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Genetic Structure and Antimicrobial Resistance of *Escherichia coli* and Cryptic Clades in Birds with Diverse Human Associations

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The manner and extent to which birds associate with humans may influence the genetic attributes and antimicrobial resistance of their commensal *Escherichia* communities through strain transmission and altered selection pressures. In this study, we determined whether the distribution of the different *Escherichia coli* phylogenetic groups and cryptic clades, the occurrence of 49 virulence associated genes, and/or the prevalence of resistance to 12 antimicrobials differed between four groups of birds from Australia with contrasting types of human association. We found that birds sampled in suburban and wilderness areas had similar *Escherichia* communities. The *Escherichia* communities of backyard domestic poultry were phylogenetically distinct from the *Escherichia* communities sourced from all other birds, with a large proportion (46%) of poultry strains belonging to phylogenetic group A and a significant minority (17%) belonging to the cryptic clades. Wild birds sampled from veterinary and wildlife rehabilitation centers (in-care birds) carried *Escherichia* isolates that possessed particular virulence-associated genes more often than *Escherichia* isolates from birds sampled in suburban and wilderness areas. The *Escherichia* isolates from both the backyard poultry and in-care birds were more likely to be multidrug resistant than the *Escherichia* isolates from wild birds. We also detected a multidrug-resistant *E. coli* strain circulating in a wildlife rehabilitation center, reinforcing the importance of adequate hygiene practices when handling and caring for wildlife. We suggest that the relatively high frequency of antimicrobial resistance in the in-care birds and backyard poultry is due primarily to the use of antimicrobials in these animals, and we recommend that the treatment protocols used for these birds be reviewed.

Escherichia coli is a generalist enteric bacterium that is able to colonize the lower intestinal tract of a range of vertebrate hosts, including most humans, other mammals, and birds. It is primarily a commensal; however, some strains are diarrheal pathogens and others can cause opportunistic extraintestinal infections in both humans and other animals (1). *E. coli* is less common in birds than mammals and can be isolated from approximately one-quarter of avian individuals (2). Birds that live close to human habitation are more likely to carry *E. coli* than birds in remote areas (2). This suggests that interactions and/or cohabitation between birds and humans as well as human activities and actions may influence avian *E. coli* communities. Here we collectively refer to these interactions and actions as "human association." In this study, we examined whether the effect of human association on commensal avian *E. coli* strains extends to their genetic attributes and antimicrobial resistance traits. The distribution of these attributes and traits in avian hosts is of importance to both avian and human health, as they may affect veterinary treatment practices and birds could be reservoirs for virulent and/or antimicrobial-resistant *E. coli* strains, to which humans may be exposed.

Human association may influence the attributes of the commensal *E. coli* communities of domestic and wild birds through several processes, including strain transmission, horizontal gene transfer, and altered selection pressures. It has been shown that strains can be shared between host species (3), and thus, birds that cohabitat or come into direct contact with people may acquire human strains via fecal-oral transmission of *E. coli* through environmental contamination or host contact (4, 5). Additionally, genes found on plasmids or other mobile genetic elements can be readily transferred between bacteria of the same or different species (6). Thus, genes found in human strains could be transferred to avian strains by horizontal gene transfer (7). Alternatively, where humans directly interact with birds, as is the case for domestic animals and wild birds brought into rehabilitation centers, human actions such as hygiene practices and antimicrobial use may result in the selection and dissemination of particular strains of *E. coli*.

The genetic attributes of *E. coli* strains vary between host species and may reflect differences in host traits, transmission dynamics, and exposure. *E. coli* strains can be classified into different phylogenetic groups that differ in their ecological characteristics and virulence-associated genes (8–10). In particular, phylogenetic group B2 strains appear to be adapted to mammalian hosts and are often associated with extraintestinal infection in humans, companion animals, and avian species (1, 11, 12). In contrast, group B1 strains appear to be generalists and are more frequently

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isolated from ectotherms, birds, and the environment (2). The extent to which human association influences the distribution of these phylogenetic groups and virulence traits in birds is largely unknown.

In contrast to the genetic attributes of *E. coli*, the effect of human association on antimicrobial resistance has been relatively well documented. The use of antimicrobials selects for corresponding antimicrobial resistance in bacterial species, such as *E. coli*, with the prevalence of antimicrobial resistance being proportional to the extent of antimicrobial use (7). As such, selection for antimicrobial-resistant strains is most acute in institutions such as hospitals, aged care facilities, and veterinary clinics, where antimicrobial use is high. These strains can then be transferred between individuals admitted to such institutions (13, 14). Additionally, antimicrobial resistance has been shown to be more prevalent in wild animals in urban areas than in those in rural or remote regions (15, 16), presumably due to the transmission of antimicrobial-resistant *E. coli* strains or genes (through horizontal gene transfer) from humans and/or domestic animals.

In this study, we determined whether the distribution of the different *E. coli* phylogenetic groups and cryptic clades, the occurrence of 49 virulence-associated genes, and/or the proportion of isolates resistant to 12 antimicrobials differed between four groups of domestic and wild birds from Australia that had contrasting extents and types of human association. Through this analysis, we have gained insights into the probable mechanisms by which these traits have been established in avian *E. coli*.

MATERIALS AND METHODS

Sample collection. Putative *E. coli* isolates were obtained from the feces of 594 Australian birds between 1994 and 2011. These birds belonged to 115 species, 81 genera, 42 families, and 17 orders (see Table S1 in the supplemental material). The birds were classified into four groups that reflected their probable association with humans. (i) Wild birds consisted of 68 birds that were sampled from wilderness areas (human population density, <500 per km^2) and so were thought to rarely associate with humans and/or livestock. (ii) Suburban birds consisted of 126 nondomesticated birds that were sampled from suburban localities and thus had some degree of human association. The human population density varied greatly between these localities, ranging from 500 to greater to 5,000 per km^2. (iii) In-care birds consisted of 246 isolates that were collected from nondomesticated birds admitted to veterinary hospitals or wildlife rehabilitation centers. (iv) Poultry consisted of 156 isolates that were collected from backyard domestic poultry; 137 of these were chickens, and the majority were sampled at the 2011 Royal Canberra National Poultry Show. The poultry included meat, egg, and fancy breeds and were kept primarily for showing by hobbyists. All poultry birds were kept in suburban backyards or on small rural properties. None of the poultry were from commercial poultry farms.

Birds were sampled from across five Australian states (Victoria, New South Wales, Queensland, Tasmania, and Western Australia [WA]) and the Australian Capital Territory (see Table S2 in the supplemental material). However, the majority of poultry and wild bird isolates came from animals in New South Wales. In general, when several samples were collected from the same bird species, they were obtained from diverse geographic locations. However, 62 long-beaked corellas and 15 rainbow lorikeets were sampled from single locations. As it could not be determined whether the *Escherichia* isolates from these birds reflected their locality, species, or extent of human association, data for all birds belonging to these species ($n = 89$) were excluded from all statistical analyses.

