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Wild Australian birds and drug resistant bacteria: Characterisation of antibiotic resistant *Escherichia coli* and *Enterococcus* spp.

Hannah G. Smith^{A,B}, Rohan H. Clarke^C, Jo-Ann Larkins^A, David C. Bean^A, Andrew R. Greenhill^A

^A School of Health and Life Sciences, Federation University, Victoria, Australia.

^B Faculty of Science, Monash University, Australia.

^C School of Biological Sciences, Faculty of Science, Monash University, Australia.

Corresponding author: Andrew R. Greenhill. andrew.greenhill@federation.edu.au

Abstract

Birds can spread microorganisms through their movement; however, it is still not fully understood how wild birds acquire and disperse antimicrobial resistant (AMR) bacteria. We sampled wild Australian birds from three geographically distinct locations for the presence of AMR strains of two clinically important species of bacteria, *Escherichia coli* and *Enterococcus* spp. A total of 121 birds were sampled, representing 15 different species of birds. Thirty *E. coli* and 54 *Enterococcus* isolates were tested for resistance to 16 antibiotics. Resistance to at least one antibiotic was common, being detected in 96% of *Enterococcus* and 60% of *E. coli* isolates. The vancomycin resistance genes *vanA* and *vanD* were detected in 22% of the enterococci (17% *vanA*, 13% *vanD*), while 14% displayed phenotypic resistance with no associated gene. Wild birds are a carrier of AMR bacteria in Australia, and are capable of harbouring a more diverse range of vancomycin resistance genes than typically seen among Australian clinical isolates.

Keywords: Zoonotic, AMR, antimicrobial, avian, passerines, *van* genes.

Introduction

Wild bird populations can be an important factor in the transmission of pathogens. Due to their highly mobile nature, birds have the potential to rapidly spread microorganisms (Reed *et al.* 2003, Bauer & Hoyer 2014, Baez *et al.* 2015) and introduce them into previously uninfected populations. In addition to pathogens, wild birds are also capable of carrying antimicrobial resistant (AMR) bacteria, which are recognised as a growing threat to global health (da Costa *et al.* 2013, Reed *et al.* 2003)). The challenge presented by the spread of AMR is enormous and growing rapidly. In humans at least 700,000 deaths are associated with AMR each year, and this is predicted to reach 10 million deaths by 2050 (Kahn 2017). AMR bacteria have been found in environments and animal populations that are not directly exposed to antibiotics (Allen *et al.* 2010), including wild animal populations (da Costa *et al.* 2013) further heightening the threat posed by antimicrobial resistance. The transmission of bacteria between wild animals and humans has been noted in areas of high human-wildlife contact, such as urban fringes (Blyton *et al.*, Goldberg 2008, Pesapane 2013). Moreover, in bacteria commonly present in various animal and human hosts, genetically similar bacteria have been detected across multiple host species (Clermont 2011).

Enterococcus spp. and *Escherichia coli* are common gastrointestinal bacteria in birds, and can be passed between wild birds and humans (Abulreesh *et al.* 2007, da Costa *et al.* 2013, Bonnedahl and Jarhult *et al.* 2014). Both are potential pathogens of humans and other animals; and importantly both are effective indicators of AMR in the environment, due to the ease with which they acquire resistance genes (Radhouani *et al.* 2012). Enterococci in particular are a serious concern in clinical settings, being ranked as the second most abundant healthcare-associated infection globally (Levitus & Perera 2018), while vancomycin-resistant *Enterococcus* (VRE) are a major cause of healthcare-associated bloodstream infections (Mahony *et al.* 2018). Of particular concern is resistance to the antibiotic

vancomycin, an antibiotic traditionally used in the treatment of severe *Enterococcus* and *Staphylococcus* infections in humans. Vancomycin was once recognised as a ‘last line of defence’ against such infections, with only one other antibiotic, teicoplanin, being available to treat a vancomycin-resistant infection; despite new antimicrobials being released, it still remains an important antibiotic for severe infections (Levitus & Perera 2018). There are multiple genes clusters that can impart resistance to vancomycin, the most common being *vanA*, *vanB*, *vanC* and *vanD*, with the *vanA* genotype imparting resistance to both vancomycin and teicoplanin (Courvalin 2006).

