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SUMMARY. The prevalence of *Escherichia coli*, *Salmonella* spp., and *Mycobacterium avium* subsp. *paratuberculosis* isolated from the feces of wild European starlings (*Sturnus vulgaris*) humanely trapped at a feedlot in central Kansas was assessed. All *E. coli* and *Salmonella* isolates recovered were tested for antimicrobial susceptibility using National Antimicrobial Resistance Monitoring System panels and the *E. coli* isolates were classified as to their content of genes associated with pathogenic *E. coli* of birds and cattle, including *cvaC, iroN2, ompTp, hlyF2, eitC, iss, iutA, ireA, papC*, *stxI*, *stxII*, *sta*, *K99*, *F41*, and *eae*. *Escherichia coli* O157:H7 and *Mycobacterium avium* subsp. *paratuberculosis* were not detected and *Salmonella* was isolated from only three samples, two of which displayed antimicrobial resistance. Approximately half of the *E. coli* isolates were resistant to antimicrobial agents with 96% showing resistance to tetracycline. Only one isolate was positive for a single gene associated with bovine pathogenic *E. coli*. An interesting finding of this study was that 5% of the *E. coli* isolates tested met the criteria established for identification as avian pathogenic *E. coli* (APEC). Thus these findings suggest that starlings are not a significant source of *Salmonella* spp., *Mycobacterium avium* subsp. *paratuberculosis, E. coli* O157, or other shiga toxin–producing *E. coli* in this feedlot. However, they may have the potential to spread APEC, an important pathogen of poultry and a potential pathogen to human beings.

RESUMEN. Presencia de *Escherichia coli, Salmonella* y *Mycobacterium avium* subesp. *paratuberculosis* en estorninos pintos silvestres en un lote de engorde de ganado en Kansas.

Se determinó la prevalencia de bacterias *Escherichia coli*, *Salmonella* spp. y *Mycobacterium avium* subsp. *paratuberculosis* que fueron aisladas a partir de muestras de excretas de estorninos pintos silvestres (*Sturnus vulgaris*) atrapados de manera humanitaria en un lote de engorde de ganado en la parte central del estado de Kansas. Todos los aislamientos de *E. coli* y de *Salmonella* que se obtuvieron, fueron analizados en su susceptibilidad a los antimicrobianos utilizando los paneles del Sistema Nacional de Monitoreo de la Resistencia Antimicrobiana. Los aislamientos de *E. coli* se clasificaron de acuerdo a la presencia de genes asociados con las cepas de *E. coli* patogénica para las aves y para el ganado, incluyendo los genes *cvaC, iroN2, ompTp, hlyF2, eitC, iss, iutA, ireA, papC*, *stxI*, *stxII*, *sta*, *K99*, *F41* y *eae*. No se detectó la presencia de *Escherichia coli* O157:H7 y de *Mycobacterium avium* subsp. *paratuberculosis.* Se aisló *Salmonella* únicamente de tres muestras; dos de las cuales mostraron resistencia antimicrobiana. Aproximadamente la mitad de los aislamientos de *E. coli* fueron resistentes a los agentes antimicrobianos y un 96% de ellos mostraron resistencia a la tetraciclina. Solo un aislamiento fue positivo a la presencia de un solo gene asociado con *E. coli* patógena para los bovinos. Un hallazgo interesante de este estudio fue que el 5% de los aislamientos de *E. coli* estudiados reunieron los criterios establecidos para la identificación como *E. coli* patogénica para las aves (con las siglas en ingles APEC). Estos hallazgos sugieren que los estorninos pintos de este lote de engorde de ganado no representan una fuente importante de *Salmonella* spp., *Mycobacterium avium* subsp. *paratuberculosis, E. coli* O157, o de otras cepas de *E. coli* productoras de toxinas del tipo de las Shigelas. Sin embargo, pueden jugar un papel potencial para la diseminación de *E. coli* patogénica para las aves, que es un patógeno importante en la avicultura y un patógeno potencial para los seres humanos.

Key words: European starling, *Escherichia coli*, *Escherichia coli* O157:H7, *Salmonella*, *Mycobacterium avium* subsp. *paratuberculosis*, *Mycobacterium avium* subsp. *avium*, antimicrobial resistance, multiplex PCR, avian pathogenic *Escherichia coli* (APEC)

Abbreviations: APEC = avian pathogenic *Escherichia coli*; BGA = brilliant green agar; BPW = buffered peptone water; ddH_2O = double-distilled water; EMB = eosin methylene blue agar; MAC = MacConkey agar; MLCB = mannitol lysine crystal violet brilliant green agar; NARMS = National Antimicrobial Resistance Monitoring System; RV = Rappaport–Vassiladis broth; SMAC = sorbitol MacConkey agar; TAE = Tris–acetate-ethylenediamine tetraacetic acid; TE = Tris–ethylenediamine tetraacetic acid; Tet = tetrathionate broth; TSA = tryptone soy agar

