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Source: Journal of Wildlife Diseases, 32(2) : 331-337

Published By: Wildlife Disease Association

URL: https://doi.org/10.7589/0090-3558-32.2.331

Journal of Wildlife Diseases, 32(2), 1996, pp. 331–337

EXPOSURE OF WILD WATERFOWL TO *MYCOPLASMA ANATIS*

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ABSTRACT: We developed an ELISA procedure to assess the presence of *M. anatis*-specific serum antibody in ducks. Sera from exposed and unexposed Pekin ducks (*Anas platyrhynchos*) were used to standardize the ELISA and to establish reference ranges to classify ELISA results as exposed or not exposed. We conducted serological surveys of female waterfowl in the central and eastern United States between 1988 and 1992 to assess the frequency of exposure in wild waterfowl. Adult breeding mallards (*Anas platyrhynchos*), wintering mallards, and black ducks (*Anas rubripes*) had high prevalences of exposure to *M. anatis* (25% to >80%). In comparison, none of the breeding adult canvasbacks (*Aythya valisineria*) had serum antibody levels indicating exposure. Approximately 50% of the juvenile mallards and black ducks were exposed to *M. anatis* by 8 months of age, indicating high transmission rates among wild birds.

Key words: *Mycoplasma anatis*, serology, enzyme-linked immunosorbent assay, ELISA, wild ducks, Pekin, mallard, *Anas platyrhynchos*, canvasback, *Aythya valisineria*, American black duck, *Anas rubripes*, mycoplasmas, survey.

INTRODUCTION

Mycoplasmas (*Mycoplasma* spp.) frequently have been isolated from domestic chickens (*Gallus gallus*) and turkeys (*Meleagris gallapavo*) (Jordan and Amin, 1980; Kleven, 1994). Many of these mycoplasmas cause clinical disease of the respiratory system or joint synovia (Yoder, 1991). In addition, avian mycoplasmas cause decreased productivity in captive-reared wild turkeys (Rocke et al., 1988) and domestic poultry, and suppress growth rates in young birds (Stipkovits, 1979). However, less is known about the occurrence and effects of mycoplasmas in wild ducks.

Mycoplasma anatis, M. cloacale, and other unidentified species have been isolated from wild North American ducks (Goldberg et al., 1995). *Mycoplasma anatis* has also been isolated from wild ducks in Egypt (El-Dimerdash et al., 1985) and Spain (Poveda et al., 1990; Astorga et al., 1994) and recovered from domestic and semi-domestic ducks throughout the world (Bradbury et al., 1987; El-Ebeedy et al., 1987; Ivanics et al., 1988). *Mycoplasma anatis* can be pathogenic to ducklings and eggs, causes reduced growth rates in young birds (Tian and Gou, 1989; Samuel et al., 1995), and has been associated with clinical disease in domestic ducks (Jordan and Amin, 1980; Ivanics et al., 1988). Mycoplasmas (including *M. anatis*) have been cultured from newly hatched ducklings (Goldberg et al., 1995); thus vertical transmission from hens to ducklings may occur. Because few serological tests have been developed to measure antibodies to *M. anatis,* little is known about exposure to this organism in wild ducks.

As part of our studies to assess the health status of wild waterfowl in the central and eastern United States, we obtained serum samples from mallards (*Anas platyrhynchos*), canvasbacks (*Aythya vali-*

sineria), and American black ducks (*Anas rubripes*). Our objectives for this portion of the study were to develop an enzyme-linked immunosorbent assay (ELISA) to measure serum antibody, and determine exposure of wild ducks to *M. anatis*.

MATERIALS AND METHODS

Standardization of ELISA

Serum antibody to *Mycoplasma anatis* was measured using ELISA essentially as described by Thomas and Sharp (1988), with the substitution of *M. anatis*-specific duck antisera (Goldberg et al., 1995) and *M. anatis* type strain (Number 25524, American Type Culture Collection, Rockville, Maryland, USA) as the antigen. The specificity of the duck antisera was verified in the ELISA using heterologous avian mycoplasma antigens. We tested the specificity of our ELISA and found minimal cross-reactivity with antigens from stock cultures of the type strain of *M. gallisepticum*, *M. synoviae*, *M. gallinarum*, *M. gallipovonis*, *M. iners*, and *M. iowae* (Goldberg et al., 1995).