The first AMR bacteria isolated from wildlife was recovered from pigeons (*Columba livia*) in 1975 (Bonnedahl and Jarhult 2014). Since then, AMR bacteria have been isolated in birds from every continent (Guenther *et al.* 2012, Hidasi *et al.* 2013, Bonnedahl and Jarhult, 2014, Blyton *et al.* 2015, Janecko *et al.* 2015). AMR bacteria have been isolated from a diversity of avian taxa including representatives in the families Laridae (Cizek *et al.* 2007), Passerines (Hernandez-Divers *et al.* 2008, LeJeune *et al.* 2008), Columbidae (Radimersky *et al.* 2010) and Accipitriformes (Radhouani *et al.* 2012). Three recent studies have investigated AMR in Australian birds: one investigated *E. coli* in wild and domestic birds; (Blyton *et al.* 2015); while two looked at AMR Enterobacteriaceae and vancomycin-resistant *Enterococcus* (VRE) in Silver Gulls from New South Wales (*Chroicocephalus novaehollandiae*; Dolejska *et al.* 2015, Oravcova *et al.* 2017). Whilst these studies provide much-needed insight into how wild birds are affected by anthropogenic activity and location, there remains a gap in knowledge for how AMR and VRE carriage may differ at sites with little to no anthropogenic impact.

In this study, we demonstrate that resistance to clinically important antibiotics is pervasive in wild populations, regardless of location or human impact. This study seeks to further contribute to our knowledge of AMR distribution across the country, and specifically in remote, relatively undisturbed habitats by investigating if the same AMR and VRE genotypes

are present in populations in these locations. This study aims to provide further clarification on the impact anthropogenic contact has on AMR carriage in wild Australian birds, by investigating habitats with little to no anthropogenic impact. This work was conducted under wildlife permits issued by Parks Australia, Victorian Department of Environment, Land, Water and Planning (DELWP) and Queensland Environment Protection Agency and animal ethics approvals administered through Monash University BSCI/2012/08 (offshore islands), BSCI/2012/15 (Gippsland Lakes) and BSCI/2014/03 (Torres Strait).

Methods

Sample sites and species investigated

We collected cloacal swabs from three geographically distinct locations: Adele Island (15 31' 28" S, 123 09' 20" E), NW Australia, Boigu Island (9 13' 59"S, 142 13' 00"E), Torres Strait, Queensland and the Gippsland Lakes (37 57' 52"S 147 46' 15" E), Victoria (Figure 1); where capture of wild free-flying birds was conducted for ecological research (see Woodland *et al.* 2016, Mott *et al.* 2017). Birds were captured using canon nets, mist nets or hand nets, as appropriate to the target species. In all instances sampled birds appeared healthy, displaying no signs of illness or injury. Cloacal swabs were collected using routine bird handling and sample collection methods. The swab was placed in Aimes transport media and stored at 4°C until bacterial culture could be conducted. Transit time between sample collection and culture varied between expeditions, with a 5 day transit time for the Gippsland Lakes samples, 2-12 days for the Torres Strait samples, and 4-8 days for the Adele Island samples.

Bacterial culture and identification

We selected two bacteria for our analysis: one Gram positive (*Enterococcus* spp.) and one Gram negative organism (*E. coli*). Both *E. coli* and *Enterococcus* spp. are known to acquire antibiotic resistance genes and can be important human and animal pathogens. Each swab

was used to inoculate duplicate Oxoid MacConkey II agar. Plates were incubated at 37°C and checked for growth at 24, 48 and 72 hours.