Zoonoses are caused by microbial pathogens that are adapted to several host species and can be passed from animals to humans. Wildlife, animals free to roam, may be a significant reservoir for zoonotic pathogens (4,16,18,35,37,39). Wild birds are known to carry and transmit over 40 diseases to humans and livestock (13,24,34,35,36,49). Recent studies have also highlighted wild birds as sources of pathogens associated with human disease (5,9,22,50). Some species of wild birds, such as European starlings, house sparrows, and rock doves, are adapted to urbanized settings and are attracted to livestock facilities (13,14,25,36). Feed-yards and other agricultural operations are especially attractive to avian wildlife because of the year-round availability of food and water (14,30). Thus dense avian congregations of birds around these facilities are of

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epidemiologic and economic concern for the spread of pathogens to livestock (13,17,25,36,39). Le Jeune and colleagues (35) report that the starling population in the United States is over 200 million with large roosts containing thousands of birds common in the wintertime. Starlings are considered an invasive species in the United States because of their foreign origin and aggressive behavior; they have the capability to outcompete native species, contributing to their displacement. At livestock operations, starlings are relatively common and are frequently observed as the major species of wild bird frequenting such sites. Pimentel and colleagues (44) estimated that the annual economic losses to U.S. agriculture as a consequence of starlings are approximately $800 million.

Over 300 species of wild birds migrate annually. Long-distance migration is stressful on birds, often leading to decreased immune function resulting in enhanced susceptibility to disease (45). As avian wildlife migrate, they make a series of flights interrupted with many stopovers (29). Stopover locations characteristically have dense congregations of birds, which can potentially facilitate pathogen transfer (15,43). As these birds continue on with migration, they are capable of spreading diseases over great geographic distances because of their migrating behavior (43).

Winter conditions also influence the behavior of birds in a way that favors the spread of diseases, such as compromised immune function, dense congregations, and large geographic coverage (15,25,43). Summer conditions, or warmer temperatures, however, are favorable for the survival of pathogens in the environment (1). Therefore, avian wildlife are a potential source for spreading infectious diseases to livestock year round. *Escherichia coli* O157:H7, *Salmonella, Listeria,* and *Campylobacter* are among pathogens that have been recognized as being carried by avian wildlife and are potentially pathogenic to humans and livestock (5,13,35,43,49,50). *Mycobacterium avium* subsp. *paratuberculosis* causes Johne's disease and is suspected to cause Crohn's disease in humans (23,26,27). One study isolated *Mycobacterium avium* subsp. *paratuberculosis* from three species of birds (house sparrow, European starling, and common snipe) from feedlots (12). The role of wildlife as a reservoir of *Mycobacterium avium* subsp. *paratuberculosis* is still largely undetermined and warrants further investigation (2,12).

Wild birds can also play a significant role in the spread of drug-resistant pathogens to livestock and humans (11,37). These resistant pathogens have the potential to complicate treatment of disease (28). One study examined the prevalence of antimicrobial-resistant *E. coli* in cattle compared to the prevalence detected in sparrows living in close association with the livestock (17). Antimicrobial-resistant *E. coli* isolates were detected in 24.5% of the rectal smears from the cattle and 9% of the cloacal swabs from the sparrows. These sparrows living near livestock facilities were capable of spreading drug-resistant *E. coli* great distances because of their migratory habits (17,45).

The objectives of the present study were to 1) determine the prevalence of *E. coli*, *Salmonella* spp., and *Mycobacterium avium* subsp. *paratuberculosis* in wild European starlings congregating in a beef cattle feedlot in Kansas; 2) identify virulence genes in *E. coli* with the potential to cause disease in cattle or birds; and 3) to determine the incidence of antimicrobial resistance in *E. coli* and *Salmonella* spp.

MATERIALS AND METHODS

Study area. Feces or intestinal contents of European starlings found frequenting a cattle feedlot in central Kansas were used for bacterial isolation. The feedlot capacity was approximately 17,000 head. Collection of the samples occurred during three time periods: January 2 through February 8, 2007; September 5–13, 2007; and January 10 through February 7, 2008. During the winter months, the average daily temperatures in the region ranged from −7 C to 9 C. During the summer months, the average daily temperatures ranged from 14 C to 34 C (42).

Bird and sample collection. Modified Australian crow traps were used to capture wild starlings. Once trapped, the birds were removed and fecal swabs were taken. Samples were collected from starlings that defecated while being handled, and the feces were collected on a sterile swab. Fecal swabs were transferred to sterile Cary Blair transport media (Becton and Dickinson, Sparks, MD). To ensure that birds were not sampled more than once, birds were tagged prior to release. During the winter of 2007, 212 samples were collected and stored at refrigeration temperatures until shipped.

During winter 2008, 200 gut-content swabs were also collected from starlings that were shot by feedlot operators as a means of control. Deceased starlings were immediately collected and refrigerated. Within 24 hr, their intestines were removed, and a swab was inserted into the lumen of the gut to collect fecal material. This method was also used in the summer of 2007 to sample 24 starlings to test for seasonality difference in the prevalence of organisms in the gut contents. All samples collected were shipped overnight on ice to the Logue lab at North Dakota State University for *Salmonella* and *E. coli* isolation.

