Antimicrobial resistant bacteria - *Escherichia coli*, *Klebsiella pneumoniae* carriage and transmission in avian species

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Thesis submitted in fulfilment of the requirements of the degree of Doctor of Philosophy

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Thesis Abstract

Antimicrobial resistance has emerged as an enormous threat to public health in the recent years. In particular, the Gram-negative Enterobacteriaceae family comprising several clinically important disease-causing species dominate reports of human infections have been frequently identified as carriers of genes conferring resistance to critically important antimicrobials. This further elevates the risk associated to human health as critically important antimicrobials are the last line of treatment available for treating serious life-threatening infections in humans and development of resistance towards this group of antimicrobials limits treatment options. One of the major human health crises with AMR is the propensity for Gram-negative bacteria to readily acquire and disseminate resistance genes further increasing the risk of bi-directional transmission of these bacteria into the wider environment and other animal species through anthropogenic activities or as zoonoses. This PhD thesis was designed to clarify the carriage and transmission of antimicrobial resistant bacteria in the wider environment especially amongst certain wild bird species that share proximity with humans, have access to remote ecological niches and interact with other avian species that are generally far from human contact.

The main highlights of the experiments performed and recorded in this thesis were to reveal some of the important aspects of how the resistant bacteria are hosted by the specific avian species and how they are transmitted further between the species. The study also focussed on understanding if anthropogenic activities and proximity to human populations can potentially influence the carriage rates of critically important antimicrobial (CIA) resistant bacteria. The other important aspect investigated in this study was the potential for commensal strain sharing between the different avian species that have different foraging habits but share a common environment.

The study initiated with investigation of the ecology, epidemiology and origins of CIA resistant *Escherichia coli* in Australian silver gulls. The Australia-wide cross-sectional survey of faecal flora of silver gulls demonstrated high levels of carriage of *E. coli*, resistant to two recognized critically important antimicrobials, extended spectrum cephalosporin (ceftriaxone) and fluoroquinolone

(ciprofloxacin), 21.7% and 23.8% respectively. Further, genomic investigation revealed that majority of the *E. coli* isolated from gulls largely belonged to human-associated extra-intestinal pathogenic *E. coli* clones.

The second study was designed to assess the implication of foraging and interspecies interaction of birds for carriage of *E. coli* demonstrated that gulls inhabiting areas of high anthropogenic activities can host, amplify and further disseminate the resistant *E. coli* strains to other bird species that share a common habitat for nesting and/or roosting. Genome sequencing results indicated that interspecies resistance transfer is facilitated by mobile genetic elements.

The third study was conducted to investigate, how proximity of gulls to areas with high anthropogenic activities impact the overall CIA resistant *E. coli* carriage level and if the gulls can also act as a potential reservoir of other CIA resistant human health associated bacteria. The study revealed that silver gulls are not limited to hosting and disseminating critically important antimicrobial resistant *E. coli* only. They can also serve as potential reservoir of yet another significant human health associated member of Enterobacteriaceae family, *Klebsiella pneumoniae* known to acquire and harbour critically important antimicrobial resistance genes. A noteworthy observation of the study was that the frequency of carriage of both *E. coli* and *K. pneumoniae* by the gulls diminished as the proximity of the gulls from the anthropogenic activity and densely human populated areas gradually increased, thus indicating that gulls inhabiting urban areas are more often exposed to critically important antimicrobial resistant bacteria compared to those inhabiting sparsely human populated or remote areas.

Furthermore, this study also investigated the potential of commensal *E. coli* strain sharing between three bird species, silver gulls, feral pigeons and little penguins that have different foraging habits but share the same environment. Although, the findings of this study indicated limited strain sharing of commensal *E. coli* and overall, very low levels of CIA resistance in the *E. coli* isolated from each bird species, this was attributed to not using enrichment and selective agar media to facilitate isolation of the resistant bacteria. This study also indicated that the resistant strains may not be dominant amongst the total *E. coli* population.

Overall, the key findings of these studies indicate and further substantiate that silver gulls are ecological sponges with a propensity to indiscriminately acquire and disseminate CIA resistant bacteria. The detection of human health associated clinically important strains of *E. coli* and *Klebsiella pneumoniae* from gulls also indicates that anthropogenic activities and proximity of these birds to urban areas that are densely populated by humans contributes to the carriage levels and frequency CIA resistant bacteria. The foraging sites of urban birds on human leftovers, from hospital effluents and wastewater treatment plants represent the major exposure sites from where the avian species can acquire CIA resistant bacteria. It is also noteworthy that although the urban birds are implicated as carriers of CIA resistant *E. coli,* these strains are not always dominant and are detected at low CFU. To further select and encourage growth of these underrepresented CIA resistant commensal *E. coli* strains from amongst the dominant *E. coli* population, use of enrichment and selective agar medium is highly recommended. Considering the low representation of CIA resistant strains amongst the dominant commensal population, it would be worthwhile carrying out further investigations to determine if the risk of exposure to humans has been overestimated.

Thesis Declaration

I certify that this work contains no materials which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no materials previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Acknowledgements

I take this opportunity to express my gratitude to all the important people who have been by my side and have supported me during this entire journey of PhD.

I would like to express my deepest gratitude to Dr Mary Barton and Dr Sam Abraham for putting their trust on me and for opening the door to this wonderful and forever mesmerizing world of knowledge. Words cannot express my gratitude to Dr Roy Kirkwood and Dr Mark O'Dea for their invaluable patience and feedback throughout my journey. I am deeply indebted to Dr Rebecca Abraham for her guidance and support during some of the important experimental work. This endeavour would not have been possible without the support of Dr James Nicholas Dunlop who was instrumental in samples collection for the experiments. I would like to express my deepest appreciation to Dr David Jordan, Dr Marc Stegger, Dr Shafi Sahibzada, Dr David Hampson for their guidance and suggestion during my writing work.

I am grateful to Dr Tanya Laird, Dr Zheng Zhou Lee, Dr Terence Lee, Samantha Gunasekera, Alec Vincent Truswell, Dr Ali Harb and Dr Bertha Rusdi for offering their help and supporting me at different stages during my PhD journey.

I am also thankful to my supervisors Dr Robert Smits, Dr Brian Luxford and Dr Rebecca Morrison at Rivalea Australia for encouraging, providing support and every assistance that I ever needed during the process of completing my thesis. I would like to extend my sincere thanks to my team members Mandy Clancy, Donna Nesbit and Salma Amani who always appreciated my work and encouraged me.

At personal level, I believe that none of this would have been possible without the support, encouragement, and affection of my family. I am thankful to my husband who stood beside me and supported me in every step of my life and my two little angels who are my biggest stress reliefs. Their belief in me kept me motivated during this entire journey.

Publications

1. Mukerji S, O'Dea M, Barton M et al. Development and transmission of antimicrobial resistance among Gram-negative bacteria in animals and their public health impact. Essays Biochem 2017; 61: 23–35.
2. Mukerji S, Stegger M, Truswell AV et al. Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. *J Antimicrob Chemother* 2019;74(9):2566-2574.
3. Mukerji S, Gunasekera S, Dunlop JN et al. Implications of foraging and interspecies interactions of birds for carriage of *Escherichia coli* strains resistant to critically important antimicrobials. *Appl Environ Microbiol* 2020;86(20): e01610-20.
4. Mukerji S, Sahibzada S, Abraham R et al. Proximity to human settlement is directly related to carriage of critically important antimicrobial-resistant *Escherichia coli* and *Klebsiella pneumoniae* in Silver Gulls *Vet Microbiol* 2023; 280: 109702.doi: 10.1016/j.vetmic.2023.109702.

Oral Presentation (selected from abstract submission)

Mukerji S, Dunlop N, Laird T, Abraham R J, Barton M, O'Dea M, Abraham S. Transmission of critically important antimicrobial resistant *E. coli* between Silver Gulls, Feral Pigeons and Little Penguins occupying different ecological niches within an urban environment. Australian Society of Antimicrobials, Annual Scientific Meeting, Adelaide 2019.

Background

Several studies have provided evidence that wildlife can be an important indicator of antimicrobial resistance in bacteria in the environment. Due to their wider access to the environment, wildlife have the potential to acquire and disseminate resistance within and between different species. Studies have also highlighted the potential of anthropogenic inputs contributing to the carriage of antimicrobial resistant bacteria in wildlife. Avian species are considered to play a key role as vectors in carrying and spreading antimicrobial resistant bacteria. Various studies across the globe have detected many clinically significant humans associated pathogenic bacteria such as *E. coli* and *Klebsiella pneumoniae* strains in avian species. Gulls have been implicated as major carriers of these resistant bacterial strains. The resistance genes detected included ESBLs, AmpCs, OXA-48, mcr-1, fluoroquinolone resistance genes thus resulting in these bacteria demonstrating resistance against the critically important antimicrobials (CIA) that are available as last resort for treating severe life-threatening infections in human.