***E. coli* isolation and DNA extraction.** A single putative *E. coli* isolate was obtained from each sample by streaking the sample onto a MacConkey agar plate (17) that was then incubated at 35°C. Colonies were subsequently tested for citrate utilization by growth on minimal citrate agar plates. Putative *E. coli* isolates (lactose positive, citrate negative) were later confirmed by genetic analysis to be either *E. coli* or one of the cryptic clades (see "Phylogenetic group assignment" below). Isolates were maintained as freezer cultures (1 ml of lysogeny broth culture and 0.5 ml glycerol) and stored at −80°C.

DNA extractions were performed by plating the freezer cultures onto MacConkey agar plates (incubated at 35°C) and subsequently inoculating singles colonies into lysogeny broth that was then incubated for 19 h at 35°C with shaking (150 rpm). DNA was extracted from the cultures using the DNAzol genomic DNA isolation reagent (Molecular Research Center Inc.) according to the manufacturer's instructions. Precipitated DNA was resuspended in Tris-EDTA buffer.

Phylogenetic group assignment. *E. coli* strains can be classified into seven different phylogenetic groups or subspecies (A, B1, B2, C, D, E, and F) (8). Additionally, five cryptic clades of *Escherichia* that are phenotypically indistinguishable from but genetically distinct from *E. coli* have recently been identified (18). Phylogenetic group membership was assigned by the quadruplex PCR method, as detailed in the work of Clermont et al. (19). All isolates that were identified as cryptic clade members in the quadruplex PCR were then screened to determine which cryptic clade they belonged to according to the method of Clermont et al. (20). All isolates that failed to produce a product for the quadruplex PCR were screened for the housekeeping genes *gadA* and *uidA*. Isolates that failed to yield a product for both *uidA* and *gadA* were considered not to be *E. coli*. All PCRs were performed as described by Blyton et al. (21).

We established whether the distribution of the different phylogenetic groups varied with the birds' extent of human association using multinomial log-linear regression models. The models were fitted using the nnet package (22) in R (v.2.15.2) (23). The response variable in the analysis was the phylogenetic group of each isolate (A, B1, B2, clade, D, or minor phylogenetic group), and the explanatory variable was the birds' human association category (wild, suburban, in-care, or poultry). As the birds in each of the human association categories differed in their characteristics, we then explored whether any additional variables could explain the distribution of the phylogenetic groups separately for each human association category. For the poultry isolates, the explanatory variables were the taxonomic order to which the birds belonged. For the isolates from wild, suburban, and in-care birds, the explanatory variables included the birds' (i) habitat (canopy, ground, or water), (ii) state or territory, (iii) diet (carnivore, granivore, herbivore, insectivore, nectarivore, omnivore), (iv) body mass ($\log_{10}$), and (v) taxonomic order. We then visualized the effects of the significant explanatory variables using regression trees fitted using the package Party (24) in R.

Virulence genes. The *E. coli* and cryptic clade isolates were screened for the presence or absence of 49 virulence genes that belong to the variable portion of the *Escherichia* genome (25). While these genes were initially described for their association with intestinal or extraintestinal virulence in humans (26), they have subsequently been found to increase colonization success, persistence, and abundance within host individuals and occurrence in host populations of mammals (27–32). The genes were divided into 11 primer pools, and the isolates were screened by multiplex PCR (see Table 1 for gene names, functions, and references). The PCR mixes and amplification conditions were those described by Blyton et al. (32), except for the annealing temperatures, which were as follows: for pools 1 to 7, 63°C; for pools 8 and 9, 57°C; and for pools 10 and 11, 50°C.

To determine whether the *E. coli* and cryptic clade isolates sampled from birds in the different human association categories differed in their propensity to possess particular virulence genes, we fitted generalized linear models (family = binomial) using the lme4 package (33) in R. A separate analysis was performed for each virulence gene. The response variables were whether or not an isolate possessed a particular gene. The primary explanatory variable in the analyses was the birds' human association category. However, the occurrence of many of the virulence genes is known to vary between the different phylogenetic groups (34). There-

TABLE 1 Virulence genes screened by multiplex PCR

Function	Gene(s) (primer pool no., reference)
Actin polymerization	*ipaC* (9, 35)
Bacteriocin	ColE1 (4, 55), ColK (6, 55), *micV* (6, 55), ColIa (6, 55), ColE6 (6, 55), ColM (7, 55), *micH47* (9, 55), ColB (10, 55)
Capsule	K1 (10, 41), *kpsMT*II (2, 41)
Cell lysis	*ehx* (8, 56)
Iron uptake	*ireA* (1, 41), *iroN* (8, 43), *sitA* (3, 42), *iutA* (3, 57), *fyuA* (5, 41), *eitA* (1, 58)
Outer membrane protein	*ompT* (2, 43), *eaeA* (8, 56)
Toxin	*hylA* (1, 60), *vat* (2[a]), *cdtA* (3[b]), *astA* (7, 61), stx_1 (8, 56), stx_2 (8, 56), *lta* (10, 62), *cdtB* (11, 41), *stlA* (11, 62)
Epithelial invasion	*ibeA2* (4, 41)
Adhesion, fimbriae, and pili	*iha* (1, 43), *afa dra* (1, 41), *focH* (2, 10), *sfa foc* (2, 63), *papA* (3, 41), *fimH* (3, 41), *C1936* (4, 10), *C2395* (4, 10), *ppdD* (4, 10), *yehA* (5, 10), *aufA* (5, 10), *ygiL* (5, 10), *yfcV* (5, 10), *sfaS* (5, 41), *lpfA* of *Shigella* (3, 64), *lpfA* of strain LF82 (7, 64), *bfp* (9, 35), *agn43* (7, 65)
Plasma vapor deposition	*pcvD* (9, 66)

[a] Primers F-TCAGGACACGTTCAGGCATTCAGT and R-GGCCAGAACATTTGCTCCCTTGTT were used.
[b] Primers F-TGCCGCTCTGACAGGTGGACTTA and R-GCCTTTAAAAACGGGGTGATACA were used.

fore, the phylogenetic group of the isolates was also included as an explanatory variable. For a particular gene, only phylogenetic groups and human association categories for which that gene occurred at a frequency of between 5% and 95% were included in the analysis (see Table 3).

Different types of *E. coli* pathogens can be genetically identified by their virulence gene profiles. The set of genes screened for in this study included genes that allowed us to identify if any of the isolates were enteroaggregative, typical and atypical enteropathogenic, enteroinvasive, enterotoxigenic, enterohemorrhagic, or Shiga toxin-producing *E. coli* isolates, as defined by Robins-Browne et al. (35).