Suspected *E. coli* or *Enterococcus* were subcultured: up to six colonies were selected from each plate. Presumptive identification was conducted by biochemical tests (Catalase, Oxidase and Gram Stain) and confirmation by PCR. *Enterococcus* isolates were identified to genus and species level by using established protocols that target the 16S rRNA gene (Ryu *et al.* 2013). Confirmation of *E. coli* was conducted following protocols of Khan *et al.* (2007) that targeted a 450bp fragment in the Internal Transcribed Spacer (ITS) region.

Isolates were tested for AMR using the Kirby-Bauer disk diffusion test following CLSI guidelines. Antibiotics appropriate for the clinical treatment of each organism were chosen (Clinical and Laboratory Standards Institute 2012).

Antimicrobial resistance testing and van gene detection

E. coli isolates were tested for susceptibility to amikacin (10 µg), co-trimoxazole (1.25/23.75 µg), streptomycin (10 µg), nalidixic acid (30 µg), imipenem (10 µg), ceftazidime (30 µg), ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg), amoxicillin-clavulanic acid (30 µg) and ciprofloxacin (5 µg). *Enterococcus* isolates were tested against erythromycin (15 µg), tetracycline (30 µg), chloramphenicol (30 µg), ampicillin (10 µg), streptomycin (300 µg), gentamicin (120 µg), ciprofloxacin (5 µg) and vancomycin (30 µg). If multiple isolates were obtained from a single swab, susceptibility testing was conducted on all isolates. Those isolates with identical resistance patterns were assumed to be clonal; thus only one representative isolate was included in data analysis. The presence of the vancomycin resistance genes *vanA*, *vanB*, *vanC* and *vanD* in the *Enterococcus* isolates was determined by PCR (Depardieu *et al.*, 2004).

Results

Bird species

Samples were collected from 121 birds. In the Gippsland Lakes region of Victoria (a major human population centre) 26 Greater Crested Terns (*Thalasseus bergii*) were sampled. In the Torres Strait (a human population of 300-400 on the island), 46 individual birds were sampled, representing 10 species (two Coraciiformes, one Cuculiforme and six Passeriformes; Table 1). From Adele Island (no permanent human population) 49 birds were sampled, representing four species of seabird (all Suliformes; Table 1).

Bacterial isolates

From 121 sampled birds, 15 (12%) were positive for *E. coli* (12 Greater Crested Tern, two Varied Honeyeater (*Gavicalis versicolor*), one Rainbow Bee-eater (*Merops ornatus*) and 14 (11%) for *Enterococcus* (5 Greater Crested Tern, one Willie Wagtail (*Rhipidura leucophrys*), one Horsfield's Bronze-Cuckoo (*Chrysococcyx basalis*), two Varied Honeyeater, two Sacred Kingfisher (*Todiramphus sanctus*), one Rainbow Bee-eater, one Masked Booby (*Sula dactylatra*), one Lesser Frigatebird (*Fregata ariel*), Table 1). A total of 71 presumptive *Enterococcus* isolates and 73 presumptive *E. coli* isolates were obtained, all of which were confirmed as *Enterococcus* spp. or *E. coli* by PCR. It is important to note that bacterial recovery may have been adversely affected by the time in transit between swab collection and culture.

For both *E. coli* and *Enterococcus* spp., multiple bacterial isolates from a single bird with identical AMR phenotypes were excluded, on the assumption the multiple isolates were clonal. This resulted in 30 distinct *E. coli* isolates and 54 distinct isolates of *Enterococcus* spp. *Enterococcus* spp. was isolated from birds in all three study sites, while *E. coli* was recovered from birds in two study sites (Gippsland Lakes and Boigu Island, Torres Strait). Of

the *Enterococcus* isolates, 45 (83%) were identified as *E. faecalis*, 2 (4%) as *E. faecium*, and 7 (13%) could not be speciated.

Antimicrobial resistance and van genes

Of the 15 birds from which *E. coli* was isolated, AMR bacteria were detected in nine birds (seven Greater Crested Terns, two Varied Honeyeaters and one Rainbow Bee-Eater (Table 2). Of the *E. coli* isolates, 34% demonstrated resistance to streptomycin, 34% to ampicillin, 7% to amikacin, 7% to amoxicillin, 7% to gentamicin, 4% to chloramphenicol, 4% to ceftazidime, and 4% to tetracycline. All isolates were sensitive to imipenem, nalidixic acid, co-trimoxazole and ciprofloxacin. Multi-drug resistance (resistance to >1 drug) was recorded in 23% of *E. coli* isolates, in both sites where *E. coli* was detected in birds.