CIA resistant *E. coli*, *K. pneumoniae* have been detected in various bird species including gulls, eagle-owl, lesser kestrel, common buzzard, cattle egret but the carriage of antimicrobial resistant bacteria in gulls has been studied most extensively. This is because gulls are often found to be inhabiting areas that are densely populated by humans. Also, being migratory birds, they often share roosting location with other bird species. Furthermore, though gulls are supposedly opportunistic marine feeders, but they have also been found to scavenge on human leftovers, wastewater treatment plants and hospital effluents which elevates their chances of exposure to resistant bacteria. Gulls are also frequent visitors to agricultural land, potentially interacting with the livestock and food producing animals that occupy these locations. The combination of all these above factors and their accessibility to the wider environmental network including densely populated areas as well as remote locations make them a preferred choice of avian species to study the acquisition, amplification and dispersion of the CIA resistant bacteria that are of concern from public health perspective.

Hypothesis:

1) This project hypothesised that Australian silver gulls play a key role in acquiring, amplifying and disseminating CIA resistant *E. coli*.
2) The silver gulls can potentially transmit these CIA resistant bacteria to other bird species that congregate and interact in a common shared environment.
3) The anthropogenic activities and proximity to human plays an important role in determining the level and frequency of CIA resistant *E. coli* and other clinically significant human associated lineages of Gram-negative bacteria by the silver gulls.
4) As with transmission of CIA resistant *E. coli,* the commensal *E. coli* strains that inhabit the gut of silver gulls are shared between different bird species sharing a common environment.

Objectives:

The overall objectives of the experiments performed in this thesis were

1) To investigate the ecology, epidemiology and origins of CIA resistant *E. coli* strains carried by Australian sliver gulls and perform a comparative analysis from a global perspective.
2) Furthermore, to determine if these resistant strains and their resistance-encoding mobile genetic elements (MGE) can be transmitted to other bird species that interact with silver gulls.
3) To investigate if proximity to human settlement can influence the carriage frequency of CIA resistant *E. coli* and other clinically significant human associated Gram-negative bacteria such as *Klebsiella pneumoniae*.
4) The final objective was to determine whether just like the resistant pathogenic *E. coli* clones, the commensal *E. coli* strains are also shared between bird species during their interactions while inhabiting a common environment.

CHAPTER 1

Development and transmission of antimicrobial resistant among Gram-negative bacteria in animals and their public health impacts.

(LITERATURE REVIEW)

Statement of Authorship

Title of Paper	Development and transmission of antimicrobial resistance among Gram-negative bacteria in animals and their public health impact
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Shewli Mukerji, Mark O'Dea, Mary Barton, Roy Kirkwood, Terence Lee and Sam Abraham. Development and transmission of antimicrobial resistance among Gram-negative bacteria in animals and their public health impact. Essays in Biochemistry (2017) 61 23–35 DOI: 10.1042/EBC20160055

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Name of Principal Author (Candidate)	Shewli Mukerji		
Contribution to the Paper	Performed the available literature search on the topic. Did the compilation, analysis and interpretation of the already available data. Wrote the manuscript. Responded to comments and suggestion of co-authors and journal editor.		
Overall percentage (%)	94%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14th June 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Signature		Date	8/7/2022

Essays in Biochemistry (2017) **61** 23–35
DOI: 10.1042/EBC20160055

Review Article

Development and transmission of antimicrobial resistance among Gram-negative bacteria in animals and their public health impact

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Gram-negative bacteria are known to cause severe infections in both humans and animals. Antimicrobial resistance (AMR) in Gram-negative bacteria is a major challenge in the treatment of clinical infections globally due to the propensity of these organisms to rapidly develop resistance against antimicrobials in use. In addition, Gram-negative bacteria possess highly efficient mechanisms through which the AMR can be disseminated between pathogenic and commensal bacteria of the same or different species. These unique traits of Gram-negative bacteria have resulted in evolution of Gram-negative bacterial strains demonstrating resistance to multiple classes of antimicrobials. The evergrowing resistance issue has not only resulted in limitation of treatment options but also led to increased treatment costs and mortality rates in humans and animals. With few or no new antimicrobials in production to combat severe life-threatening infections, AMR has been described as the one of the most severe, long-term threats to human health. Aside from overuse and misuse of antimicrobials in humans, another factor that has exacerbated the emergence of AMR in Gram-negative bacteria is the veterinary use of antimicrobials that belong to the same classes considered to be critically important for treating serious life-threatening infections in humans. Despite the fact that development of AMR dates back to before the introduction of antimicrobials, the recent surge in the resistance towards all available critically important antimicrobials has emerged as a major public health issue. This review thus focuses on discussing the development, transmission and public health impact of AMR in Gram-negative bacteria in animals.

Introduction

Gram-negative bacteria are a part of the commensal microflora of mammals and typically colonize the gastrointestinal tract of humans and animals. However, some of the species cause opportunistic infections in both humans and animals [1]. Enterobacteriaceae are a particularly important family of Gram-negative bacteria containing disease-causing species such as *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Proteus* spp., *Enterobacter* spp., *Citrobacter* spp. and *Yersinia pestis*. In addition, other Gram-negative species such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Campylobacter* spp. are commonly involved in disease and some environmental organisms such as *Acinetobacter* spp. can also cause serious infections in susceptible hosts [1,2]. The ability of Gram-negative bacteria to rapidly develop and disseminate resistance between the same as well as different strains contributes to these organisms emerging as a potential threat to human and animal health. Table 1 outlines many of the important diseases caused by Enterobacteriaceae, the majority of which are clinically managed with antimicrobial treatment.

Received: 03 November 2016
Revised: 26 January 2017
Accepted: 27 January 2017

Version of Record published:
3 March 2017

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Table 1 Major Enterobacteriaceae species responsible for human and animal infections[1]

Gram-negative bacteria	Human infections	Animal infections
E. coli	Hospital-acquired pneumonia, gastroenteritis, septicaemia, neonatal meningitis and urinary tract infection	Young animals: enteric colibacillosis (pigs), neonatal diarrhoea, septicaemia or toxaemia. Adult animals: urinary tract infection (dogs), mastitis (cattle) and endometritis (horse)
Enterobacter spp.	Septicaemia, urinary tract infection	Occasional mastitis in cows and sows
K. pneumoniae	Pneumonia, lung abscess, meningitis, otitis externa, urinary tract infection	Occasional mastitis (with severe inflammatory response)
Salmonella serotypes	Typhoid, diarrhoea, gastroenteritis	Enteritis, septicaemia and abortion in cattle, salpingitis in poultry, diarrhoea in chicks. Enterocolitis, septicaemia in pigs, horses, dogs and sheep
Shigella spp.	Shigellosis (bacillary dysentery)	Not reported

[1]Based on the data from references [1-3].

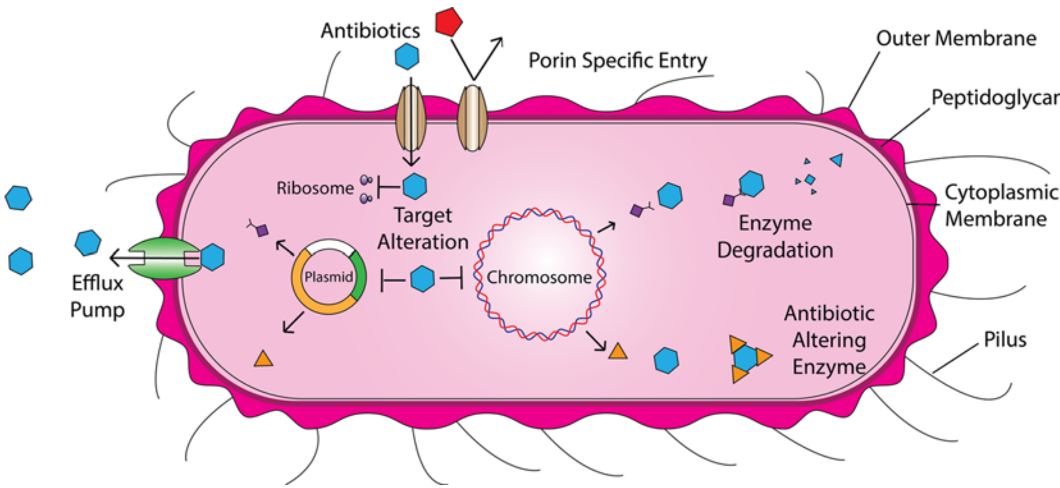

Figure 1. Illustration of major antimicrobial resistance (AMR) mechanisms utilized by Gram-negative bacteria – enzymatic degradation (examples – penicillin and cephalosporins), target alteration (example – fluoroquinolones), porin specificity and efflux pumps (examples – tetracycline, cephalosporins, fluoroquinolones and penicillins)