Negative reference serum was obtained from an uninfected 3-wk-old mallard raised in an isolation room during our experimental *M. anatis* studies (Samuel et al., 1995). We were unable to recover mycoplasmas from a tracheal swab, or tracheal or lung tissues of this bird or any other birds ($n = 41$) raised in the same isolation room, using the procedures of Goldberg et al. (1995). Positive reference sera were obtained from two 6-wk-old mallards initially infected in ovo with a field isolate of *M. anatis* (Samuel et al., 1995) and hyperimmunized by intratracheal inoculation at 3 wk of age. *Mycoplasma anatis* was recovered from the tracheal swabs from both birds by the methods of Goldberg et al. (1995). Blood was collected by jugular venipuncture into untreated syringes and the serum was separated by centrifugation ($1500 \times G$ for 20 min). The positive reference antisera were pooled and frozen at −20 C.

All ELISA tests were conducted following the procedure of Thomas and Sharp (1988). Briefly, after minimal sonication to disperse aggregated cells, *M. anatis* antigen was diluted to 5 μg mycoplasma protein/ml in 0.1 M Na_2CO_3 (pH 9.6), and 0.1 ml of diluted antigen (0.5 μg protein) was then placed in wells of a 96-well microtitration plate (Linbro, Flow Laboratories, Inc., McLean, Virginia, USA). Following incubation at 37 C overnight, plates were washed three times with 0.15 M NaCl containing 0.1% (weight/volume) Tween 20 (Sigma Chemical Company, St. Louis, Missouri, USA). Next, 0.1 ml duck antisera diluted appropriately in phosphate buffered saline containing 0.05% (weight/volume) Tween 20 (PBS-T, pH 7.2) was placed in microtitration wells and incubated for 90 min at 20 to 22 C. After washing as described above, affinity purified goat anti-duck Immunoglobulin G-horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) diluted 1:500 in PBS-T was added to microtitration wells (0.1 ml/well) and incubated at 20 to 22 C for 1 hr, followed again by washing. Color reagent (0.1 ml) containing 0.4 mM 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (Sigma Chemical Co.) and 1.5 mM H_2O_2 in 0.05 M citrate (pH 4) was then placed into each well. Color was developed for 45 min at 20 to 22 C, and the enzyme reaction was stopped by addition of 0.2 ml of 0.1 M hydrofluoric acid in 0.001 M ethylene-diamine-tetraacetic acid. Absorbance (A_{405}) was measured on a microtitration plate reader (MR580, Dynatech Laboratories, Alexandria, Virginia). The dual wavelength mode was set at 410 nm for test and 450 nm for reference wavelengths, and a blank was used on a control well, which was included in all assay steps, but was not coated with antigen. The mean absorbance (optical density, OD) measurement of quadruplicate test sera was calculated and values were expressed as the percent positivity relative to the positive reference sample; the ELISA value percent (EV%) = [(Test OD − Negative reference OD)/(Positive reference OD − Negative reference OD)] × 100 (de Savigny and Voller, 1980; Wright et al., 1993).

Reference ranges for ELISA reactivity (EV%) were established for classifying sera from individual wild ducks as exposed or not exposed. Two groups of domestic Pekin ducks were used to establish the classification criterion. One group (five males, 34 females) of 18-wk-old Pekin ducks was obtained from a research facility of a commercial duck producer (Maple Leaf Farms, Inc., Milford, Indiana, USA). Based on preliminary ELISA serology, Pekin ducks from this source were deemed unlikely to have been exposed to *M. anatis* and this group is referred to as the reference negative group. A second group (21 males, 42 females) of 10-mo-old Pekin ducks, the reference positive group, was also obtained from a Maple Leaf Farms closed commercial Pekin duck production flock which had ELISA reactivity in pilot studies. After collection of blood and tracheal swabs, ducks were necropsied to collect tissue samples (trachea, lung, and reproductive organs) for isolation of mycoplasmas. Ducks from these two groups were evaluated independently with swab and tissue samples processed as described by Goldberg et al. (1995).

Primary microbiological isolation and passage of mycoplasmas was attempted using the medium formulation of Jordan (1983). Additionally, with the exception of nine Pekin ducks from the reference positive group, duplicate isolation attempts from swab and tissue samples were made using SP-4 medium (Whitcomb, 1983). Sera collected from the reference negative and positive groups were assayed for *M. anatis*-specific antibody in ELISA on two separate occasions. We averaged the replicate ELISA EV% measurements for Pekin ducks in the reference negative ($\bar{x}_1 = 14.6$, $\bar{x}_2 = 12.4$) and reference positive ($\bar{x}_1 = 81.1$, $\bar{x}_2 = 78.8$) groups.