Antimicrobial resistance. The *E. coli* and cryptic clade isolates were screened for their resistance to 12 antimicrobials using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disk diffusion method (v.3.0) (36). These antimicrobials included three cephalosporins (cefazolin, cefotaxime, and ceftazidime), two penicillins (ampicillin and amoxicillin-clavulanic acid), and two quinolones (ciprofloxacin and nalidixic acid) (Table 2). Isolates were scored as resistant, intermediate, or susceptible to an antimicrobial on the basis of BD's clinical inhibition zone diameter breakpoints. The inhibition zone diameters were measured using the automated colony counting and zone measuring instrument ProtoCol (v.3; Synbiosis). Isolates that were classified as intermediate according to their inhibition zone diameters were generally grouped with the resistant isolates for analysis. However, isolates classified as intermediate to nitrofurantoin, ampicillin, or cefazolin were grouped with the susceptible isolates, as there was no discernible break between the zone diameter distribution of those isolates and that of the susceptible isolates.

To determine whether the *E. coli* and cryptic clade isolates sampled from the different bird-human association categories varied in their resistance to the antimicrobials, we performed two sets of analyses using generalized linear models (family = binomial) fitted in the lme4 package in R. In the first set of analyses, we fitted separate models for each antimicrobial to which resistance was detected in more than 5% of isolates. The response variables in these analyses were whether an isolate was resistant or susceptible to a particular antimicrobial. In the second analysis, we assessed whether or not an isolate was multidrug resistant. Isolates were classified as multidrug resistant if they were resistant to two or more classes of antimicrobials. In both sets of analyses, the primary explanatory variable was the birds' human association category. However, because antimicrobial resistance has been found to be less prevalent among phylogenetic group B2 strains (37, 38), phylogenetic group was also included as an explanatory variable.

RESULTS

Phylogenetic group. The putative *E. coli* isolates were assigned to the different phylogenetic groups and cryptic clades in the following proportions: B1, 40.0% (n = 237 isolates); B2, 17.9% (n = 106); A, 17.5% (n = 104); D, 6.9% (n = 41); clade V, 4.3% (n = 26); E, 4.2% (n = 25); F, 3.7% (n = 22); clade III, 2.5% (n = 15);

TABLE 2 Antimicrobial resistance of Australian bird *E. coli* and cryptic clade isolates

Antimicrobial (abbreviation)	Class	No. of resistant isolates	Frequency of resistance (%)	*P* value of explanatory variables[a]	
				Phylogenetic group	Human association
Tetracycline (TE)	Polyketide	82	13.9	**<0.001**	**0.002**
Ampicillin (AM)	Penicillin	47	8.0	**0.007**	0.091
Trimethoprim-sulfamethoxazole (SXT)	Folate pathway inhibitor	44	7.5	**0.028**	0.083
Nalidixic acid (NA)	Quinolone	24	4.1	NT	NT
Ciprofloxacin (CIP)	Fluoroquinolone	20	3.4	NT	NT
Gentamicin (GM)	Aminoglycoside	11	1.9	NT	NT
Amoxicillin-clavulanic acid (AMC)	β-Lactam and β-lactamase inhibitor	10	1.7	NT	NT
Cefotaxime (CTX)	Cephalosporin	6	1.0	NT	NT
Cefazolin (CZ)	Cephalosporin	5	0.8	NT	NT
Ceftazidime (CAZ)	Cephalosporin	3	0.5	NT	NT
Nitrofurantoin (FM)	Nitrofuran	3	0.5	NT	NT
Ertapenem (ETP)	Carbapenem	2	0.3	NT	NT

[a] Statistical analyses did not include data for isolates from long-billed corellas, rainbow lorikeets, or the Western Australian wildlife rehabilitation clinic. *P* values of <0.05 are shaded and shown in bold. NT, not statistically tested.

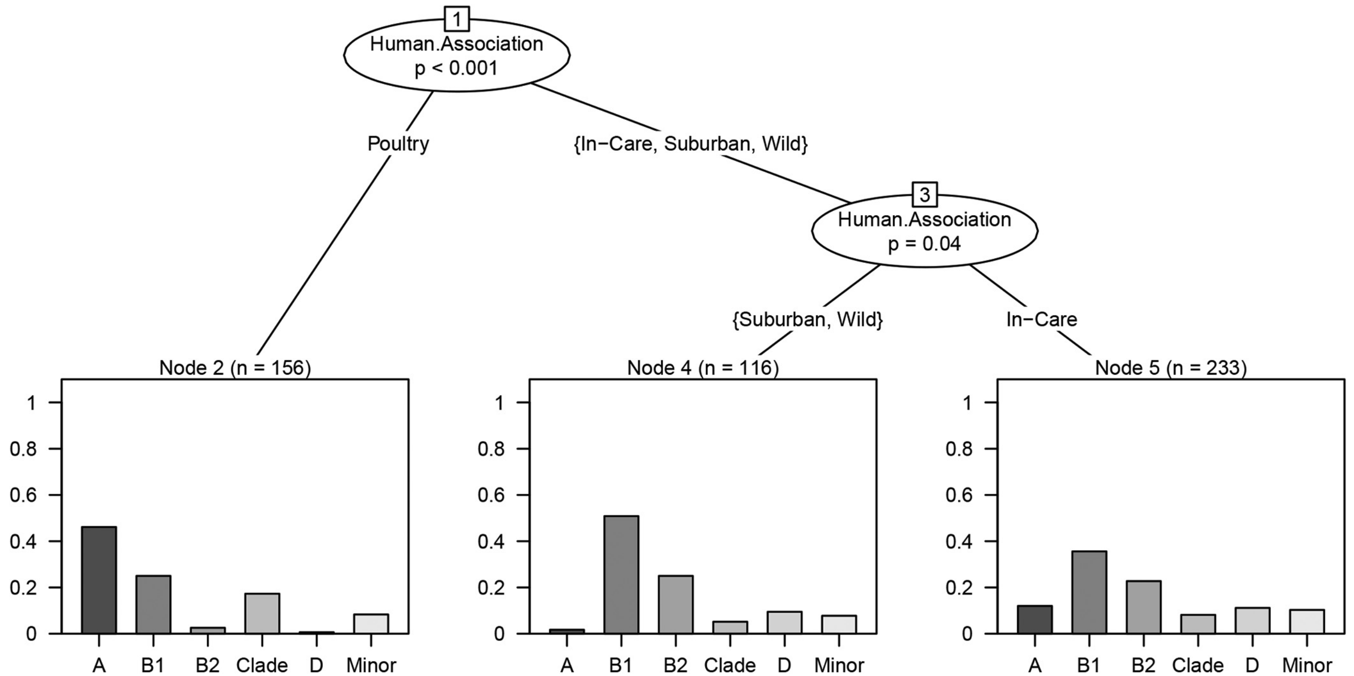

FIG 1 Regression tree of how the distribution of the different phylogenetic groups differed between the different human association categories. The *y* axes represent proportion of isolates.

clade IV, 1.5% ($n = 9$); C <1% ($n = 4$); clade I, <1% ($n = 3$); and clade II, <1% ($n = 1$).