Antimicrobial treatment

The modern era of clinical use of antimicrobials began with Paul Ehrlich and Salvarsan in 1907 [4]. The discovery of penicillin by Sir Alexander Fleming and sulfonamides during the 1930s further accelerated the clinical use of antimicrobials [5]. The advent of antimicrobials enabled effective treatment of potentially fatal infections and revolutionized clinical treatment procedures in humans. The use of antimicrobials also led to emergence of AMR within the span of a few years and to overcome this issue, newer antimicrobial classes were introduced [6,7]. With the success of combating bacterial infections in humans, antimicrobials were then deployed to treat and control bacterial infections in animals. Table 2 lists the different antimicrobials available to treat Gram-negative bacterial infections in humans and animals. With the extensive use of antimicrobials in humans and animals, bacteria developed resistance to evade the effect of commonly used antimicrobials.

Mechanisms of AMR in Gram-negative bacteria

Gram-negative bacteria, particularly within the Enterobacteriaceae family, have developed several mechanisms to evade the effects of antimicrobials. Some strains possess multiple mechanisms resulting in multidrug resistance (Figure 1). The mechanisms that can lead to resistance include:

(i) Enzymatic degradation or modification**:** This mechanism involves the inactivation of antimicrobials with specific enzymes secreted by the bacteria, such as β-lactamase enzymes that hydrolyse the β-lactam ring thus

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Essays in Biochemistry (2017) **61** 23–35
DOI: 10.1042/EBC20160055

Table 2 Importance ratings and summary of use of common antimicrobials available to treat Gram-negative bacterial infections. Importance ratings are based on the WHO and Australian Antimicrobial Resistance Standing Committee ranking[1]

Antimicrobial class	WHO	AMRSC	Drug name	Use in humans	Use in animals in Australia (AVA)
Penicillin	CI	Low	Amoxycillin, ampicillin	Respiratory tract infections, surgical and endocarditis prophylaxis	Used to treat a range of infections in pigs, poultry, cattle, sheep, horses, cats and dogs
Penicillin and β-lactamase inhibitor	CI	Medium	Amoxycillin–clavulanate	Surgical prophylaxis, Gram-negative, aerobic and anaerobic infections	Used in pigs, cattle, sheep, horses, dogs and cats
		High	Ticarcillin–clavulanate, piperacillin–tazobactum	Treatment of *Pseudomonas aeruginosa* infections, mixed aerobic and anaerobic infections, skin and soft tissue infection	Not registered
Second generation cephalosporins and cephamycins	HI	Medium	Cefaclor, cefuroxime–Axetil, cefoxitin	Alternative for patients allergic to penicillin. Used for surgical prophylaxis and respiratory tract infection. Cephamycins have anti-anaerobe activity	Cefuroxime is used as second line mastitis treatment in cattle
Third and fourth generation cephalosporins	CI	High	Ceftriaxone, cefotaxime ceftazidime cefepime	Severe pneumonia, meningitis, pseudomonal infections, neutropenic sepsis	Ceftiofur is registered for treatment of respiratory disease in cattle and it is used off-label in other livestock; cefovecin is used in companion animals
Carbapenems	CI	High	Imipenem, meropenem, doripenem ertapenem	Treatment of multi-resistant, serious Gram-negative and mixed infections	Not registered
Monobactams	CI	High	Aztreonam	Resistant Gram-negative infections and also used as an alternative for patients with severe β-lactam allergy	Not registered
Tetracyclines	HI	Low	Doxycycline, minocycline	Minor respiratory tract infections and supportive in the treatment of pneumonia	Used in livestock and companion animals
Glycylcyclines	CI	High	Tigecycline	Mostly used for treating multi-resistant Gram-positive infections and some Gram-negative infections	Not registered
Aminoglycosides	CI	Medium	Gentamicin	In combination, used in the treatment of pseudomonal infection, endocarditis (gentamicin)	Gentamicin is used in horses, cats and dogs – not registered for livestock
		High	Amikacin	Treatment of Gram-negative organisms resistant to gentamicin, tobramycin	Not registered
Quinolones	CI	High	Norfloxacin, ciprofloxacin, moxifloxacin	Used in treating complicated urinary tract infections. Ciprofloxacin is an oral treatment for resistant Gram-negative bacteria. Moxifloxacin is used for managing serious respiratory tract infections in patients allergic to penicillin	Not registered for livestock. Marbofloxacin, enrofloxacin registered for use in dogs and cats; enrofloxacin is used off-label in horses
Polymyxins	CI	High	Polymyxin B, colistin	Topical application of polymyxin B helps to treat Gram-negative infections. Colistin is reserved for the treatment of multi-resistant Gram-negative infections	Polymyxin B is used topically in cattle, sheep, horse, dogs and cats

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Table 2 Importance ratings and summary of use of common antimicrobials available to treat Gram-negative bacterial infections. Importance ratings are based on the WHO and Australian Antimicrobial Resistance Standing Committee ranking[1] (Continued)

Antimicrobial class	WHO	AMRSC	Drug name	Use in humans	Use in animals in Australia (AVA)
					Colistin is not registered
Phenicols	HI	Low	Chloramphenicol	Used in topical-eye preparation and occasionally for treatment of bacterial meningitis	Not registered for use in food producing animals
Macrolides[2]	CI	Low	Erythromycin	Treatment of minor Gram-positive infections	Used in treating pigs, cattle, sheep
Sulfonamides and DHFR inhibitors	HI	Medium	Trimethoprime/ sulfamethoxazole	Minor infections, treatment and prophylaxis of UTI	Used in treating pigs, poultry, cattle, horse, dogs and cats

AMRSC, Australian Antimicrobial Resistance Standing Committee; AVA, Australian Veterinary Association Limited; CI, critically important; DHFR, dihydrofolate reductase; HI, highly important.
[1]Based on the data from references [8-10].
[2]Macrolides are classified under critically important category as per WHO rating due to their effectiveness against *Campylobacter* infections particularly in children.

inactivating penicillins and cephalosporins [11]. Examples of enzymes that lead to enzymatic degradation of cephalosporins are AmpC β-lactamases, extended-spectrum-β-lactamases (ESBLs) and carbapenemases [12].

(ii) Target modification**:** This mechanism of resistance occurs through alteration of the target site of the antimicrobial, which in turn prevents the binding of the antimicrobial to the bacterial cell thus disabling the antimicrobial activity [12]. Fluoroquinolone resistance as a result of modification to the gene that encodes the enzymes DNA gyrase and DNA topoisomerase IV, the two main targets of the antimicrobial, is an example of target modification and structural gene mutation (point mutation) [12-14].

(iii) Reduction in cell permeability and expression of efflux pumps: Altering cell permeability or increasing the efflux action is another mechanism of resistance observed in Gram-negative bacteria. The outer membrane structure of Gram-negative bacteria is unique due to the presence of outer membrane proteins or porins, which are selective and help in regulating or restricting the entry of harmful substances into the cell. The antimicrobials have porin specificity and are required to pass through the water-filled channels to gain entry into the target sites [15]. Reduction or loss of porin changes the cell wall permeability and limits or prevents the entry of antimicrobials into the cell [12]. Due to this mechanism the antimicrobial agent cannot reach desirable concentrations within the bacterial cell and antimicrobial activity is inhibited. The efflux mechanism prevents the antimicrobial agent from achieving bactericidal concentration within the bacterial cell by removing the antimicrobial from the cell [12]. An increase in efflux activity can be due to chromosomally encoded efflux pumps such as resistance-nodulation division (RND) efflux pumps within the bacterial cell or by acquisition of specific genes (often plasmid encoded), an example being tetracycline resistance [16,17]. RND efflux systems ArcAB-TolC of *E. coli* and MexAB-OprM of *Pseudomonas aeruginosa* have been studied widely and are known to be effective against wide range of antimicrobials including cephalosporins, fluoroquinolones, penicillins and chloramphenicol [17,18].