Field methods and sample collection

We obtained serum samples from breeding mallards and canvasbacks in the central United States, and from wintering mallards and black ducks in the eastern United States. Study locations and field methods are summarized in Goldberg et al. (1995). Breeding mallards were captured near Kulm, North Dakota (46°25′N, 98°55′W) and Hitterdal, Minnesota (USA) (47°00′N, 96°15′W) prior to spring nesting attempts in 1988, 1989, and 1990. Breeding canvasbacks at Agassiz National Wildlife Refuge (NWR), near Thief River Falls, Minnesota (48°18′N, 96°00′W) were sampled in the spring of 1990. In the fall and winter of 1990 to 1991, 1991 to 1992, and 1992 to 1993, serum samples were collected from juvenile (<12 mo old) mallards and black ducks at the Ottawa NWR near Oak Harbor, Ohio (USA) (41°37′N, 83°10′W). Adult (>12 mo old) black ducks were sampled at the Tennessee NWR near New Johnsonville, Tennessee (USA) (35°50′N, 87°50′W) during the winters of 1990 to 1991 and 1991 to 1992; juvenile black ducks were sampled during 1991 to 1992. All sera were stored at −20 C until testing. Only sera from female birds were used in this study.

Statistical analysis

We conducted a quadratic discriminant analysis (SAS, 1989) to statistically classify the average ELISA antibody EV%s of the positive (exposed) and negative (unexposed) reference groups of Pekin ducks. We used the discriminant analysis to establish a cut-off value that provided a 95% probability of correctly classifying positive titers to increase the specificity of our test. The ELISA values from our wild bird serum samples were classified as exposed or unexposed using criteria from this evaluation. We compared mean ELISA antibody levels among reference groups using a *t*-test (Zar, 1984). McNemar's test for related samples (Daniel, 1978) was used to compare the proportions of *M. anatis* recovered from the same tissues using Jordan's or SP-4 media. We analyzed seroprevalence data from wild birds using logistic regression methods (Hosmer and Lemeshow, 1989). Due to the differences in species and ages of wild birds sampled, analyses were conducted separately for summer (breeding ducks) and winter data. Exact 95% confidence intervals on reported seroprevalence data were calculated based on the *F* distribution (Zar, 1984).

RESULTS

We were unable to recover mycoplasmas from tissues or swabs of 39 Pekins in the unexposed (reference negative) group. In the exposed (reference positive) group, *M. anatis* was isolated from 21 (33%) of 63 birds. We compared Jordan's and SP-4 media using duplicate samples from 54 Pekins in the exposed group. None of the ovaries from 35 females were positive for *M. anatis* using either media. For males, the frequency of positive cultures from reproductive organs (vas deferens and testes) was higher (McNemar's test, $Z = 2.24$, $P < 0.05$) using Jordan's medium (five of 19 birds; 26%) than SP-4 (0 of 19 birds). *Mycoplasma anatis* was recovered most frequently from tracheal swabs, using either Jordan's (nine of 54 birds; 17%) or SP-4 media (nine of 54 birds; 17%). Trachea and lung tissues had lower frequency of *M. anatis* recovery; four (7.4%) of 54 tracheas using Jordan's, two (3.7%) of 54 tracheas using SP-4, three (5.6%) of 54 lungs using Jordan's, and one (1.9%) of 54 lungs using SP-4. Despite a higher frequency of isolation using Jordan's medium, we isolated *M. anatis* from four tracheal swabs and one lung tissue using SP-4, but not Jordan's medium. In contrast, we recovered *M. anatis* in Jordan's medium from four tracheal swabs, two tracheal tissues, and three lung tissues where *M. anatis* was not recovered in SP-4 medium. Overall, isolations of *M. anatis* were made more often (composite $Z = 2.33$ based on McNemar's tests for each comparison; $P < 0.05$) in Jordan's medium (21 of 216 samples;

TABLE 1. Seroprevalence and 95% confidence interval (C.I.) to *Mycoplasma anatis* in breeding adult hen mallards and canvasbacks from Minnesota and North Dakota based on ELISA.