To test for the prevalence of *Mycobacterium* species, cutting boards were placed under perches in the traps to collect fecal material. During each winter, 32 samples were collected. Fecal material was collected on days above freezing to avoid any potential for cellular damage of the organism as a result of freezing temperatures. Boards were placed in the trap on the morning of the day of collection and removed in the afternoon when the feces were scraped off using a rubber spatula and/or paint scrapper. All fecal material collected was placed into a 150-ml Nalgene® screw top bottle (Nalge Nunc, Rochester, NY) and stored at refrigeration temperatures until shipped. All samples for *Mycobacterium* analysis were shipped overnight to the North Dakota Veterinary Diagnostic Lab at North Dakota State University.

Bacteriology. On arrival at the lab, all fecal swabs were logged and sample numbers were assigned. The swabs were removed from the transport medium and snipped at the tip into 5 ml of buffered peptone water (BPW; Difco). All samples were incubated for 18–24 hr at 37 C. A secondary enrichment was used to select for *Salmonella*, with 0.5 ml and 0.1 ml of the BPW transferred to 10 ml of tetrathionate (Tet) enrichment and Rappaport–Vassiliadis broth (RV broth), respectively. The Tet broth was incubated at 37 C for 18–24 hr and the RV broth at 42 C for 18–24 hr. Ten microliters of the secondary enrichment was struck to brilliant green agar (BGA) and mannitol lysine crystal violet brilliant green agar (MLCB) and incubated at 37 C for 18–24 hr.

Escherichia coli and *E. coli* O157:H7 were selected from the BPW primary enrichment by striking out 10 µl of the broth onto MacConkey agar (MAC) and eosin methylene blue agar (EMB) for *E. coli* and onto sorbitol MacConkey (SMAC) supplemented with cefiximine and tellurite for *E. coli* O157:H7. All *E. coli* plates were incubated for 18–24 hr at 37 C.

Following incubation, all plates were examined for suspect colonies based upon morphological characteristics. *Salmonella* colonies appearing dark purple to black on MLCB or pale pink to white on BGA with a rose-colored background were selected. *Escherichia coli* colonies with a green metallic sheen on EMB or pink on MAC were considered suspect. On SMAC plates, *E. coli* O157:H7 colonies that appeared straw-yellow or clear-colored were selected. All suspect *Salmonella* and *E. coli* colonies were picked and transferred to tryptone soy agar (TSA) to purify the colonies and incubated for 18–24 hr at 37 C.

Suspect *Salmonella* and *E. coli* isolates were identified using the Sensititre Microplate System (AP 80; Trek Diagnostics, Westlake, OH). After the plates were inoculated, they were incubated for 18–24 hr at 37 C and read using the autoreader. Latex agglutination assays (DrySpot; Oxoid, Basingstoke, UK) were used to confirm suspect *E. coli* O157:H7 colonies. Isolates that were positive by the agglutination assay were sent for serotyping to the *E. coli* Reference Center at Pennsylvania State University.

Table 1. Primers used in multiplex PCR for detection of bovine pathogenic *E. coli*.

Virulence factor	Description	Primer sequence (5′ to 3′)	Amplicon size (bp)	Reference
StxI	Shiga toxin I	F: TTCGCTCTGCAATAGGTA R: TTCCCCAGTTCAATGTAAGAT	555	(21)
intimin	Attachment protein	F: ATATCCGTTTTAATGGCTATCT R: AATCTTCTGCGTACTGTGTTCA	425	(21)
F41	Attachment protein	F: GCATCAGCGGCAGTATCT R: GTCCCTAGCTCAGTATTATCACCT	380	(21)
K99	Attachment protein	F: TATTATCTTAGGTGGTATGG R: GGTATCCTTTAGCAGCAGTATTTC	314	(21)
Sta	Heat stable enterotoxin	F: GCTAATGTTGGCAATTTTTATTTCTGTA R: AGGATTACAACAAAGTTCACAGCAGTAA	190	(21)
StxII	Shiga-like toxin II	F: GTGCCTGTTACTGGGTTTTTCTTC R: AGGGGTCGATATCTCTGTCC	118	(21)

Routine cultivation methods for *Mycobacterium avium* subsp. *paratuberculosis* followed instructions from the National Animal Disease Control method (48). After the samples were prepared, procedures were then followed from Trek™ Diagnostic Systems, Inc. (Cleveland, OH) ESP Liquid Culture System II for the detection of *Mycobacterium avium* subsp. *paratuberculosis*. The Kinyoun method for acid-fast staining was then used on all samples (8). Samples that were acid-fast negative were considered to be *Mycobacterium avium* subsp. *paratuberculosis* negative. PCR was done on all samples identified as acid-fast positive.

National Antimicrobial Resistance Monitoring System (NARMS). All *E. coli* and *Salmonella* isolates were tested for antimicrobial resistance using the NARMS (CMV1AGNF; Trek Diagnostics). Each plate tested the susceptibility of the strain to 15 antimicrobials. Antimicrobials tested included amikacin (concentration range 0.5–64 μg/ml), amoxicillin/clavulanic acid (1–32 μg/ml), cefoxitin (0.5–32 μg/ml), ceftiofur (0.12–8 μg/ml), ceftriaxone (0.25–64 μg/ml), ciprofloxacin (0.015–4 μg/ml), nalidixic acid (0.5–32 μg/ml), gentamicin (0.25–16 μg/ml), trimethoprim/sulphamethoxazole (0.12–4 μg/ml), streptomycin (32–64 μg/ml), kanamycin (8–64 μg/ml), chloramphenicol (2–32 μg/ml), sulfizoxazole (16–256 μg/ml), ampicillin (1–32 μg/ml), and tetracycline (4–32 μg/ml) (41).