(iv) Modification of metabolic pathway: Alterations or changes in the metabolic pathways (by secreting modified enzymes) within the bacterial cell can also result in resistance to an antimicrobial agent. Genes encoding such enzymes are often found on plasmids. Trimethoprim resistance is an example of this mechanism where the target site of trimethoprim is altered and bypassed by synthesis of the plasmid mediated additional trimethoprim-resistant DHFR [19,20].

Despite being accelerated by antimicrobial use, AMR is also a natural phenomenon in bacteria and resistance to antimicrobials in Gram-negative bacteria can either be an inherent characteristic or an acquired trait [21]. It is well documented that many resistance mechanisms emerged in bacteria long before antimicrobials were first used, for example bacterial penicillinase was identified before penicillin was introduced to the market [22]. It is worth noting that in studies of DNA extracted from 30000 years old permafrost cores, resistance genes to β-lactams, tetracycline and vancomycin (a last-line antimicrobial for treating multidrug-resistant, Gram-positive bacterial infections) were

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Essays in Biochemistry (2017) **61** 23–35
DOI: 10.1042/EBC20160055

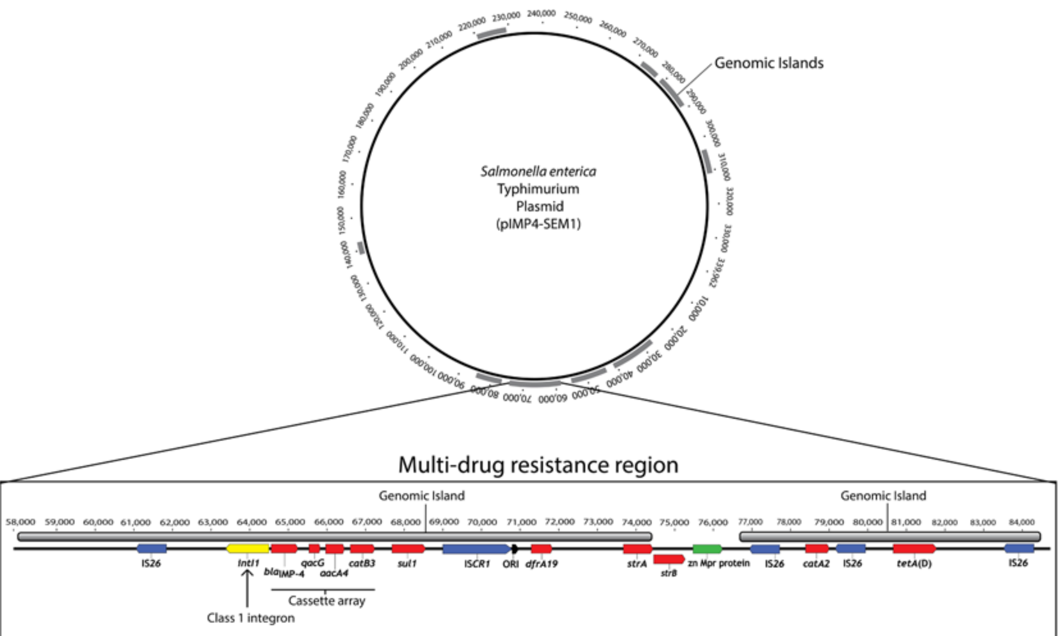

Figure 2. Diagrammatic representation of recently identified IncHI2 plasmid (pIMP4-SEM1) from *Salmonella enterica* Typhimurium in cat with multidrug-resistant region (red), class 1 integron (yellow) and insertion sequences (IS) (blue) adopted from [30]

found [23]. Multidrug-resistant bacteria were also discovered in caves that had been isolated for millions of years [24]. The acquisition of AMR can be either through gene mutation or via horizontal gene transfer [21].

Horizontal gene transfer and mobile genetic elements

Horizontal gene transfer is the mechanism by which foreign DNA is acquired or transferred among bacteria and is one of the major mechanisms, by which bacteria acquire and transfer AMR. Horizontal gene transfer is facilitated by mobile genetic elements such as plasmids, transposons and integrons. The mobile genetic elements play a vital role in dissemination of AMR since these genetic structures have the ability to transfer multiple genes responsible for resistance to different classes of antimicrobials in a single event [5,12,25].

Plasmids

Plasmids are extrachromosomal, autonomous mobile genetic elements and are separate from bacterial chromosomes. Conjugative plasmids may serve as a carrier for several bacterial genes including resistance-conferring genes from one bacterial cell to other [25].

Transposons

Transposons, also referred to as jumping genes, are genes that can move within, attach to or detach from plasmids or bacterial chromosomes. Transposons can carry resistance genes that can ride on plasmids for transfer between bacterial cells thus disseminating resistance. Since, transposons generally do not have specific affinity or preference for a binding site on the plasmid or chromosome, this is a highly efficient and effective mechanism of resistance gene transfer [14].

Integrons

Integrons are another mobile genetic element that can capture AMR genes. Integrons are structures that integrate gene cassettes that are transcribed in the integron from a promoter [26]. An integron comprises an *intI* gene, *attI* recombination site and a Pc promoter (Figure 2). The gene cassettes responsible for conferring AMR are integrated at specific sites on the integron. The gene cassettes comprise a gene, a ribosome-binding site and an *attC* recombination site and retain a conserved region at their ends. Antimicrobial-resistant species can possess hundreds of such gene

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DOI: 10.1042/EBC20160055

cassettes resulting in multi-resistance [27], for example the class 1 RI with gene cassette array *dfrA 15-aadA1* in *Shigella dysenteriae* demonstrates resistance against streptomycin, spectinomycin and trimethoprim [28].

Multidrug-resistance regions

Multi-resistant regions are collections of resistance genes that were originally located on the chromosomes of different and unrelated bacterial species merging in a single position on a chromosome or plasmid of the recipient bacterial cell [27]. The mobile genetic elements that transfer these resistance genes between DNA molecules such as IS, transposons, integrons and gene cassettes are known as integrative and mobile elements, an example being the SGI1 region in *Salmonella* spp. and AbaR in *Acinetobacter baumannii* [27]. The multidrug-resistance regions (MRRs) carry genes that confer resistance to multiple classes of antimicrobials including critically important antimicrobials and are a key factor in the rapid dissemination of AMR in both humans and animals [29] (Figure 2). A recently discovered plasmid isolated from *S. enterica* Typhimurium, pIMP4-SEM1, found in a cat in Australia is an example of a plasmid that includes a class I integron and several MRRs and heavy metal resistances. This IncHI2 plasmid (pIMP4-SEM1) carried resistance to nine antimicrobial classes including carbapenems (critically important antimicrobials). The carbapenem-resistance gene (bla_{IMP-4}) was on a $bla_{\text{IMP-4}}$-*qacG-aacA4-catB3* cassette array (Figure 2) associated with class 1 integron [30].

Development and transmission of AMR in animals

In animals, antimicrobials are used for the treatment of specific bacterial infections, prophylaxis and growth promotion by minimizing bacterial infections or selecting certain species of gut bacteria, which increases weight gain. Some countries have banned the use of antimicrobial growth promotants, some allow a restricted range and others have limited or no controls over such use [32]. Many of the antimicrobials used for prophylaxis and therapeutic purposes in animals belong to the same classes used in human medicine. As a result, the AMR seen among bacterial isolates from animals becomes a public health concern as these antimicrobial-resistant bacteria can transfer easily from animal to human via direct contact or via the food chain [33].

Figure 3 shows the different routes of AMR transmission between human and animals. The major transmission routes of AMR between humans and animals are by direct contact or through the food chain. Antimicrobial resistant zoonotic bacteria such as Shiga-toxin producing *E. coli* (STEC), *Salmonella* spp. and *Campylobacter* spp. may transfer between hosts and cause infections [33]. In addition, non-pathogenic commensal *E. coli* that are resistant to antimicrobials may transfer and disperse AMR genes to human pathogens by horizontal gene transfer. Mobile genetic elements such as plasmids, transposons and integrons that carry AMR genes can move from humans to animals and vice versa via the environment [34].