Location	Species	Year	Prevalence (%)[a]	95% C.I.
Minnesota	Mallard	1988	11/16 (69)	41%–89%
Minnesota	Mallard	1989	5/6 (83)	36%–100%
Minnesota	Mallard	1990	16/19 (84)	60%–97%
N. Dakota	Mallard	1988	1/1 (100)	3%–100%
N. Dakota	Mallard	1989	13/18 (72)	47%–90%
N. Dakota	Mallard	1990	10/12 (83)	52%–98%
Minnesota	Canvasback	1990	0/19 (0)	0%–18%

[a] Prevalence: number positive/ number sampled (%).

9.7%) than SP-4 (12 of 216 samples; 5.6%), where both media were used.

Mean ELISA EV% for the reference negative group ($\bar{x}$ = 13.5, n = 39, SD = 5.09) was lower (t = 15.8, $P < 0.001$) than the reference positive group ($\bar{x}$ = 79.9, n = 63, SD = 32.6). Based on the quadratic discriminant analysis, an ELISA cutoff value of 27.3% would best classify the EV% of the Pekin ducks into positive and negative reference groups.

For summer samples, differences in exposure to *M. anatis* were most pronounced between species; exposure in canvasback ducks was lower than in mallards (0% vs. 78%, $P < 0.001$) (Table 1). No differences in prevalence ($P > 0.5$) were found between locations or years in breeding mallards.

Adult black ducks sampled in the winter had a higher exposure to *M. anatis* (P = 0.049) during 1990 to 1991 than during 1991 to 1992. Adult black ducks had similar prevalence of exposure (P = 0.11) between sampling locations in Ohio and Tennessee. Juvenile ducks had elevated frequencies of exposure (averaging 50%) by their first winter (Table 2). Juvenile exposure also varied among our annual samples ($P < 0.005$); exposure was highest during 1992 to 1993. Juvenile black ducks and juvenile mallards had similar exposure ($P \geq 0.80$). However, juvenile black ducks sampled in Tennessee during 1991 to 1992 had a lower prevalence of exposure than juveniles sampled in Ohio ($P < 0.005$). Other temporal and spatial changes in prevalence of exposure were difficult to evaluate because adults and juveniles were not consistently sampled at all locations each year.

TABLE 2. Seroprevalence and 95% confidence interval (C.I.) to *Mycoplasma anatis* in wintering mallards and black ducks from Ohio and Tennessee based on ELISA.

Location	Species	Year	Age[a]	Prevalence (%)[b]	95% C.I.
Ohio	Black duck	1990 to 1991	Ad	1/4 (25)	1%–81%
Ohio	Black duck	1990 to 1991	Juv	13/34 (38)	22%–56%
Ohio	Black duck	1991 to 1992	Juv	12/35 (34)	19%–52%
Ohio	Black duck	1992 to 1993	Juv	31/42 (74)	58%–86%
Ohio	Mallard	1990 to 1991	Juv	12/28 (43)	24%–63%
Ohio	Mallard	1991 to 1992	Juv	20/38 (53)	36%–69%
Ohio	Mallard	1992 to 1993	Juv	26/45 (58)	42%–72%
Tennessee	Black duck	1990 to 1991	Ad	15/21 (71)	48%–89%
Tennessee	Black duck	1991 to 1992	Ad	10/24 (42)	22%–63%
Tennessee	Black duck	1991 to 1992	Juv	4/23 (17)	5%–39%

[a] Ad, adult; Juv, juvenile.

[b] Prevalence: number positive/number sampled (%).

DISCUSSION

We developed an *M. anatis*-specific ELISA procedure to assess the presence of serum antibody in ducks. Sera from exposed and unexposed domestic Pekin ducks were used to standardize the ELISA. One Pekin duck from the exposed (reference positive) group had inconsistent, low ELISA antibody EV% from the replicate assays (5.6% and 30%) and our attempts to culture *M. anatis* from this bird were unsuccessful. This bird was not included in any of our evaluations because of the low and inconsistent EV% results. We suspect this bird was either not exposed, it did not become infected, or it did not produce antibodies to *M. anatis* infection. Determination of the most appropriate cut-off value for classification depends on the true proportion of positive and negative birds in the sampled population. To be conservative, we increased the ELISA cut-off value to 31.6% to give an estimated 95% probability that Pekins exceeding this EV% belong to the positive group. This modification was designed to increase the specificity of the test (Wright et al., 1993) so that only birds which were highly likely (≥95%) to be exposed to *M. anatis* were considered positive. This higher cut-off level was used to classify wild ducks as either exposed or not exposed. Based on our serological survey of wild duck populations in the central and eastern United States, there was a high frequency of exposure in both adult and juvenile birds.