Escherichia coli and *Salmonella* isolates were considered resistant if growth occurred above the breakpoint. The breakpoint was defined as the minimal drug concentration level above which an isolate should not grow; this level is usually set by NARMS. The following breakpoints used in this study were: amikacin ≥64 μg/ml, amoxicillin/clavulanic acid ≥32/16 μg/ml, cefoxitin ≥32 μg/ml, ceftiofur ≥8 μg/ml, ceftriaxone ≥64 μg/ml, ciprofloxacin ≥4 μg/ml, nalidixic acid ≥32 μg/ml, gentamicin ≥16 μg/ml, trimethoprim/sulphamethoxazole ≥4/76 μg/ml, streptomycin ≥64 μg/ml, kanamycin ≥64 μg/ml, chloramphenicol ≥32 μg/ml, sulfizoxazole ≥512 μg/ml, ampicillin ≥32 μg/ml, and tetracycline ≥16 μg/ml (41).

DNA extraction and PCR. PCR analysis was carried out on all *E. coli* isolates to detect virulence genes associated with bovine and avian pathogenic strains. A solution of 40 μl of single cell lysing buffer, (consisting of 1 ml of Tris–ethylenediamine tetraacetic acid [TE] buffer [10 mM Tris-HCl and 1 mM ethylenediamine tetraacetic acid] and 10 μl of proteinase K [5 mg/ml]), was dispensed into microtubes. One isolated colony was picked from the TSA plates with an inoculating needle and suspended in the solution. The tubes were then vortexed and placed in a thermocycler (Eppendorf, Hamburg, Germany) and heated to 80 C for 10 min followed by cooling to 55 C for 10 min. Sterile double-distilled water (ddH_2O; 80 μl) was added to the tubes and centrifuged for 30 sec. The supernatant was removed and pipetted into a new tube and the pellet discarded. All DNA supernatants were stored at −20 C until needed for PCR.

Two multiplex PCR procedures were used to assess the isolated strains for virulence genes associated with *E. coli* of bovine origin and those associated with avian species (Tables 1, 2). Both multiplex PCR procedures were used to test the prevalence of these genes in 206 *E. coli* isolates.

A 50-μl PCR solution was prepared that included 5 μl of template DNA, sterile ddH_2O, 1× PCR buffer (GoTaq; Promega, Madison, WI), 0.2mM dNTP each, 0.6 mM of each primer (see Tables 1, 2) and 1.25 U of *Taq*. The mixture was heated in a thermocycler under the following conditions for the bovine-associated genes: 94 C for 5 min; 30 cycles at 94 C for 30 sec, 50 C for 45 sec, and 70 C for 90 sec; a final cycle at 70 C for 10 min; and holding at 4 C (21).

PCR preparation for the detection of the avian-associated genes followed the same protocol as that for bovine virulence genes above. PCR conditions for avian pathogenic genes were as follows: 95 C for 2 min; 25 cycles at 94 C for 30 sec, 63 C for 30 sec, and 68 C for 3 min; with a final cycle at 72 C for 10 min and holding at 4 C. To ensure each amplified gene could be detected, two rounds of PCR were conducted on each *E. coli* isolate for the avian genes. The first run consisted of primers for the genes *cvaC*, *iutA*, *ireA,* and *papC* and the second run consisted of primers for the genes *ompTp*, *eitC*, *iroN2*, *hlyF2,* and *iss*.

All PCR products were separated in 1.5% agarose by horizontal gel electrophoresis run in 1× Tris-acetate-ethylenediamine tetraacetic acid (TAE) buffer at 100 V for 2 hr for the cattle-associated genes and a 2% gel run in 1× TAE buffer at 130 V for 1.5 hr for the avian-associated genes. Sizes of the amplicons were compared against a 1-kb DNA marker (Promega). Positive and negative controls possessing the genes of interest were included in each PCR run. These strains included *E. coli* B44 for genes *F41*, *K99,* and *Sta*; *E. coli* 2671i for genes *StxI* and *StxII*; *E. coli* 3474i for *eae* and intimin (lab strains); and *E. coli* DH5α for the negative control. *Escherichia coli* O1:K2 was used for all the avian genes except *cvaC*, where a *Salmonella* lab strain containing *cvaC* was used; ddH_2O was used for the negative control.