Of the 14 individual birds from which *Enterococcus* spp. were isolated, AMR bacteria were detected in 13 birds (Five Greater Crested Terns, two Sacred Kingfishers, one Varied Honeyeater, one Rainbow Bee-Eater, one Willie Wagtail, one Horsfields Bronze-Cuckoo, one Lesser Frigatebird, and one Masked Booby (Table 2). Of these isolates, 83% demonstrated resistance to streptomycin, 50% to erythromycin, 48% to gentamicin, 30% to vancomycin, 26% to ciprofloxacin, 13% to ampicillin, 6% to chloramphenicol, and 2% to tetracycline. Resistance was observed to every antibiotic tested. Multi-drug resistance was recorded in 72% of *Enterococcus* isolates, and was detected in all three study sites.

Nine of the *Enterococcus* isolates carried *van* genes: six carried *vanA* and six *vanD*, with three of the isolates containing both *vanA* and *vanD*. No *vanB* or *vanC* genes were detected.

VRE was isolated from six bird species, with *vanA* carried by one Willie Wagtail, two Sacred Kingfishers, one Masked Booby, and *vanD* carried by one Willie Wagtail, one Sacred Kingfisher and one Greater Crested Tern (all of which also carried *vanA*). Vancomycin resistance was also present in a single Greater Crested Tern, Varied Honeyeater and Lesser

Frigatebird, but a *van* gene was not identified in these instances. Vancomycin resistance was detected in all three study sites.

Discussion

We have demonstrated that wild birds in three geographically distinct locations around the periphery of the Australian continent harbour AMR bacteria. This adds to our understanding of the prevalence of AMR bacteria in Australian wild birds, demonstrating that resistance is widespread across bird taxa, and is present in remote and isolated habitats.

Direct comparisons to previous studies are not without their limitations, due to our small sample sizes. The proportion of AMR *E. coli* isolates was generally higher in this study than in similar studies conducted in countries such as Portugal (24% of isolates; Radhouani *et al.* 2012) and the Czech Republic (1.5% of isolates; Radimersky *et al.* 2010). However, a different study of wild European Starlings (*Sturnus vulgaris*) at cattle feedlots found 49% of *E. coli* isolates displayed AMR, with 60% of those multidrug resistant (Gaukler *et al.* 2009).

The overall proportion of multidrug resistance (29%) in Gaukler *et al.* was comparable to our findings (23% of *E. coli*), despite differences in habitat and likely exposure to antibiotics.

Multidrug resistant strains of *E. coli* were observed in our study, with isolates resistant to ≥ 3 antibiotics (17% of isolates resistant to two antibiotics and 3% resistant to three antibiotics), higher than the proportion of multi-drug resistant isolates found by Blyton *et al.*, detecting a single multi-drug resistant isolate in the wild birds sampled (1.5%).

As was the case with *E. coli*, the prevalence of AMR enterococci was generally higher in this study than in similar studies. In this study reduced susceptibility to all eight antibiotics tested was observed. The proportion of isolates that were resistant to ≥ 1 antibiotics (82% of isolates) was higher than in similar studies: 31% in feral pigeons in the Czech Republic

(Radimersky *et al.* 2010), and 46% of enterococci from wild birds in the Azores Archipelago (Santos *et al.* 2013). Three-quarters of the *Enterococcus* isolates were resistant to ≥ 2 antibiotics in this study, compared to only 8% of multi-resistant enterococci isolates from Azores Archipelago (Santos *et al.* 2013), and no multi-drug resistance being identified in pigeons in the Czech Republic.