According to the results of multinomial regression analysis, the human association category of the birds had a significant effect on the phylogenetic group membership of the sampled *Escherichia* isolates (likelihood ratio statistic = 206.7, $P < 0.001$). The phylogenetic group distribution of the isolates differed between all human association categories except between wild and suburban birds (for wild versus suburban birds, $P = 0.49$; Fig. 1). The majority of wild and suburban bird isolates ($n = 116$) belonged to phylogenetic group B1 (57.5%), with B2 being the second most abundant phylogenetic group (22.8%). The major fraction of in-care bird isolates ($n = 233$) was also B1 (35.7%); however, group A, clade, group D, and the minor phylogenetic groups accounted for a larger proportion of the in-care bird isolates than they did of those from the suburban or wild birds. The poultry isolates ($n = 156$) were the most phylogenetically distinct group, being dominated by phylogenetic group A (46.1%) and including a comparative abundance of clade strains (17.3%; Fig. 1).

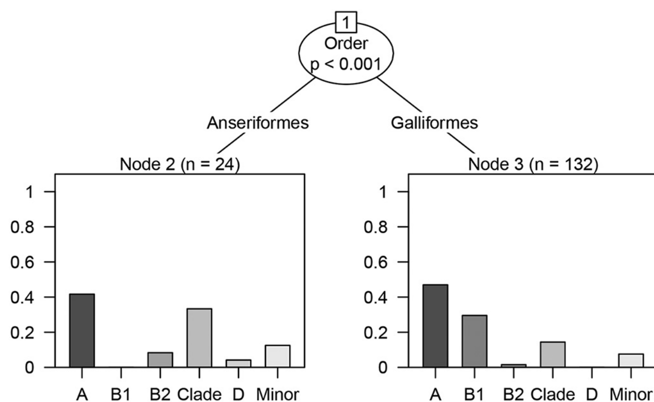

FIG 2 Regression tree of how the distribution of the different phylogenetic groups differed with the birds' taxonomic orders within the poultry. The *y* axes represent proportion of isolates.

Among the poultry isolates, the various taxonomic orders of birds had significantly different phylogenetic group distributions (likelihood ratio statistic = 23.5, $P < 0.001$). The major fraction of *Escherichia* spp. from both the Anseriformes (ducks and geese) and Galliformes (chickens, quail, and guinea fowl) were phylogenetic group A. However, the Anseriformes ($n = 24$) possessed a very high proportion of clade strains (33.3%), whereas in the Galliformes ($n = 132$), the second most abundant phylogenetic group was B1 (Fig. 2).

Among isolates from in-care birds, the phylogenetic group distribution varied by state or territory (likelihood ratio statistic = 63.6, $P < 0.001$). Isolates from Tasmania, sampled from a veterinary clinic and a wildlife rehabilitation center ($n = 49$), had a higher proportion of clade strains (28.6%) than isolates from the other states or territory (Fig. 3). These clade strains belonged predominantly to cryptic clade V. Additionally, the isolates from Western Australia ($n = 45$), sampled from a wildlife rehabilitation clinic, had a higher proportion of the minor phylogenetic groups (26.5%) than did the other in-care bird isolates. All except one of these minor phylogenetic group isolates belonged to phylogenetic group F.

Among the suburban and wild bird isolates, none of the explanatory variables were significantly associated with the distribution of the different phylogenetic groups.

Virulence genes. Among the 49 genes screened for, 7 were not detected in any isolate (*afa dra*, ColK, ColE6, *ehc*, stx_2, *ipaC*, and *bfp*) and 17 were found in less than 5% of strains (*pcvD*, *lta*, *stlA*,

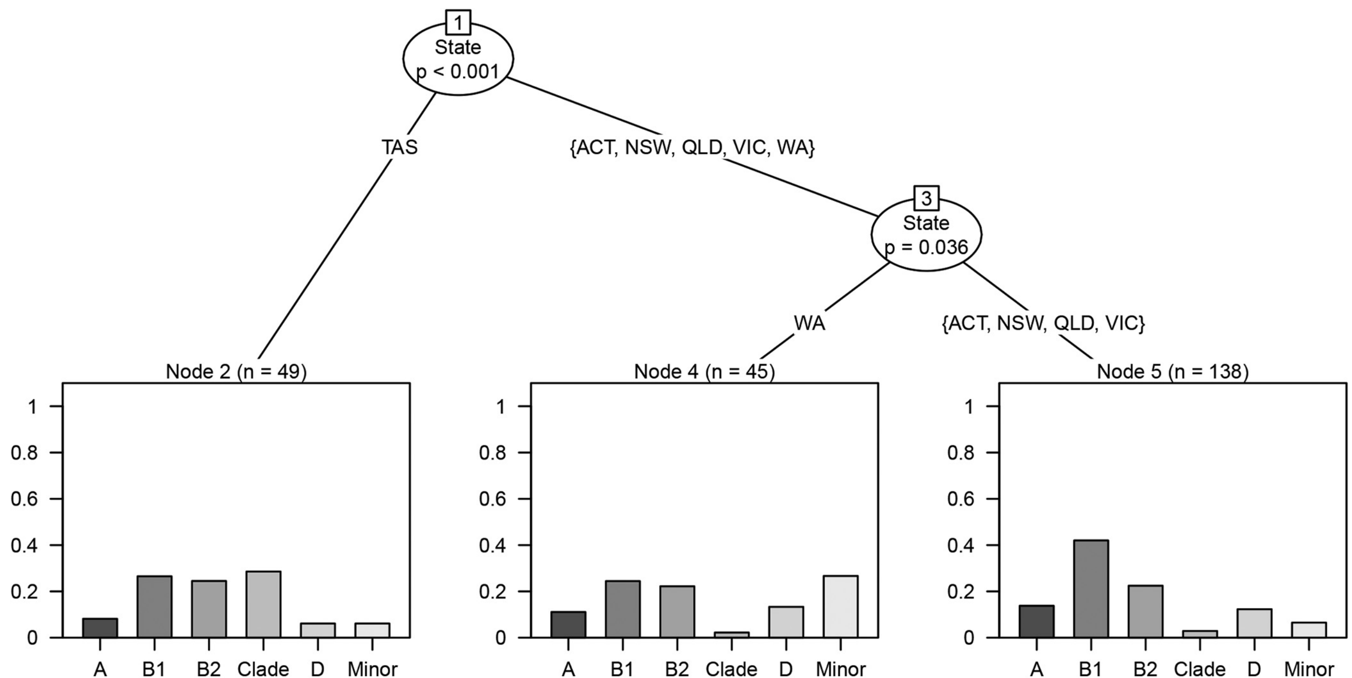

FIG 3 Regression tree of how the distribution of the different phylogenetic groups differed between the Australian states or territory within the in-care human association category. The *y* axes represent proportion of isolates. TAS, Tasmania; ACT, Australian Capital Territory; NSW, New South Wales; QLD, Queensland; VIC, Victoria; WA, Western Australia.

stx_1, *sfaS*, *papA*, *focH*, ColE1, *hylA*, *cdtB*, *iha*, *ireA*, *sfa foc*, MicH47, K1, MicV, and *eaeA*). One gene (*fimH*) was found at a very high frequency (96.6% of isolates).