***Mycobacterium* samples.** Liquid samples from the enrichment were placed on a shaker for 20 min at 2000 rpm. One milliliter was transferred to bead-beater tubes containing 0.1-mm glass beads and centrifuged for 10 min at 16,000 × *g*, and 200 μl of the supernatant was transferred to a new tube. Extraction procedures followed the QIAamp® DNA mini kit instructions (Qiagen, Valencia, CA). The DNA was stored at −20 C until needed for PCR. In searching for *Mycobacterium avium* subsp. *paratuberculosis*, the PCR procedure followed the Tetracore® *Mycobacterium paratuberculosis* DNA test kit PCR instructions (Tetracore, Rockville, MD). All acid-fast positive samples were sent to the College of Veterinary Medicine at the University of Minnesota, Minneapolis, MN, for further testing. PCR was run on the samples to differentiate between *Mycobacterium avium* subsp. *paratuberculosis* and *Mycobacterium avium* subsp. *avium* following methods from Motiwala *et al.* (40).

Statistical analysis. A logistic regression (STRATA) was run on the sex, age, and weight of the host birds and the season of isolation to determine which variable (or variables) had a significant effect on *E. coli* detection. Descriptive statistics were given to summarize the results of bacterial and antimicrobial findings and PCR product.

RESULTS

Of the 434 samples tested, 206 (48.2%) were *E. coli*–positive. *Escherichia coli* O157:H7 was not detected. The agglutination assay

Table 2. Primers used in multiplex PCR for detection of APEC.

Virulence factor	Description	Primer sequence (5′ to 3′)	Amplicon size (bp)	Reference
cvaC	Protein for colicin V synthesis	F: CACACACAAACGGGAGCTGTT R: CTTCCCGCAGCATAGTTCCAT	679	(32,46)
iroN2	Siderophore receptor gene	F: AATCCGGCAAAGAGACGAACCGCCTGTA R: GTTCGGGCAACCCCTGCTTTGACTTTGA	552	(32,46)
omp Tp	Outer membrane protein, protease precursor	F: TCATCCCGGAAGCCTCCCTCACTACTAT R: TAGCGTTTGCTGCATGGCTTCTGATAC	496	(32)
hlyF2	Avian hemolysis	F: GGCCACAGTCGTTTAGGGTGCTTACC R: GGCGGTTTAGGCATTCCGATACTCAG	423	(32)
eitC	ABC iron transporter, ATP-binding protein	F: CAACTGGGCGGGAACGAAATCAGGA R: TCAGTTCCGCGCTGGCAACAACCTAC	379	(32)
iss	Increase serum survival gene	F: CAGCAACCCGAACCACTTGATG R: AGCATTGCCAGAGCGGCAGAA	323	(46)
iutA	Ferric aerobactin receptor	F: GGCTGGACATCATGGGAACTGG R: CGTCGGGAACGGGTAGAATCG	302	(46)
ireA	Iron-responsive element	F: GATGACTCAGCCACGGGTAA R: CCAGGACTCACCTCACGAAT	254	(46)
papC	Encodes for P pilus	F: GTGGCAGTATGAGTAATGACCGTTA R: ATATCCTTTCTGCAGGGATGCAATA	205	(46)

detected three suspect (weakly positive by agglutination) isolates of *E. coli* O157:H7; however, after serotyping, these isolates were identified as *E. coli* serotypes O29:H51, O11:H47, and O117:H32. During winter 2007, 52.8% of the samples collected were positive for *E. coli*; summer 2007 had a detection rate of 83.3% and winter 2008 had a detection rate of 38.0%. Only three samples tested positive for *Salmonella*; two of these isolates were *Salmonella choleraesuis* subsp. *arizonae*, which is commonly associated with swine. The third isolate was also confirmed as *Salmonella* but was not serotyped. One *Salmonella choleraesuis* subsp. *arizonae* isolate was detected during winter 2008, and the remaining two isolates were detected during summer 2007.

Mycobacterium avium subsp. *paratuberculosis* was not isolated from the 64 samples of fecal material collected. During winter 2007, 18.8% and again in 2008, 28% of the samples were positive for acid-fast organisms, a characteristic of *Mycobacterium* species. Further identification was pursued on these isolates from the second winter and revealed that they did indeed belong to the *Mycobacterium avium–Mycobacterium intracellulare* complex; however, the subspecies remains questionable. The subspecies of these isolates is either *avium* or *hominisuis*, with a strong suspicion of subsp. *avium* because they were isolated from wild birds (Sreevatsan, pers. comm.).

Epidemiology. Age, sex and weight were not significant variables in determining whether *E. coli* was detected in European starlings ($P > 0.05$). However, a significant difference was observed in seasonal effects, from summer 2007 to winter 2008 ($P < 0.01$). Fecal material collected from starlings during the summer months was four times more likely to be positive for *E. coli* than from winter 2008 (Table 3).

Table 3. Comparisons of sex, age, weight, and season influencing the probability of *E. coli* detection from European starlings 2006–08.