The movement of food animals and animal products across continents further extends the spread of AMR thus making it an issue not just for an individual country but a worldwide concern [35]. Aside from routes such as international movement of people and food products, migratory birds are also considered to play a role in the transmission of antimicrobial-esistant bacteria among continents [36]. Another possible transmission route is the movement of AMR from humans to animals. There are reported cases where humans that work with animals are likely to have transferred resistant bacteria to animals, particularly wildlife but also possibly pets and food-producing animals. This has been demonstrated in a study where the isolation of multiresistance was found to be the highest in pets followed by farm animals and lowest in wild animals [37,38]. In animal production systems, antimicrobial-resistant bacteria can be amplified due to antimicrobial selection pressure from the various uses of antimicrobials. Once amplified, these antimicrobial-resistant bacteria can pose a potential public health risk to humans as indicated. In addition to food animals, use of antimicrobials in companion animals (dog, cat, horse) has also contributed to the movement of resistant bacteria to humans [39].

Resistance to critically important antimicrobials

One of the major concerns in the transmission of AMR between animals and humans is the transmission of resistance to critically important antimicrobials. Due to the emergence of multidrug-resistant bacteria that cannot be treated with first-line antimicrobials, the last-line drugs (critical) should be preserved to treat infections caused by resistant bacteria. As a result, ranking parameters for antimicrobials have been developed and are regularly revised by the World Health Organization (WHO) and other bodies [10]. The antimicrobial rankings have been developed from a human health perspective and require adherence to strict guidelines when used for infection management in food animals. According to the WHO guidelines [10], antimicrobials are rated as: (i) critically important when they are the only treatment option available for treating severe life-threatening infections in humans and do not have any

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Essays in Biochemistry (2017) **61** 23–35
DOI: 10.1042/EBC20160055

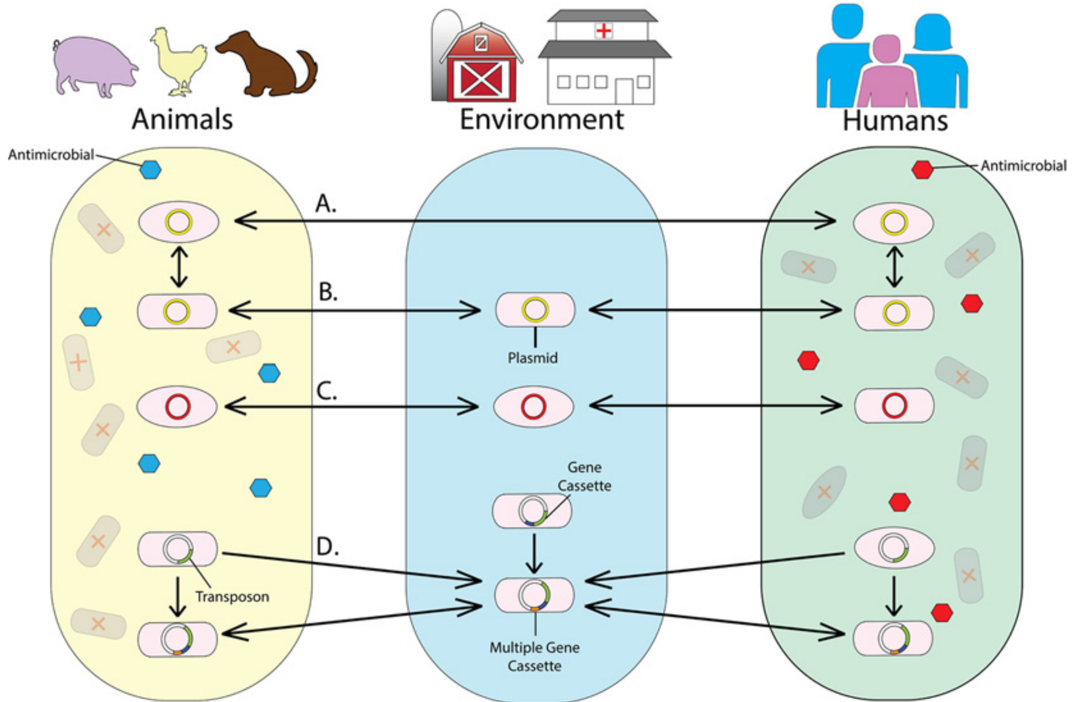

Figure 3. Transmission of AMR between human and animals – (A) Direct transmission of antimicrobial-resistant bacteria between human and animals. (B) Transmission of resistant determinant genes encoded either on chromosomes or plasmids. (C) Mobile genetic elements (plasmids) carrying and disseminating resistance genes. (D) Transmission of resistance via transposons, IS, integrons assembled on gene cassettes and multi-resistant regions carrying array of gene cassettes resulting in multiple AMR in bacteria within same host or between animal and humans. Illustration adopted from [31]

Table 3 Major critically important antimicrobials: targets, transmissible AMR genes and mechanisms

Critically important antimicrobials	Target	Transmissible AMR genes[1]	Resistance mechanism
Third generation cephalosporins	Peptidoglycan synthesis	bla_{TEM}, bla_{SHV}, $bla_{CTX\text{-}M}$, bla_{CMY}	Enzymatic degradation, altered target, efflux
Carbapenem	Peptidoglycan synthesis	bla_{VEB}, bla_{KPC}, bla_{GES}, bla_{OXA}, bla_{IMP}, bla_{VIM}, bla_{NDM}	Enzymatic degradation, altered target, efflux
Fluoroquinolones	DNA replication	*qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrVC*, *aac(6′)-Ib-cr*, *qepA*	Target modification, efflux, inactivation
Colistin	Outer and cytoplasmic membrane	*mcr*	Target modification, efflux

[1] All resistance genes mentioned are plasmid-borne genes.

alternatives or substitutes, (ii) highly important antimicrobials form the second line therapy and have limited but alternative options in case of resistant infections and (iii) important antimicrobials are the first-line therapy but due to their overuse have marked resistance issues [33].

The major critically important antimicrobials used to treat multidrug-resistant Gram-negative bacteria include fluoroquinolones, extended-spectrum cephalosporins, carbapenems and colistin [10]. The macrolide class of antimicrobials has also recently been classified as critically important by the WHO [10]. Although macrolides are predominantly used for treating infections caused by Gram-positive bacteria, they have also been found to be highly effective against certain Gram-negative bacteria such as *Campylobacter* spp. Macrolides are the preferred choice of treatment over fluoroquinolones for serious *Campylobacter* infections [10], especially in children. However, since it is generally accepted that macrolides have very limited use in treating infections caused by Gram-negative bacteria, further examination of resistance mechanisms against macrolides is not discussed in this review. Table 3 lists the different critically important antimicrobial classes and the corresponding bacterial genes conferring resistance to each class.

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DOI: 10.1042/EBC20160055

Fluoroquinolone resistance

Fluoroquinolones are used for the treatment of various infections including urinary tract, respiratory tract and gastrointestinal infections, but excessive use of this class of antimicrobials due to their safety and convenience of dosing has resulted in increased resistance issues. Enrofloxacin for animal treatment was first introduced in Europe and the United States of America in the late 1980s and resistance was first reported in veterinary isolates of *Salmonella* spp. in the mid 1990s [40]. A study of *Campylobacter* spp. isolated from humans and poultry showed increase in resistance to enrofloxacin from 0 to 11% and 0 to 14% respectively, between 1982 and 1989 [41]. Subsequent studies have reported a correlation between use of fluoroquinolones in food-producing animals and emergence of resistance in human infections [42,43]. Plasmid-borne fluoroquinolone resistance has now been found in animal isolates [44,45]. High-level fluoroquinolone resistance is conferred due to mutation in chromosomal quinolone resistance determining regions (QRDRs) DNA gyrase (*gyrA, gyrB*) and DNA topoisomerase IV genes (*parC, parE*) [46]. Studies have also demonstrated plasmid-mediated quinolone resistance by *qnr* genes [46]. Seven fluoroquinolone resistant genes have been identified and reported so far – *qnrA*, *qnrB*, *qnrS*, *qnrC*, *qnrD* and *qnrVC*. In addition to *qnr* genes, the presence of aminoglycoside resistance gene *aac(6′)-Ib* and also an efflux pump *qepA* also can result in quinolone resistance [47]. Importantly, it has been observed that the plasmids carrying *qnr* genes are often carriers of genes for ESBLs thus conferring multidrug resistance to two critically important antimicrobials to the members of the Enterobacteriaceae family [48-50]. Human self-medication in developing countries and overuse of fluoroquinolones in food animals has led to further emergence and dissemination of resistance. One study evaluating the impact of fluoroquinolone use in swine, poultry, human and various environmental sources within farms in China reported very high prevalence of resistance [51].