The effect of the birds' human association category on the propensity of their *E. coli* and cryptic clade isolates to possess a particular trait could be assessed for 31 virulence factors that occurred at an intermediate frequency (5% to 95%) in at least one of the phylogenetic groups. There was a significant main effect of human association category on the frequency of 5 virulence genes (*ompT*, *sitA*, *yehA*, *kpsMT*II, and *fyuA*) after accounting for any effects of the phylogenetic groups. Among the 24 genes that were assessed across multiple phylogenetic groups, the frequency of 15 genes differed significantly between the phylogenetic groups assessed (Table 3).

Among the five genes for which the human association category had a significant effect on their frequency, three (*sitA*, *kpsMT*II, and *yehA*; Fig. 4) were more prevalent among isolates from in-care birds than among isolates from birds in the other human association categories. The prevalence of *sitA* was significantly higher in isolates from in-care birds than those from birds in all other human association categories (Fig. 4). The prevalence of *kpsMT*II was significantly higher in isolates from in-care birds than those from suburban birds or poultry (Fig. 4). The prevalence of *yehA* was significantly higher in isolates from in-care birds than those from poultry (for in-care versus poultry isolates, $P = 0.003$; Fig. 4). Additionally, *ompT* was significantly more prevalent among poultry isolates than among isolates from birds in all other human association categories (Fig. 4) and among isolates from in-care birds than those from suburban birds ($P = 0.028$). Finally, *fyuA* was significantly less prevalent among isolates from suburban birds than those from birds in all other human association categories (Fig. 4).

The virulence gene profiles indicated that none of the study isolates were typical enteropathogenic, enteroinvasive, enterotoxigenic, enterohemorrhagic, or Shiga toxin-producing *E. coli* isolates. A single isolate from an in-care noisy minor (*Manorina melanocephala*) was a probable enteroaggregative pathogen, as it possessed *pcvD*. Twenty-nine bird isolates were potential atypical enteropathogenic *E. coli* isolates, as they possessed the *eaeA* gene. The *eaeA* gene occurred predominantly in isolates of phylogenetic groups B1 and B2, but it also occurred in isolates of phylogenetic group A. There was no evidence that the frequency of *eaeA* varied between the isolates from birds in the different human association categories (Table 3).

Antimicrobial resistance. Antimicrobial resistance was observed for all tested antimicrobials (Table 2). Resistance to tetracycline (13.9%), ampicillin (8.0%), and trimethoprim-sulfamethoxazole (7.5%) was relatively common. Although rare, resistance to the critically important expanded-spectrum cephalosporins (cefotaxime, 1.0%; ceftazidime, 0.5%) was also observed. Antimicrobial resistance was relatively common among isolates from poultry and in-care birds (rates of resistance to one or more antimicrobials, 29.0% and 25.5%, respectively). In contrast, antimicrobial resistance was relatively uncommon among isolates from suburban and wild birds (rates of resistance to one or more antimicrobials, 4.8% and 3.0%, respectively).

We observed a high prevalence of antimicrobial resistance among isolates from a wildlife rehabilitation clinic in Western Australia (WA). In particular, 12 phylogenetic group F isolates from this clinic were multidrug resistant. All these isolates were resistant to trimethoprim-sulfamethoxazole, nalidixic acid, and a fluoroquinolone (ciprofloxacin). Ten of these isolates were also resistant to gentamicin, ampicillin, and tetracycline, and one isolate was resistant to amoxicillin-clavulanic acid. Gentamicin resistance occurred only in these phylogenetic group F isolates. Both nalidixic acid and ciprofloxacin resistance was also rare outside

TABLE 3 Occurrence of virulence genes in Australian bird *E. coli* and cryptic clade isolates

			Groups with frequency between 5% and 95%		*P* value of explanatory variables[a]	
Gene	No. of positive isolates	Overall frequency (%)	Phylogenetic group(s)[b]	Human association	Phylogenetic groups	Human association
agn43	216	36.4	All	All	**0.01**	0.46
astA	65	10.9	All	All	**<0.001**	0.40
ompT	205	34.6	All	All	**<0.001**	**<0.001**
sitA	193	32.5	All	All	**<0.001**	**<0.001**
ColIa	132	22.3	A, B1, B2, D, M	All	**<0.001**	0.25
ColM	95	16.0	A, B1, B2, D, M	All	**<0.001**	0.63
iroN	92	15.5	A, B1, B2, D, M	All	**<0.001**	0.57
iutA	38	6.4	A, B2, D, M	All	**0.04**	0.11
C1936	423	71.3	A, B2, M	All	**<0.001**	0.43
yehA	472	79.6	A, D, M	All	0.39	**0.02**
cdtA	297	50.1	A, D, M	All	**<0.001**	0.71
eitA	32	5.4	A, D, M	All	0.12	0.18
MicV	29	4.9	A, D, M	All	0.10	0.09
ColB	76	12.8	B1, B2, D, M	All	0.35	0.10
eaeA	29	4.9	B1, B2	All	0.16	0.55
lpfA from strain LF82	156	26.3	B1, B2	All	0.06	0.74
*kpsMT*II	106	17.9	B2, clade, D, M	All	0.58	**0.01**
lpfA from *Shigella*	36	6.1	B2, clade, M	All	**0.04**	0.21
ibeA	84	14.2	B2, clade	All	**0.001**	0.79
fyuA	129	21.8	B2, D, M	All	**<0.001**	**0.02**
ygiL	95	16.0	B2, D, M	All	**<0.001**	0.30
ppdD	524	88.4	B2, D, M	In-care, suburban, wild	**<0.001**	0.11
K1	20	3.4	B2, D	All	0.86	0.23
MicH47	20	3.4	B2, M	All	0.88	0.38
ayfA	79	13.3	B2	In-care, suburban, wild	NA	0.18
C2395	50	8.4	B2	In-care, suburban, wild	NA	0.30
focH	6	1.0	B2	In-care, suburban, wild	NA	0.99
iha	9	1.5	B2	In-care, suburban, wild	NA	0.22
sfa foc	11	1.9	B2	In-care, suburban, wild	NA	0.47
vat	82	13.8	B2	In-care, suburban, wild	NA	0.89
ireA	11	1.9	E	All	NA	0.34

[a] *P* values of <0.05 are shaded and shown in bold. NA, not applicable.
[b] Clade, clades I, II, III, IV, and V; M, minor phylogenetic groups C, E, and F.

the WA clinic, occurring in only five and two isolates, respectively. However, among the WA clinic isolates ($n = 45$), 5 phylogenetic group B2 isolates and 1 phylogenetic group B1 isolate, in addition to the 12 above-mentioned group F isolates, were nalidixic acid and ciprofloxacin resistant. Due to the unusually high prevalence of antimicrobial resistance detected at the WA rehabilitation clinic, we excluded the data for that clinic's 45 isolates from our statistical analyses.