Variable	n	Odds ratio	SE	*P*-value
Sex	214	0.829	0.231	$P > 0.05$
Age	214	1.115	0.309	$P > 0.05$
Weight	214	1.029	0.152	$P > 0.05$
Season	438	0.744	0.074	$P < 0.01$
Summer	438	4.469	2.523	$P < 0.01$
Winter 2008	438	0.548	0.11	$P < 0.01$

NARMS. Of the 206 isolates of *E. coli* examined, 49% showed resistance to at least one antimicrobial agent (Fig. 1). Drug-resistant *E. coli* were detected in approximately 60% of the isolates from the winter 2007 and summer 2007. Only 20% of the isolates from winter 2008 were resistant. Resistance to seven different antimicrobials was found during winter and summer 2007, whereas resistance to only five antimicrobials was observed during winter 2008 (Fig. 2, Table 4). Twenty-eight *E. coli* isolates showed resistance to sulfizoxazole at a breakpoint of ≥256. The current breakpoint for this antimicrobial is ≥512; therefore, it remains inconclusive if these isolates are indeed resistant to sulfizoxazole due to the limitation of the plates. *Escherichia coli* isolates showed a higher prevalence of resistance to tetracycline and ampicillin than any other drugs. Many of the isolates resistant to tetracycline showed resistance levels double that of the breakpoint concentration (Fig. 3).

Samples collected from starlings that were positive for *Salmonella* were also positive for *E. coli*. Both *Salmonella choleraesuis* subsp. *arizonae* isolates were observed to be resistant to cefoxitin (≥32 μg/ml) and tetracycline (≥16 μg/ml). One of these *Salmonella* isolates was also resistant to amoxicillin/clavulanic acid (≥32/16 μg/ml). The *E. coli* isolated from this starling was also positive for the avian pathogenic genes *eitC* and *iss*. The *E. coli* isolates retrieved from the

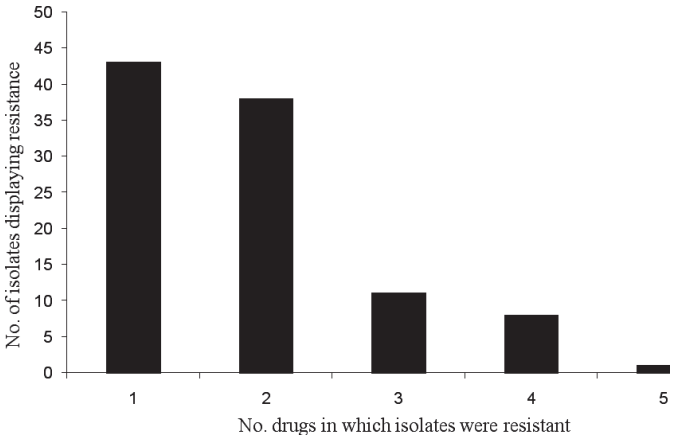

Fig. 1. Summary of *E. coli* isolates displaying drug resistance recovered from European starlings (2006–08).

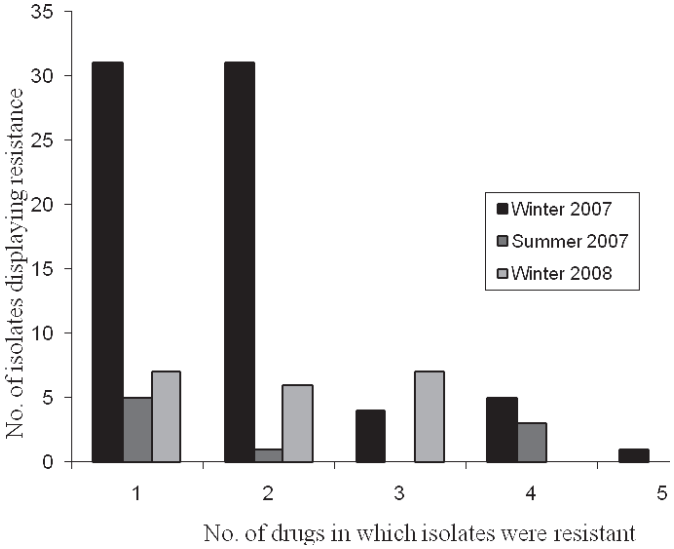

Fig. 2. Profile of *E. coli* isolates from European starlings displaying drug resistance from summer of 2007 and winters of 2007 and 2008.

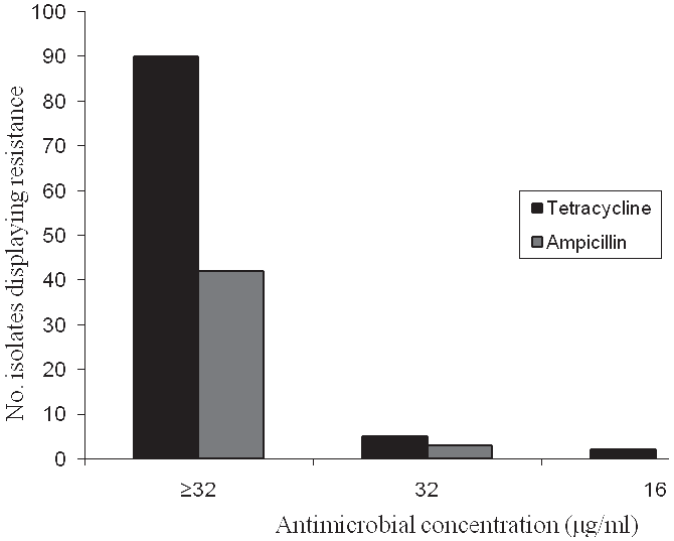

Fig. 3. Range of antimicrobial resistance levels observed to tetracycline (breakpoint ≥16) and ampicillin (breakpoint ≥32) in *E. coli* recovered from European starlings.

starlings with *Salmonella choleraesuis* subsp. *arizonae* were susceptible to all antimicrobials tested. The unserotyped *Salmonella* isolate was susceptible to all antimicrobials, but the *E. coli* serotype O29:H51 isolated from the same starling was resistant to ampicillin and tetracycline. The *E. coli* isolate serotyped as O117:H32 was resistant to both ampicillin and tetracycline, and *E. coli* isolate O11:H47 was resistant to tetracycline.