Jordan's medium was more effective for *M. anatis* isolation than SP-4, and we recovered *M. anatis* most frequently from tracheal swabs. However, using SP-4 medium, we recovered *M. anatis* isolates from three birds (from two tracheal swabs and one lung tissue) which were culture-negative using Jordan's medium. Similar to our results with *M. anatis,* Fritz et al. (1991) found a variation of Jordan's medium better suited for isolation of *M. gallisepticum* from chickens than a modified SP-4 medium. If possible, both Jordan's and SP-4 media and several tissue types should be used for *M. anatis* isolation.

We frequently found *M. anatis* in the testes and vas deferens of male Pekins, but were unable to isolate *M. anatis* from the ovaries of female Pekins in our infected group; however, we have frequently isolated *Mycoplasma* spp. (including *M. anatis*) from the ovaries of wild ducks and from newly hatched ducklings (Goldberg et al., 1995). It is possible that the males may serve as a source of female infection during copulation, as occurs for other mycoplasmas (Yamamoto, 1991). Infection of ovaries or sexual transmission could facilitate vertical transmission during egg formation, with ensuing increases in egg mortality and reductions in duckling growth (Samuel et al., 1995).

Mycoplasma anatis has caused clinical disease, embryo mortality, and reduced growth in domestic ducklings, but little is known about its occurrence or consequences in wild ducks. We found evidence that wild adult mallards and black ducks commonly are exposed to *M. anatis*; 68% of the birds we sampled had serum antibody levels, indicating previous exposure. Based on antibody titers of juvenile birds, exposure frequently occurs within the first 8 mo after hatch, thus environmental routes of exposure may be common. Nesting mallards had a similar frequency of exposure among years, but sample sizes were generally too small for detecting annual variation. We found annual changes in exposure of juveniles sampled in the fall and winter; however, no consistent patterns were evident and we do not know what factors may influence this variation.

In contrast, none of the canvasbacks we sampled had antibody levels demonstrating prior exposure, implying differential species exposure to *M. anatis.* Although our sample of canvasbacks was small, we also were unable to isolate *M. anatis* from tracheal swabs of these birds (Goldberg et al., 1995), supporting our conclusion of infrequent exposure in this group of canvasbacks. As an alternative explanation, neg-

ative results in our sample of canvasbacks may be due to the inability of our ELISA to detect antibodies in the sera of species not closely related to mallards. Species other than mallards also can develop antibodies to *M. anatis*. Astorga et al. (1994) found the prevalence of exposure for waterfowl in Spain to range from 0 to 13%, using a hemagglutination-inhibition test. In their survey, antibody response to *M. anatis* occurred in several species of Anatidae, including diving ducks.

In domestic ducks, *M. anatis* can cause diseases, affecting respiratory and reproductive systems, which may lead to economic losses through decreased productivity and poor growth. Based on our data, we believe exposure to *M. anatis* is common among the mallard and black ducks sampled, and many juveniles are exposed during their first year. However, the consequence of *M. anatis* infection in wild birds is still uncertain. In particular, little is known about the various routes of exposure, how and when infection occurs, the frequency of vertical or sexual transmission and its effect on hatching success of wild birds, and what factors may influence transmission. Further research will be needed to address these questions, as well as to determine the prevalence of exposure in other species of waterfowl.

ACKNOWLEDGMENTS

We thank the numerous biologists who provided field and laboratory assistance to the project. T. E. Rocke offered frequent assistance and support during the project. C. J. Brand and L. Sileo initiated our field work and collected samples. D. Shadduck, A. Kamel, S. Smith, L. Umbright, and M. Narlock assisted in the collection and processing of tissue samples. F. J. Dein provided pathology and animal care support. D. Xiang provided assistance with statistical analysis. T. E. Rocke, S. H. Kleven, and C. J. Brand reviewed and improved the manuscript. Field work in Ohio was supported by Region 3 of the U.S. Fish and Wildlife Service, Ohio Division of Wildlife, and the Winous Point Research Committee.

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Received for publication 5 July 1995.