The frequency of resistance to three antimicrobials outside the WA wildlife clinic was sufficient for meaningful statistical analysis. Resistance to one of these antimicrobials (tetracycline) varied significantly by human association category, after accounting for any effect of phylogenetic group (Table 2). Tetracycline resistance was the most common in isolates from poultry (25.8%) and also was relatively common in isolates from in-care birds (15.2%) but was rare in isolates from suburban birds (2.4%) and wild birds (3.0%) (Fig. 4). Resistance to each of the three antimicrobials (tetracycline, ampicillin, and trimethoprim-sulfamethoxazole) tested in the statistical analyses was also found to vary significantly by phylogenetic group, after accounting for any effect of human association category (Table 2). Resistance to each of these antimicrobials was the highest among phylogenetic group D isolates and the lowest among phylogenetic group B2 isolates (Fig. 5).

The prevalence of multidrug resistance varied significantly between the human association categories (data for WA clinic isolates were excluded; $P = 0.009$; Fig. 5). Multidrug resistance was relatively prevalent among isolates from the in-care birds and poultry (12.2% and 11.2%, respectively). In contrast, only a single multidrug-resistant isolate was detected in each of the suburban and wild birds (2.1% and 1.5%, respectively). The prevalence of multidrug resistance also varied significantly between the different phylogenetic groups ($P = 0.004$; Fig. 6). The incidence of multidrug resistance was the highest among phylogenetic group D isolates, and multidrug resistance was the least common among phylogenetic group B2 isolates.

DISCUSSION

In this study, we assessed how the genetic attributes and antimicrobial resistance of commensal avian *E. coli* and cryptic clade

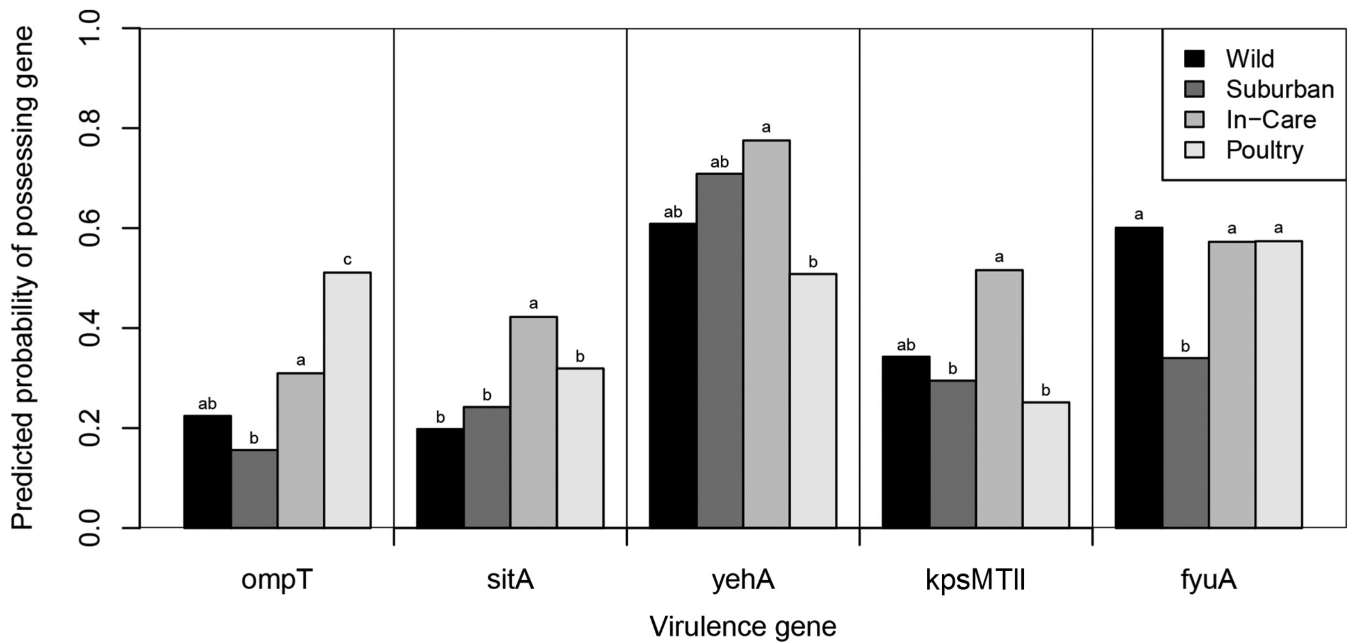

FIG 4 Predicted probability of virulence gene presence in relation to human association category. Predictions were made from the generalized linear regression models. The frequency of each phylogenetic group within each human association category was set to the overall mean for the Australian bird *E. coli* and cryptic clade isolates for the predictions. Probabilities for *yehA* were predicted for phylogenetic groups A and D and the minor phylogenetic group only. Probabilities for *kpsMT*II were predicted for phylogenetic group B2, cryptic clades, group D, and the minor phylogenetic groups (C, E, and F) only. Probabilities for *fyuA* were predicted for phylogenetic groups B2 and D and the minor phylogenetic groups (C, E, and F) only. Results for human association categories with different letters were significantly different from each other ($P > 0.05$).

isolates were influenced by the birds' type of human association, categorized into three wild bird groups, consisting of (i) birds from wilderness areas, (ii) birds from suburban locations, and (iii) birds receiving care in veterinary clinics or other wildlife rehabilitation facilities, and one domesticated bird group, consisting of backyard poultry. We found that the *Escherichia* spp. of backyard domestic poultry were phylogenetically distinct from the *Escherichia* spp. sourced from all other categories of birds. We found