***Escherichia coli* PCR.** Only one *E. coli* isolate was positive for one gene associated with illness in cattle. The isolate contained the *eae* gene and was also resistant to tetracycline; no genes associated with avian pathogenicty were found in this isolate. *Escherichia coli* isolates were considered to be avian pathogenic *E. coli* (APEC) if at least four genes associated with APEC pathogenicity were present (33). At least one of these genes was detected in 43 *E. coli* isolates. Of these isolates, 12 had more than four genes associated with APEC pathogenicity and four showed drug resistance also (Table 5).

Table 4. Prevalence of antimicrobial resistance in *E. coli* recovered from European starlings.

	% Resistance		
Antimicrobial[A]	Winter 2007 (n = 115)	Summer 2007 (n = 15)	Winter 2008 (n = 76)
Amikacin (≥64)	0	0	0
Ceftiofur (≥8)	0	0	0
Ceftriaxone (≥64)	0	0	0
Ciprofloxacin (≥4)	0	0	0
Gentamicin (≥16)	0.9	0	0
Amoxicillin/clavulanic acid (≥32/16)	0	0	0
Cefoxitin (≥32)	0	0	0
Nalidixic Acid (≥32)	0	0	0
Trimethoprim/ sulphamethoxazole (≥4/76)	4	6	0
Streptomycin (≥64)	3	27	4
Kanamycin (≥64)	0	6	14
Chloramphenicol (≥32)	3	27	9
Sulfizoxazole (≥512)	0	0	0
Ampicillin (≥32)	27	40	1
Tetracycline (≥16)	57	87	24

[A]Number in parentheses indicates the breakpoint of the antimicrobial (μg/ml).

DISCUSSION

Escherichia coli prevalence was detected at significantly different rates throughout this study. During winter 2007, samples were collected from starlings while they were defecating. Sampling techniques for summer and winter 2008 were collection of fecal material from inside the intestinal lumen. During both of these seasons, samples were not collected until a day after the birds had died. Since the rate of recovery of *E. coli* differed so much between these two seasons although the same sampling technique was used, diet may be responsible for the change. Over the course of the year, the starlings' diet changes, as does their intestinal flora and the intestine of the bird. During spring and summer, starlings mainly eat protein matter such as invertebrates. The intestine of the starling is shorter when the diet is high in protein. In contrast, during the winter when starlings primarily eat grains, the intestine is much longer (6,20). The difference in seasonal diet is most likely the cause for the difference in the prevalence of *E. coli* isolated during the summer (83.3%) compared to winter 2008 (38.0%). *Escherichia coli* was isolated from (52.8%) of the samples collected from winter 2007. The difference in recovery rate between the two winters is most likely a result from sampling techniques. Fresh fecal material was collected on swabs during the winter of 2007, whereas intestinal swabs were collected after the bird had died which appeared to result in better overall recovery rates.

Overall, approximately half of the *E. coli* isolates recovered showed resistance above the breakpoint to the tested antimicrobials. Resistance to seven antimicrobials, including gentamicin, streptomycin, trimethoprim/sulphamethozazole, kanamycin, chloramphenicol, ampicillin, and tetracycline was observed. Antimicrobials to which all *E. coli* isolates were susceptible included amikacin, ceftiofur, ceftriaxone, ciprofloxacin, amoxicillin/clavulanic acid, cefoxitin, and nalidixic acid. Approximately 28% of the *E. coli* isolates showed resistance to sulfizoxazole at a breakpoint of ≥256 μg/ml. Because of the limitations of the NARMS panel, it remains inconclusive if these isolates are indeed resistant to sulfizoxazole.

Table 5. Prevalence of APEC genes in *E. coli* recovered from European starlings.

Bird	*cvaC*	*iroN2*	*hlyF2*	*eitC*	*iss*	*aerJ*	*papC*	*ireA*	*ompTp*	Total genes	Drug resistance
1	+	+	+	−	+	−	−	−	−	4	0
2	+	+	+	−	+	−	−	−	−	4	0
3	−	+	+	−	+	−	+	−	−	4	2
4	+	+	+	−	+	−	−	−	−	4	1
5	+	+	+	−	+	−	−	−	−	4	0
6	+	+	+	−	+	−	−	−	+	5	0
7	+	+	+	−	+	−	−	−	−	4	0
8	+	+	+	−	+	−	−	−	+	5	0
9	+	+	+	−	+	−	−	−	−	4	1
10	+	+	+	−	+	−	−	−	−	4	0
11	+	+	+	−	+	−	−	−	−	4	2
12	−	+	+	+	+	−	−	−	+	5	0

Tetracycline resistance was present in 96% and ampicillin resistance was present in 45% of the isolates.

