)	4 (12.5)	0	2 (1.0)	1 (0.5)
MG, MS	6 (18.8)	2 (6.3)	6 (2.9)	0
MG, MS, IBV	10 (31.2)	0	1 (0.5)	0
MS, ILTV	4 (12.5)	0	3 (1.5)	0
MG, IBV	0	0	2 (1.0)	0

Numbers in parentheses are percentages.
* $p = 0.17$.
† $p = 0.001$.

nosuppression was not different between *A. paragallinarum* PCR–positive and –negative birds. Birds from small flocks with depression or acute death, particularly those that are <1 y old, should be tested with a PCR assay to determine if *A. paragallinarum* is a health concern to the flock.

Small-flock birds frequently have at least partial access to outdoor environments and airborne spread of respiratory agents.[7,8] During this same testing period, 42.2% (65 of 154) of commercial poultry samples were positive for *A. paragallinarum*; however, only birds with respiratory signs were selected for testing from these flocks. Although the direction of disease transmission between small flocks and commercial flocks was outside the scope of our study, it is conceivable that such a highly contagious agent could originate from either population or from unowned chickens allowed to roam free. Vaccination is common in commercial poultry operations; however, available vaccines contain inactivated *A. paragallinarum* strains, and therefore shedding post-vaccination is less likely than with modified-live vaccines. Furthermore, birds from small flocks tend to be kept into late adulthood, making colonized backyard birds a greater risk for shedding to susceptible chickens. Although data about vaccine and antimicrobial use in these small flocks was not collected, most of these operations have little or no veterinary oversight, so routine veterinary care is not practiced commonly. Monitoring of convenience samples such as these can better inform risks to commercial flocks and assess the efficacy of health maintenance plans for susceptible commercial operations. Screening of commercial chickens using a PCR assay, even those submitted because of non-respiratory mortality, may be warranted to ensure that *A. paragallinarum* is not contributing to flock disease.

Acknowledgments

We thank all of the pathologists and technicians who collected samples from autopsy cases that were used in this study.

Declaration of conflicting interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Kristin A. Clothier https://orcid.org/0000-0002-8180-6385

References

1. Badouei MA, et al. Isolation and molecular identification of *Avibacterium paragallinarum* in suspected cases of infectious coryza. Turk J Vet Anim Sci 2014;38:46–49.
2. Blackall PJ. Infectious coryza: overview of the disease and new diagnostic options. Clin Microbiol Rev 1999;12:627–632.
3. Blackall PJ, et al. Reclassification of *Pasteurella gallinarum*, [*Haemophilus*] *paragallinarum, Pasteurella avium*, and *Pasteurella volantium* as *Avibacterium gallinarum* gen. nov, comb. nov., *Avibacterium paragallinarum* comb. nov., *Avibacterium avium* comb. nov., and *Avibacterium volantium* comb. nov. Int J Syst Bacteriol 2015;55:353–362.

4. Blackall PJ, et al. Infectious coryza and related diseases. In: Swain DA, ed. Diseases of Poultry. 13th ed. Ames, IA: Wiley, 2013:859–873.
5. Chen X, et al. Development and application of DNA probes and PCR tests for *Haemophilus paragallinarum*. Avian Dis 1996;40:398–407.
6. Corney BG, et al. Rapid and sensitive detection of *Avibacterium paragallinarum* in the presence of other bacteria using a 5' Taq nuclease assay: a new tool for diagnosing infectious coryza. Avian Pathol 2008;37:599–604.
7. Grunkemeyer VL. Zoonoses, public health, and the backyard poultry flock. Vet Clin North Am Exot Anim Pract 2011;14: 477–490.
8. Mete A, et al. Causes of mortality in backyard chickens in northern California: 2007–2011. Avian Dis 2013;57: 311–315.
9. Morales-Erasto V, et al. Coinfection of *Avibacterium paragallinarum* and *Ornithobacterium rhinotracheale* in chickens from Peru. Avian Dis 2016;60:75–78.
10. Paudel S, et al. Coinfection of *Avibacterium paragallinarum* and *Gallibacterium anatis* in specific-pathogen-free chickens complicates clinical signs of infectious coryza,which can be prevented by vaccination. Avian Dis 2017;61:55–63.
11. Requena D, et al. Genome sequence and comparative analysis of *Avibacterium paragallinarum*. Bioinformation 2013;9: 528–536.