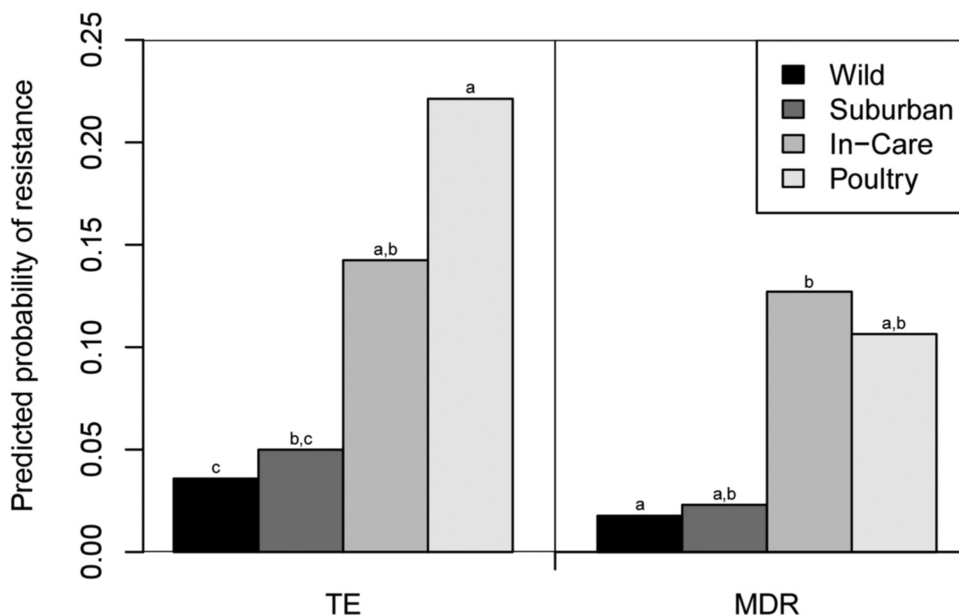

FIG 5 Predicted probabilities of an *Escherichia* isolate being resistant to tetracycline (TE) or multidrug resistant (MDR). Predictions were made from the generalized linear regression models. The frequency of each phylogenetic group within each human association category was set to the overall mean for the Australian bird *E. coli* and cryptic clade isolates for the predictions. The data used for the predictions did not include those from the Western Australian wildlife rehabilitation clinic. Results for human association categories with different letters were significantly different from each other ($P < 0.05$).

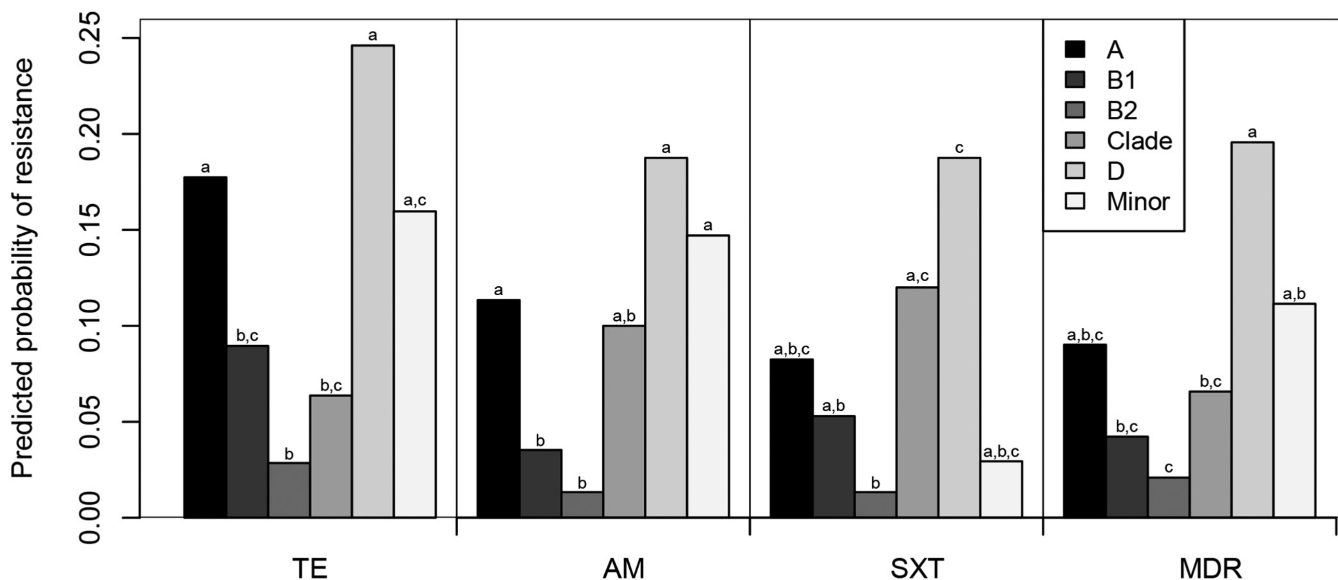

FIG 6 Predicted probabilities of an *Escherichia* isolate being resistant to an antimicrobial or multidrug resistant (MDR). TE, tetracycline; AM, ampicillin; SXT, trimethoprim-sulfamethoxazole. Predictions were made from the generalized linear regression models. Where the human association category was also a significant predictor of resistance, the human association categories were equally represented within each phylogenetic group for the predictions. The data used for the predictions did not include those from the Western Australian wildlife rehabilitation clinic. Results for phylogenetic groups with different letters were significantly different from each other ($P < 0.05$).

that *Escherichia* spp. from in-care birds possessed particular virulence-associated genes more often than *Escherichia* spp. from wild and suburban birds. Furthermore, *Escherichia* spp. from both the backyard poultry and in-care birds were more likely to be multidrug resistant than isolates from wild birds. In contrast, we found little difference between the *Escherichia* spp. isolated from wild and suburban birds. These differences and similarities between the avian *E. coli* and cryptic clade isolates from these different human association categories likely reflect differences in host traits, transmission dynamics, exposures, and selection pressures on the respective *Escherichia* communities between the groups. We discuss these potential processes for each human association category in detail below.

Wild and suburban bird *E. coli* and cryptic clade isolates. The similarity between the *E. coli* and cryptic clade isolates from suburban and wild birds was surprising, given the findings of previous studies on the effects of human habitation on the prevalence of antimicrobial resistance. Several previous studies that compared animals in the same or different countries have found that animals in remote regions carry *E. coli* isolates with a lower prevalence of antimicrobial resistance than isolates carried by animals living close to human settlements (15). However, many of these studies primarily investigated mammalian hosts (for example, see reference 16), and in urban environments, the rate of transmission of strains or their genes between humans and animals may be less for birds than for mammals. Two studies that looked specifically at antimicrobial resistance in birds focused on gulls and geese that nested around waste or agricultural waters (39, 40). These waters likely had more extensive *E. coli* contamination from humans and domestic animals than the suburban environment of Australian cities. Thus, the low prevalence of antimicrobial resistance in both the suburban and wild bird *Escherichia* spp. evaluated in this study may reflect minimal exposure to human and domestic animal strains in both these groups.