Many livestock producers add subtherapeutic amounts of antimicrobials to feed rations to prevent illness (28). Carson and colleagues (7) found a higher detection rate of antimicrobial resistance in *E. coli* isolated from cattle that were closer to going to market, such as those in feedlots, as compared to calves. One of the antimicrobials with the highest prevalence reported in their study was tetracycline, which was also true in our study. Wild birds also encounter antimicrobials from agricultural runoff and human sewage (19). Because these starlings inhabit feedlots, the drug resistance observed in our isolates was probably associated with exposure to livestock feed containing antibiotics.

Escherichia coli was isolated from all three starlings that were positive for *Salmonella*. Starlings with *Salmonella choleraesuis* subsp. *arizonae* displayed drug resistance; however, the *E. coli* isolates from the same birds did not. *Salmonella choleraesuis* subsp. *arizonae* is pathogenic and typically infects swine, but can also cause disease in humans (10). Because *Salmonella choleraesuis* subsp. *arizonae* is not commonly isolated from other wild bird species, it is possible that these starlings frequented a facility where swine were present and where they were exposed to these resistant bacteria. A radiotelemetry study on the same population of starlings found that some of these birds also frequented a vacant swine operation, so these starlings were capable of potentially spreading pathogens to more than cattle feedlots (unpubl. data).

The unserotyped *Salmonella* was susceptible to all antimicrobials; however, the *E. coli* O29:H51 isolate recovered from the same starling was resistant to ampicillin and tetracycline. *Escherichia coli* O29:H51 serotypes have been identified as enteroinvasive types by producing shiga-like toxins (3), whereas *E. coli* O117:H32 serotypes have been identified as enterohemorrhagic types, similar to *E. coli* O157:H7 (3). This isolate was also resistant to both ampicillin and tetracycline. *Escherichia coli* serotype O11:H47 is probably non-pathogenic but was found to be resistant to tetracycline. Because the *E. coli* O29:H51 showed resistance to antimicrobials but the *Salmonella* isolate retrieved from the same starlings did not, the *E. coli* strains may have been picked up at a separate time; confirming the wild and possible transient nature of their host. However, exposure to antimicrobials at the feedlot cannot be ruled out as a factor in contributing to the antimicrobial resistance observed.

No *E. coli* O157:H7 type strains were recovered in this study which in itself was not surprising given the overall low prevalence of pathogens in wild birds (4,24,35,50). Le Jeune and colleagues (35) also found a relatively low prevalence of *E. coli* O157:H7 in starlings frequenting dairy farms (2.2%), whereas a similar low prevalence ranging from 0.9% to 2.9% was observed in wild birds (primarily gulls) in a United Kingdom study by Wallace and colleagues (50). PCR results showed that the *E. coli* isolated from starlings were nonpathogenic to cattle and are probably not cattle-related. Only one *E. coli* isolate was found to possess intimin, a gene associated with pathogenicity. Intimin causes intestinal lesions by attaching to and effacing the microvilli (21). Because bovine pathogenic *E. coli* were detected at such a low rate, these starlings did not appear to be a significant reservoir for spreading *E. coli*–related diseases to other feedlots.

PCR results did, however, reveal a higher detection rate of APEC than bovine pathogenic *E. coli*. Although 42% of the *E. coli* isolates contained at least one virulence gene, only 12 starlings were considered to possess pathogenic potential by possession of four or more virulence genes (33). All *E. coli* isolates deemed potentially pathogenic possessed the genes *iroN2, hlyF2,* and *iss.* The increased serum survival gene (*iss*) has been frequently recovered from birds with avian colibacillosis, and correlates with disease (31). A similar observation by Gibbs and colleagues (24) found *E. coli* recovered from yellow-headed blackbirds possessed traits commonly associated with pathogenesis in poultry. Although the *iutA* gene is commonly found in APEC (46), none of the isolates in this study were positive for *iutA* or *ireA*. The roles of these virulence genes in *E. coli* are still largely undetermined and warrant further study (46,47). These results suggest that the virulence genes *iroN2, hlyF2,* and *iss* are common in wild birds and are more likely associated with disease pathogenesis.

Mycobacterium avium subsp. *paratuberculosis* was not detected from any of the fecal samples. Infections with *Mycobacterium avium* subsp. *paratuberculosis* tend to be common in ruminant animals and can also occur in other species, including birds (12). A total of 15 acid-fast positive samples were found. Further identification of nine of these isolates showed that they were probably *Mycobacterium avium* subsp. *avium*, but there is a chance that they were *Mycobacterium avium* subsp. *hominisuis*. If these isolates are *Mycobacterium avium* subsp. *hominisuis*, then starlings pose a potential risk for spreading disease to humans (38). In summary, these results suggest that starlings are not a significant threat for spreading *E. coli* O157:H7, bovine pathogenic *E. coli, Salmonella*, or *Mycobacterium avium* subsp. *paratuberculosis* to cattle. A greater risk is probably associated with wild starlings' potential for spreading APEC and *Mycobacterium avium* subsp. *avium* to poultry and other avian wildlife.

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