While vancomycin has never been available for use in livestock, a chemically similar antibiotic (Avoparcin) was only banned from use in Australian agriculture in 2007 (FSANZ 2007). This, in conjunction with the first clinical report of VRE in Australia in 1994 (Bell *et al.* 1998), could be a possible reason for why VRE seems to be present in many different bird communities; the extended presence of VRE in both clinical and agricultural settings may have allowed for the spread of such bacteria into the surrounding environment, even geographically isolated environments. VRE in Australian birds has only previously been described on two occasions: in Silver Gulls in Australia in both Sydney and Five Islands, NSW (Dolejska *et al.* 2015, Oravcova *et al.* 2017). Our study isolated VRE from 16 of the *Enterococcus* isolates, in six species (Willie Wagtail, Sacred Kingfisher, Varied Honeyeater, Masked Booby, Greater Crested Tern and Lesser Frigatebird) and from all study sites.

Elsewhere, VRE has been reported to be present in a number of avian species, including Black-headed Gulls in Sweden (*Chroicocephalus ridibundus*; Sellin *et al.* 2000), and Rooks (*Corvus frugilegus*; Oravcova *et al.* 2013), and feral pigeons in Europe (Radimersky *et al.* 2010). Our findings compliment those of Oravcova *et al.* (2017), and demonstrate that VRE occurs in a range of taxonomically distinct Australian bird species that are geographically dispersed, and in association with both high and low human population densities. In addition to the *vanA* and *vanB* genotypes discovered by Oravcova *et al.*, this study shows that *vanD* genotypes also are present in Australian birds. This genotype is not currently seen in the clinical population (Lee *et al.* 2018, Coombs *et al.* 2013). While Silver Gulls often live in

close proximity to humans and may be exposed to clinically important bacteria through behaviour such as feeding on refuse (Smith *et al.* 1993), many of the identified carriers of VRE in this study are unlikely to have the same exposure due to habitat or foraging requirements and behavioural responses (Jackson *et al.* 1993, Mott *et al.* 2017). It is possible that the birds acquired resistance from environmental contamination (Berglund *et al.* 2015), or through interactions with other wildlife that may interact with anthropogenic environments (Smith *et al.* 1993).

This study clearly demonstrates that AMR bacteria are present in a diversity of wild bird species at distant points around Australia. Whilst acknowledging limited replication across just three sites, given that one of these sites (Adele Island) is remote from human population centres, and another (Boigu Island) is sparsely populated, our results suggest that direct anthropogenic contact may only be one of a number of drivers of AMR and VRE at regional scales. It is possible that proximity to the Asia-Pacific is a contributing factor to this trend (Molton *et al.* 2013). Of particular interest is that *vanA* was detected in our isolates. In Australia *vanB* is most commonly associated with clinical VRE (Christiansen *et al.* 2010); whereas in China *vanA* has been shown to be common (Zhao *et al.* 2010). Four of the six *vanA* isolates were obtained from Boigu Island, Torres Strait. Although these birds do not migrate to Asia, there is potential for cross colonisation by migratory birds that breed in eastern Asia and travel through, or remain in, the Torres Strait during the non-breeding season. Given the absence of direct AMR exposure pathways for the bird species assessed here, more work needs to be done to determine where and how such AMR bacteria are colonizing Australian birds.

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Table 1. Australian bird species from which swabs were collected, and the proportion of sampled birds positive for carriage of *E. coli* and *Enterococcus* spp.