***E. coli* and cryptic clade isolates recovered from birds in care.** It is difficult to interpret the finding that four virulence-associated genes (*kpsMT*II, *sitA*, *ompT*, and *yehA*) were overrepresented in *Escherichia* spp. from birds in care. These genes code for a diverse range of traits. *kpsMT*II encodes group 2 capsules (41), *sitA* encodes a protein involved in iron uptake (42), *ompT* encodes an outer membrane protein (43), and *yehA* encodes a putative fimbrial adhesion protein (10). Given the large number of statistical tests performed in this study, the results of individual analyses should be treated with caution. However, the overall finding of a greater virulence gene content in the *Escherichia* spp. from birds in care is convincing. The birds brought to these institutions were orphaned, had sustained an injury, or were sick. It is conceivable that the sick birds may have been carrying *Escherichia* spp. that more often possessed virulence-associated genes, as these genes are associated with disease-causing *E. coli* strains in humans. However, we do not possess individual-level information on the birds, so we were unable to determine whether those strains contributed to the birds' condition. Alternatively, the birds may have acquired *Escherichia* spp. possessing those virulence-associated genes from the environment at the rehabilitation centers.

The higher prevalence of multidrug resistance in the *Escherichia* spp. from the in-care birds than in isolates from wild and suburban birds is likely the result of selection for and acquisition of antimicrobial-resistant *Escherichia* spp. in those institutions. Nosocomial antimicrobial-resistant infections have been well described in humans and provide an indication of how prevalent they may be in animals where less information is available. It has been estimated that approximately 5% of people admitted to a hospital in the United States during 2002 acquired an infection while in the hospital (44). Furthermore, during 2006 and 2007, 16% of hospital-acquired infections were caused by multidrug-resistant organisms (44). Our findings show that the *Escherichia* isolates recovered from in-care birds were distinct from those

recovered from wild or suburban birds in the same regions. In particular, we found that geographic location (state or territory) influenced the phylogenetic distribution of the strains from the in-care birds but not those from the wild or suburban birds and that a larger proportion of isolates from the in-care birds belonged to phylogenetic group A or D, the minor phylogenetic groups, and the cryptic clades. These results support the suggestion that birds at veterinary clinics and rehabilitation centers may acquire locally circulating *Escherichia* strains.

The circulation of several multidrug-resistant *E. coli* strains in veterinary teaching hospitals has been reported in Australia and the United States (13, 14). In both instances, the *E. coli* strains caused extraintestinal infections in dogs, were highly clonal, and were isolated from the hospital environment. Nucleotide sequencing data have shown that 11 of the phylogenetic group F isolates with similar multidrug resistance profiles from the WA clinic in our study were clonal (unpublished data). Therefore, it appears that there was a single phylogenetic group F strain circulating among the birds at the WA wildlife rehabilitation center. These phylogenetic group F isolates were resistant to at least 5 of the 12 antimicrobials tested, including the fluoroquinolone ciprofloxacin. Fluoroquinolone-resistant phylogenetic group F strains have previously been identified among fecal and urinary tract isolates from hospitalized dogs in Australia, with the vast majority belonging to sequence type 354 (ST354) (45). If the avian phylogenetic group F isolates from the WA clinic also belong to this sequence type, this would suggest that ST354 may be selected for in veterinary institutions, where antimicrobial use is common.

In addition to the circulation of the multidrug-resistant group F strain, there also appears to have been general selection for quinolone-resistant isolates at the WA wildlife rehabilitation clinic. Resistance to nalidixic acid and ciprofloxacin was rare outside this clinic, yet multiple phylogenetic group F, B2, and B1 isolates from the clinic were resistant to these antimicrobials. The fluoroquinolone enrofloxacin is commonly used to treat injured wildlife in Australia (46). Therefore, the prevalence of ciprofloxacin resistance observed in this clinic may have been the result of antimicrobial selection pressure mediated by enrofloxacin use.

Poultry *E. coli* and cryptic clade isolates. The phylogenetic group distribution of the *Escherichia* isolates from backyard domestic poultry was broadly consistent with the findings from previous studies. In particular, the high proportion of phylogenetic group A strains fell within the range found in other studies. In four of five studies of fecal *E. coli* isolates from food-producing poultry, the proportion of phylogenetic group A strains ranged from 38 to 78% (47–51). Escobar-Páramo and colleagues (52) also found a higher proportion of phylogenetic group A strains in domestic birds (primarily poultry) than wild birds. It is unknown whether this high occurrence of phylogenetic group A strains in domestic poultry is a result of the avian hosts' domestic lifestyle or species-specific traits.

Consistent with the findings of Clermont and colleagues (20), in this study the isolates of the cryptic clades were more likely to be isolated from poultry than the other groups of birds. Those authors (20) found that isolates of the cryptic clades were more likely to be isolated from birds sampled in France than birds sampled in Australia, with farmyard poultry representing most of the French birds sampled. Because the cryptic *Escherichia* clades have only recently been described (18), relatively little is known about their patterns of occurrence and ecology. Ingle and colleagues (53) found that the cryptic clades have an enhanced ability to form biofilms compared to *E. coli*, can replicate at lower temperatures, and may be able to persist for longer periods in the external environment. Clade strains are generally rare in fecal samples, representing less than 3% of isolates in humans (20), although particular cryptic clade lineages may be found at higher frequencies in some species (21). Interestingly, we found that the Anseriformes (ducks and geese) had a higher proportion of cryptic clade strains than the Galliformes (chickens, quail, and guinea fowl). This could potentially reflect either a greater ability of the cryptic clades to inhabit these hosts or these hosts' association with common habitat features, such as water.

The higher prevalence of tetracycline resistance and multidrug resistance in the *Escherichia* spp. from backyard poultry than in isolates from suburban and wild birds is likely to be a result of antimicrobial use in the former. Tetracycline is not used to treat infections in humans in Australia; however, it is used to treat a broad spectrum of systemic infections in livestock, including poultry (54). Furthermore, backyard poultry may be medicated with a range of antimicrobials to treat bacterial diseases, particularly respiratory infections, as well as protozoan infections, including coccidiosis (54).

Conclusions. In this study, we have shown that the extent and type of association between humans and birds influence the genetic attributes and antimicrobial resistance profiles of avian *Escherichia* communities. The similarity of antimicrobial resistance profiles and genetic attributes between *Escherichia* spp. from suburban and wild birds is reassuring, in that it suggests a low rate of transmission of *Escherichia* spp. between humans and birds in Australian urban environments. However, the relatively high prevalence of antimicrobial resistance and multidrug resistance detected among *Escherichia* spp. from in-care birds and poultry is concerning. We propose that the antimicrobial resistance observed in these groups is due primarily to the use of antimicrobials in these birds, and we advise that such use be carefully scrutinized. In particular, antimicrobials should be administered only in cases where an infection is strongly suspected or confirmed. Antimicrobial treatment should also be avoided for wildlife and poultry that have a low chance of survival. Additionally, the detection of a multidrug-resistant *E. coli* strain resistant to a fluoroquinolone circulating in a wildlife rehabilitation center reinforces the importance of adequate hygiene practices when handling and caring for sick as well as injured and orphaned wildlife.

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