Extended spectrum cephalosporin resistance

Third generation cephalosporins are classified as critically important antimicrobials and are often used to treat several life threatening infections in humans [10,52]. Apart from overuse of third generation cephalosporins in human medicine, another contributing factor that has led to further emergence and dissemination of resistance is the veterinary use of ceftiofur, an antimicrobial belonging to the same class. Ceftiofur was first introduced in the United States of America in 1998 following its earlier release in Europe. It has been demonstrated that ESBL producing human strains of *E. coli* and *K. pneumoniae* were resistant to ceftiofur and that there is a high correlation between resistance to ceftriaxone (a human third generation cephalosporin) and ceftiofur in these isolates [53]. Ceftiofur resistance was first detected in *Salmonella* isolates in Canada in the late 1990s [54] and in the United States of America at around the same time [55]. It was also recognized that ceftiofur resistant animal isolates produced AmpC (CMY-2) and ESBL enzymes [56,57]. The first gene reported to be responsible for ceftiofur resistance was $bla_{\text{CMY-2}}$ [58]. Studies have reported that use of ceftiofur in food-producing animals not only exerts selection pressure for development of ceftiofur resistance but can also select for resistance to ceftriaxone, a critically important human antimicrobial in enteric *E. coli* and *Salmonella* spp. [59]. This is due to the fact that the genes that encode resistance to ceftiofur can also provide resistance to other third generation cephalosporins such as ceftriaxone ($bla_{\text{CMY-2}}$, $bla_{\text{CTX-M}}$). On the basis of Ambler classification, β-lactamases can be classified into four classes; class A serine β-lactamases (ESBLs, penicillinases), class B metallo-β-lactamases, class C AmpC-type-β-lactamases and class D OXA β-lactamases [60,61]. The most common and widespread β-lactamases are the ESBL cephalosporinases (CTX-M type enzymes), which confer resistance to nearly all penicillins and cephalosporins [62]. *E. coli* (ST131) is a highly virulent extraintestinal pathogen (ExPEC) belonging to a pathogenic subclone *H30*-Rx and has emerged as a major threat globally within a very short time span. The fluoroquinolone resistance and ESBL production resulting in multidrug resistance in *E. coli* (ST131) is attributed to a CTX-M-15 encoding mobile genetic element [63]. Other β-lactamases frequently encountered in Enterobacteriaceae can be chromosomal (AmpC) or plasmid mediated (pAmpCs) enzymes. CMY is the most frequently detected AmpC type β-lactamase among Gram-negative bacteria worldwide [64-66].

Carbapenem resistance

Carbapenems are the last-line therapy for treatment of multidrug-resistant infections in humans. Failure of treatment with penicillin and cephalosporin has led to the extensive use of carbapenem in humans, which in turn has resulted in emergence of carbapenem resistance [67]. Although *K. pneumoniae* was the first pathogen detected to harbour the plasmid-encoded carbapenemases, there has been a steady surge of carbapenem resistance in *E. coli* and other members of the Enterobacteriaceae [68,69]. Apart from KPC, VIM, IMP and OXA-48 the most widely disseminated extensively drug resistant Gram-negative bacteria are the NDM-1 producing strains that are resistant to a number of

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Essays in Biochemistry (2017) **61** 23–35
DOI: 10.1042/EBC20160055

other classes of antimicrobials. Resistance to carbapenems is more frequent in humans but previously several studies have reported detection of carbapenemase producing *E. coli*, *Salmonella* spp. and *Acinetobacter* spp. in food animals (poultry, cattle and pigs) and companion animals (dogs, cats and horses) [67,70]. It should be noted that carbapenems are not registered for use in animals although off-label use occurs in cats and dogs. The source of resistance in livestock isolates requires investigation. The diversity of carbapenemase enzymes allows them to confer resistance against all cephalosporins including carbapenems. The various categories of carbapenemases are class D enzymes (OXA family) found in *Enterobacter* and *P. aeruginosa*, metallo-β-lactamases (IMP, VIM family) observed predominantly in *Klebsiella* and *Enterobacter* spp. and non-metallo-carbapenemases (SME, IMI/NMC) in *Serratia* and *Enterobacter* spp. [64]. The KPC enzymes in *K. pneumoniae* and *E. cloacae* are of major concern as they confer broad-spectrum resistance against all β-lactams including cephalosporins, monobactams and carbapenems [11]. The rise in carbapenem resistance in Enterobacteriaceae is of major concern as the members of this family are most frequently encountered in healthcare and community infections thus increasing the chances of further dissemination of carbapenem resistance in the community [71]. The plasmid-mediated carbapenemases, MBLs (IMP, VIM), are more significant in the healthcare sector worldwide when compared with chromosomal carbapenemases (IMI, NMC-A and SME) due to their inherent mobility. Studies in the past have reported NDM-1 producing *E. coli*, OXA-48 producing *E. coli* and *K. pneumoniae* in companion animals [72,73]. Further, a recent study in Australia reported carbapenemase (IMP-4) producing *S. enterica* Typhimurium from cats [30]. These findings imply that companion animals serve as potential reservoirs to enable further dissemination of carbapenemase-producing Gram-negative bacteria within the community thus making it imperative that this critically important antimicrobial be used judicially in veterinary medicine [74].

Colistin resistance

Once seldom used for treating human infections due to side effects such as nephrotoxicity [75], increased resistance demonstrated by Gram-negative bacteria towards critically important antimicrobials has led to increased reliance on polymyxin antimicrobials, polymyxin B and polymyxin E (colistin), as a last resort [76]. Colistin is predominantly used in intensive care units in intravenous administrations. Colistin is also extensively used for treating *E. coli* and *Salmonella* in addition to use as a growth promotant in pigs and poultry in some countries [70,71]. Colistin appears to have been used since the 1960s in pigs and poultry but the first resistance was reported in poultry in 2001 [77] and in pigs in 2005 [78]. It is reported to be chromosomally mediated by increased enzyme activity that results in modification of bacterial lipopolysaccharide (LPS) thus reducing the binding affinity of colistin [76]. A recent study identified and reported the plasmid mediated resistance gene *mcr-1* in *E. coli* isolated from animals and humans [76]. Another study reported a different plasmid mediated resistance gene *mcr-2* in porcine and bovine *E. coli* isolates [79]. Following the first detection of *mcr-1* in isolates from food animals in China many countries have reported the presence of *mcr-1* in isolates from human, animals and the environment [80]. In cases of human isolates, the resistance rates were highest in Asia followed by Europe and America [81]. Switzerland, China, Malaysia and France have reported *mcr-1* in environmental samples, while 28 countries have reported presence of *mcr-1* in isolates from food and other animals [80]. Since colistin is used as the last-line therapy for treating infections caused by carbapenemase-producing Enterobacteriaceae, the major implication of subsequent acquisition of *mcr-1* and *mcr-2* by these organisms is that it would make them potentially pan-drug resistant [76]. Studies have also reported coexistence of *mcr-1* with $bla_{\text{NMD-1}}$ in certain *E. coli* strains thus indicating that evolution of such strains can lead to resistance to all critically important antimicrobials [82].

Limiting critically important antimicrobial-resistant Gram-negative bacteria in livestock: a success story from Australia

Previous studies have suggested that the ecology of critically important AMR among Enterobacteriaceae isolated from Australian food-producing animals differs from that in other parts of the world [83,84]. This is attributed to Australia's unique geography, quarantine restrictions (restrictions on importation of livestock and fresh meat from 1901) and more importantly, the tight regulations governing the use of critically important antimicrobials in livestock. In Australia, it is illegal to administer fluoroquinolones (which have never been registered) and carbapenems to any food-producing animals and ceftiofur use is registered only for treatment of bovine respiratory tract infections in individual animals although off-label use in pigs also occurs. This multifaceted approach has delivered promising

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DOI: 10.1042/EBC20160055

results in minimizing the occurrence of critically important antimicrobial-resistant, Gram-negative bacteria from food producing animals.

So far in Australia, there are no reports of resistance to carbapenems among Enterobacteriaceae from livestock. A number of cross-sectional studies demonstrated absence of resistance to critically important antimicrobials among Gram-negative bacteria [85]. In addition, these studies reported extremely low levels of ceftiofur-resistant bacteria [85].

Some of the recent studies in Australia, however, have detected the carriage of carbapenem resistant *S. enterica* Typhimurium from cats and *E. coli* from seagulls [30,86]. Although the selection pressure for acquiring carbapenem resistance in these studies is highly unlikely to be due to carbapenem use as it is rarely used in treating companion animals and seagulls [74], the possibility of co-selection of carbapenem resistance due to use of other registered veterinary antimicrobials including β-lactam, fluoroquinolones and cephalosporins cannot be excluded. Studies have also reported the isolation of fluoroquinolone resistant *E. coli* ST354 from dogs, humans and chicken meat [87-89]. These studies show that antimicrobial-resistant, Gram-negative bacteria do not have defined species boundaries and illustrates the complex nature of AMR within Gram-negative bacteria involving a cycle that encompasses humans, animals and the environment.