Bird species (common name)	No. of birds sampled	Proportion birds <i>E. coli</i> positive (No.)	Proportion birds <i>Enterococcus</i> positive (No.)
<i>Thalasseus bergii</i> (Greater Crested Tern)	26	46% (n=12)	19% (n=5)
<i>Rhipidura leucophrys</i> (Willie Wagtail)	1	0%	100% (n=1)
<i>Chrysococcyx basalis</i> (Horsfields Bronze-Cuckoo)	1	0%	100% (n=1)
<i>Gavicalis versicolour</i> (Varied Honeyeater)	23	9% (n=2)	9% (n=2)
<i>Todiramphus sanctus</i> (Sacred Kingfisher)	2	0%	100% (n=2)
<i>Merops ornatu</i> (Rainbow Bee-eater)	2	50% (n=1)	50% (n=1)
<i>Myiagra alecto</i> (Shining Flycatcher)	1	0%	0%
<i>Ramsayornis modestus</i> (Brown-backed Honeyeater)	1	0%	0%
<i>Conopophila albogularis</i> (Rufous Banded Honeyeater)	1	0%	0%
<i>Xanthotis flaviventer</i> (Tawny- breasted Honeyeater)	12	0%	0%
<i>Dicrurus bracteatus</i> (Spangled Drongo)	2	0%	0%
<i>Sula dactylatra</i> (Masked Booby)	9	0%	11% (n=1)
<i>Fregata ariel</i> (Lesser Frigatebird)	20	0%	5% (n=1)
<i>Sula leucogaster</i> (Brown Booby)	16	0%	0%
<i>Fregata minor</i> (Great Frigatebird)	4	0%	0%

Table 2. Drug-resistance phenotypes of the *E. coli* and *Enterococcus* isolates that were resistant and intermediately resistant to the selected antibiotics.

Bird species	Location	Bacteria	Resistant to	n
Greater Crested Tern	Gippsland Lakes	<i>E. coli</i>	Streptomycin	4
Greater Crested Tern	Gippsland Lakes	<i>E. coli</i>	Ceftazidime	1
Greater Crested Tern	Gippsland Lakes	<i>E. coli</i>	Streptomycin, amikacin	1
Greater Crested Tern	Gippsland Lakes	<i>E. coli</i>	Chloramphenicol	1
Greater Crested Tern	Gippsland Lakes	<i>Enterococcus</i>	Streptomycin, gentamicin, erythromycin	1
Greater Crested Tern	Gippsland Lakes	<i>Enterococcus</i>	Gentamicin, streptomycin, erythromycin, ciprofloxacin	2
Greater Crested Tern	Gippsland Lakes	<i>Enterococcus</i>	Gentamicin, streptomycin, erythromycin, vancomycin	1
Greater Crested Tern	Gippsland Lakes	<i>Enterococcus</i>	Gentamicin, streptomycin, erythromycin, ciprofloxacin, vancomycin	1
Varied Honeyeater	Torres Strait	<i>E. coli</i>	Streptomycin, amikacin, amoxicillin, ampicillin	1
Varied Honeyeater	Torres Strait	<i>E. coli</i>	Streptomycin, ampicillin.	1
		<i>Enterococcus</i>	Streptomycin, ampicillin, chloramphenicol, erythromycin, vancomycin, ciprofloxacin, gentamicin	
Rainbow Bee-eater	Torres Strait	<i>E. coli</i> ,	Streptomycin, amoxicillin, gentamicin, tetracycline, ampicillin.	1
		<i>Enterococcus</i>	Streptomycin, chloramphenicol, erythromycin	
Willie Wagtail	Torres Strait	<i>Enterococcus</i>	Ciprofloxacin, gentamicin, streptomycin, ampicillin, erythromycin, vancomycin	1
Horsfields Bronze-Cuckoo	Torres Strait	<i>Enterococcus</i>	Ampicillin	1

Sacred Kingfisher	Torres Strait	<i>Enterococcus</i>	Ciprofloxacin, gentamicin, streptomycin, ampicillin, erythromycin, vancomycin, chloramphenicol	1
Sacred Kingfisher	Torres Strait	<i>Enterococcus</i>	Ciprofloxacin, gentamicin, streptomycin, ampicillin, erythromycin, vancomycin tetracycline	1
Masked Booby	Adele Island	<i>Enterococcus</i>	Ciprofloxacin, Gentamicin, Streptomycin, Erythromycin, Vancomycin	1
Lesser Frigatebird	Adele Island	<i>Enterococcus</i>	Streptomycin, Erythromycin, Vancomycin	1

Figure 1. Locations of avian swab collections during the 2013-2015 period.