These findings also imply that regulation alone cannot prevent the emergence of bacterial resistance to critically important antimicrobials in animals. Besides regulating or limiting the use of antimicrobials, it is highly imperative to effectively control infection and maintain hygiene and biosecurity to limit the transmission of resistant bacteria from one individual to another within community and healthcare settings or from animal to human in animal production. Furthermore, prevention of infection and maintenance of water bodies and drinking water supplies that serve as a major source responsible for transmission of resistant bacteria, especially in developing countries, would play a vital role in restricting the dissemination of antimicrobial-resistant Gram-negative bacteria.

Conclusions

The emergence of resistance to critically important antimicrobials in Gram-negative bacteria in humans and animals has narrowed the available treatment options making it imperative to look for alternative treatment choices either independently or in combination with existing antimicrobials. The role of antimicrobials in preventing, controlling and treating infectious diseases in both humans and animals not only makes them indispensable but also necessitates their conservation. The current scenario thus demands further judicious use of antimicrobials in both veterinary and human medicine and development of viable alternative options. The consequences of AMR are presented by way of limitation in treatment options for bacterial infections, increase in treatment cost and higher mortality rates. This complex situation demands a multidisciplinary approach for successful control and management of AMR. Development of strategies for antimicrobial stewardship in human and veterinary medicine, reviving the process of developing newer antimicrobials for introduction in the market and appraising viable alternative options to antimicrobials are required as a combined approach to combat this global issue.

Summary

- AMR is a natural phenomenon accelerated by antimicrobial use.
- AMR is a serious threat to global public health.
- Resistance is highly transferrable among different Gram-negative bacteria.
- Antimicrobial-resistant bacteria can be transferred in either direction between humans and animals.
- Gram-negative bacteria use a number of different mobile genetic elements to acquire or transfer AMR.
- Emerging AMR in animals to critically important (last line) antimicrobials is a public health concern due to horizontal gene transfer of AMR genes.

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Acknowledgements

We thank Mrs. Rebecca Jane Abraham (Murdoch University) and Dr. Stephen Page (Advanced Veterinary Therapeutics) for critical review of the manuscript and their valuable contribution

Competing interests

The author declares that there are no competing interests associated with the manuscript.

Abbreviations

AMR, antimicrobial resistance; DHFR, dihydrofolate reductase; ESBL, extended-spectrum-β-lactamase; ExPEC, Extraintestinal pathogenic *E. coli*; IS, insertion sequences; MBL, Metallo beta-lactamases; MRR, multidrug-resistance region; RND, resistance-nodulation division; ST, Sequence Type; UTI, Urinary Tract Infection; WHO, World Health Organization.

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Further to the literatures reviewed in 2017 there has been significant development in the area of antimicrobial resistance emergence in Gram-negative bacteria belonging to Enterobacteriaceae family. The fast-paced emergence of resistance has limited the treatment options available for life threatening infections caused by multi-drug resistant (MDR), extensively drug resistant (XDR), pan drug resistant (PDR) bacteria and forced the use of old antimicrobial agents such as colistin which is known to cause toxicity at high dose and fosfomycin. This has also led to discovery and addition of much awaited new antimicrobial agents like combination of beta-lactamase inhibitor such as avibactum with ceftazidime or aztreonam, meropenem/vaborbactam, ceftolozane/tazobactam, plazomicin and eravacycline (1, 2). There are studies that provide evidence that the emergence of antimicrobial resistant Gram-negative bacteria is not only a concern limited to human health, but recent years have witnessed a steady rise in antimicrobial resistant bacteria in companion animals as well. A study to evaluate the antimicrobial resistance amongst pathogens isolated from domestic animals detected isolates resistant to critically important antimicrobials including ceftazidime, ciprofloxacin, imipenem, ceftriaxone with high number of ESBL producing *E. coli* and *Klebsiella pneumoniae* amongst these animals (3).

A study compared the prevalence of antimicrobial resistance (AMR) and MDR between the *E. coli* strains isolated from wild birds and mammal faecal samples collected from different sampling sites which included sewage treatment plant, farm site with waste of antibiotic treated livestock and a central site with no sources of anthropogenic wastes (4). The study observed that the prevalence of MDR amongst the antimicrobial resistant isolates was higher in mammals than compared to wild birds. This was not only due to transfer of mobile genetic elements like plasmids and transposons but also because of commonly occurring chromosomal mutations (4). The highest number of MDR isolates were detected in the sampling site near sewage treatment plant thus implicating the potential anthropogenic influence. The study also detected high prevalence of colistin resistant isolates that were MDR thus elevating the concerns around public health risks further (4).

Another study detected carriage of several clinically significant bacterial species with resistance to colistin, fluoroquinolone or third generation cephalosporin in wild animal and birds including commensal *E. coli* strains from wild boars demonstrating resistance to two – four antimicrobial agents (5). Further the extended spectrum beta-lactamse (ESBL) producing isolate dominated in all animal species indicating a possible connect to human and livestock waste. CTX-M-1, CTX-M-15 and CTX-M-9 ESBL genes were most prevalent (5). The prevalence of critically important antimicrobial resistance in wild animals and particularly wild birds such as ducks and geese are of concern because of their accessibility to the wider environment. MDR *E. coli* and Staphylococcus spp were recovered in a study investigating the potential of AMR bacteria carriage and dissemination by migratory wild birds (6). In this study Salmonella isolates were also recovered from the cloacal swabs of these migratory birds although none of these isolates demonstrated MDR (6). Comparison of faecal samples between wild birds and rodents demonstrated that the frequency of AMR *E. coli* isolate carriage was significantly higher in wild birds thus implicating them as environmental reservoir of resistance (7, 8).

Considering the widespread dispersal and prevalence of antimicrobial resistant clinically important Gram-negative bacteria in different environmental niches it is imperative to contain the transmission routes. Also, it is important to take more calculative decisions or further limit the use of now available newer and much awaited antimicrobial agents in order to reduce the selection pressure and resistance development.

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CHAPTER 2

Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins.

Statement of Authorship

Title of Paper	Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins		
Publication Status	☑ Published	☐ Accepted for Publication	
	☐ Submitted for Publication	☐ Unpublished and Unsubmitted w ork w ritten in manuscript style	
Publication Details	Shewli Mukerji, Marc Stegger, Alec Vincent Truswell, Tanya Laird, David Jordan, Rebecca Jane Abraham, Ali Harb, Mary Barton, Mark O'Dea and Sam Abraham. Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins. J Antimicrob Chemother 2019; 74: 2566–2574 doi:10.1093/jac/dkz242		

Principal Author

Name of Principal Author (Candidate)	Shewli Mukerji		
Contribution to the Paper	Performed experiments and recorded the data. Did the compilation, analysis and interpretation of the data generated. Wrote the manuscript. Responded to comments and suggestion of co-authors and journal editor.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14th June 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised and assisted with data interpretation. Provided guidance and inputs in manuscript writing. Is the Corresponding author for this published article. Responded to editor's comment for revision.		
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Contribution to the Paper	Supervised and assisted with data interpretation. Provided guidance and inputs in manuscript writing. Helped in performing the whole genome sequencing of the selected isolates. Responded to editor's comment for revision.		
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Name of Co-Author	Dr Marc Stegger		
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Contribution to the Paper	Assisted in performing the antimicrobial disk sensitivity testing and provided suggestions in manuscript writing.		
Signature		Date	11th June 2022
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Contribution to the Paper	Performed all the laboratory work for whole genome sequencing, library preparation. Helped in collating data needed for creating phylogenetic trees for the manuscript.		
Signature		Date	22/06/2022
Name of Co-Author	Dr Mary Barton		
Contribution to the Paper	Supervised, provided guidance and inputs/comments to improvise the manuscript.		
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Contribution to the Paper	Provided guidance and inputs/comments to improvise the manuscript		
Signature		Date	8th June 2022
Name of Co-Author	Ali Harb		
Contribution to the Paper	Assisted in performing the antimicrobial disk sensitivity testing.		
Signature		Date	22nd June 2022

J Antimicrob Chemother 2019; **74**: 2566–2574
doi:10.1093/jac/dkz242 Advance Access publication 9 July 2019

Journal of Antimicrobial Chemotherapy

Resistance to critically important antimicrobials in Australian silver gulls (*Chroicocephalus novaehollandiae*) and evidence of anthropogenic origins

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Received 5 November 2018; returned 5 March 2019; revised 21 March 2019; accepted 6 May 2019

Objectives: Antimicrobial resistance (AMR) to critically important antimicrobials (CIAs) amongst Gram-negative bacteria can feasibly be transferred amongst wildlife, humans and domestic animals. This study investigated the ecology, epidemiology and origins of CIA-resistant *Escherichia coli* carried by Australian silver gulls (*Chroicocephalus novaehollandiae*), a gregarious avian wildlife species that is a common inhabitant of coastal areas with high levels of human contact.

Methods: Sampling locations were widely dispersed around the perimeter of the Australian continent, with sites separated by up to 3500 km. WGS was used to study the diversity and molecular characteristics of resistant isolates to ascertain their epidemiological origin.

Results: Investigation of 562 faecal samples revealed widespread occurrence of extended-spectrum cephalosporin-resistant (21.7%) and fluoroquinolone-resistant (23.8%) *E. coli*. Genome sequencing revealed that CIA-resistant *E. coli* isolates (*n*=284) from gulls predominantly belonged to human-associated extra-intestinal pathogenic *E. coli* (ExPEC) clones, including ST131 (17%), ST10 (8%), ST1193 (6%), ST69 (5%) and ST38 (4%). Genomic analysis revealed that gulls carry pandemic ExPEC-ST131 clades (O25:H4 *H*30-R and *H*30-Rx) and globally emerging fluoroquinolone-resistant ST1193 identified among humans worldwide. Comparative analysis revealed that ST131 and ST1193 isolates from gulls overlapped extensively with human clinical isolates from Australia and overseas. The present study also detected single isolates of carbapenem-resistant *E. coli* (ST410-bla_{OXA-48}) and colistin-resistant *E. coli* (ST345-*mcr-1*).

Conclusions: The carriage of diverse CIA-resistant *E. coli* clones that strongly resemble pathogenic clones from humans suggests that gulls can act as ecological sponges indiscriminately accumulating and disseminating CIA-resistant bacteria over vast distances.

Introduction

Antimicrobial resistance (AMR) is regarded as one of the greatest threats to human and animal health.[1] Resistance to critically important antimicrobials (CIAs) such as extended-spectrum cephalosporins (ESCs), fluoroquinolones (FQs), colistin and carbapenems amongst Enterobacteriaceae and other Gram-negative bacteria is a major public health concern.[2,3] This is attributed to co-associated resistance to other classes of antimicrobials and limited therapeutic options to treat infections caused by CIA-resistant bacteria in both humans and animals.[1]

Globally, emergence and dissemination of CIA resistance in livestock, wild birds and companion animals is a major concern due to the potential for direct or indirect transfer of such resistant bacteria to humans.[4,5] Examples of this include widespread occurrence of ESC- and FQ-resistant *E. coli* in livestock and companion animals,[6,7] recent reports of plasmid-mediated colistin resistance genes (*mcr*)

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in livestock *E. coli*[8] and the detection of ESC-, FQ- and carbapenem-resistant *E. coli* in wildlife such as herring gulls, hybrid deer and wild boars.[9–13] In particular, wildlife reservoirs of these resistant elements may be involved in pathways of dissemination into livestock and humans, which have not yet been investigated.

Australia is a country that has low levels of CIA resistance among livestock.[14–17] This is attributed to Australia's unique geography, quarantine restrictions, including bans on importation of livestock and fresh meat from 1901, and conservative regulations governing the use of CIAs including ESCs, FQs and carbapenems in food-producing animals.[14,16] However, recent studies have demonstrated that the status quo is changing, with recent reports of carbapenem-resistant *Salmonella enterica* serovar Typhimurium carrying bla_{IMP-4} from cats[18] and the carriage of carbapenem resistance in *E. coli* from a single, large, off-shore gull colony in New South Wales.[19] Another study identified two ciprofloxacin-resistant *E. coli* isolates from Australian pigs,[20] implying that despite strict biosecurity and absence of antimicrobial selection pressure, food-producing animals can still be at risk.

The report of carbapenem resistance in sources such as gulls raises public health concern regarding the role of these and other wild birds as reservoirs and in the potential transmission of CIA-resistant bacteria in the community. In this study, we performed a cross-sectional survey of gull faecal flora from across Australia to identify the carriage of CIA-resistant *E. coli*. In addition, comparative genomic analysis was performed to identify the origins of these CIA-resistant *E. coli* clones isolated in the current study from a global perspective.

Materials and methods

Sample collection

Swabs (n=562) were taken from freshly voided faeces from silver gulls (December 2015–April 2017) and transported in Amies charcoal medium (Copan). Sampling occurred in all Australian states (excluding the Northern Territory and Australian Capital Territory) and consisted of sites where silver gulls congregated on beaches and foreshores. The sampling locations included Geraldton (August 2016), Perth (Bibra Lake and Cottesloe; November 2016), Bunbury (November 2016) and Busselton (November 2016) in Western Australia; Adelaide (February 2017) in South Australia; Hobart (October 2016) in Tasmania; Wollongong (November 2015), Sydney (August 2016), Batemans Bay (December 2016), Ulladalla (December 2016) and Kiama (December 2016) in New South Wales (December 2016); Sandgate (March 2017), Gold Coast (March 2017) and Manley (March 2017) in Queensland; and Melbourne (April 2016 and April 2017) and Geelong (April 2016) in Victoria.

Bacterial isolation

Swabs were incubated in buffered peptone water (3 mL; ThermoFisher) for 4 h followed by aerobic culturing (37°C; 16–20 h) on three selective agar plates to identify FQ-resistant (MacConkey agar infused with 1 mg/L ciprofloxacin, ThermoFisher), ESC-resistant (Brilliance ESBL, ThermoFisher) and carbapenem-resistant (Brilliance CRE, ThermoFisher) *E. coli*. Presumptive *E. coli* (one colony per plate) were subcultured and species identity was confirmed by MALDI-TOF MS (Bruker). If the first isolate per plate was not identified as *E. coli*, another presumptive *E. coli* (if present) was subcultured and entered into the study.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed on 284 *E. coli* isolates using disc diffusion susceptibility testing as per CLSI guidelines and recommended breakpoints for interpreting phenotypic antimicrobial resistance were applied.[21] The 12 antimicrobials used were: ampicillin, ciprofloxacin, imipenem, meropenem, trimethoprim/sulfamethoxazole, tetracycline, gentamicin, ceftriaxone, streptomycin, cefoxitin, amoxicillin/clavulanate and chloramphenicol. *E. coli* ATCC 25922 was used as a control as per CLSI guidelines during antimicrobial susceptibility testing.[21]

Isolates in which the transmissible colistin resistance gene *mcr-1* was detected were subjected to MIC determination using the broth microdilution method as per ISO 20776-1:2006 CLSI using 96-well microtitre plates (Thermo Fisher, Australia).[21]

WGS

Genome sequencing was undertaken on 284 *E. coli* isolates using Illumina Chemistry (Nextera XT) as previously described.[22] Generated sequence reads were deposited in the NCBI Sequence Read Archive database under accession number PRJNA486855.

Data analysis

Raw reads were *de novo* assembled using SPAdes[23] and antimicrobial resistance genes, serotypes, multilocus STs and virulence factors inferred with the aid of the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/). SNPs within QRDRs were identified using Snippy v3.2.[23] SNP analysis for all data sets except ST131 was conducted using the Nullarbor platform[23] and parsed through Gubbins v2.3.1[24] to remove recombination. The phylogenetic analysis was performed using PhyML v3.0.[25] For analysis regarding ST131, sequence reads from international *H*27 and *H*30 isolates (n=58)[26] and all 40 available Australian ST131 isolates (38 human isolates, one companion animal and one unknown source) identified in EnteroBase (http://enterobase.warwick.ac.uk) with accessible raw sequence data on 19 March 2017 were included for SNP detection using NASP,[27] as previously described.[26] Selected isolates were removed due to low sequence depth to increase core genome size prior to purging using Gubbins v2.3.1. Phylogenetic analysis was performed using PhyML v3.0 with Smart Model Selection,[25] rooted with the *fimH* 41 (*H*41) cluster and visualized using iTol.[28]

Results

CIA-resistant E. coli carriage among gulls

Of the total number of samples collected (n=562) from across eastern (n=384), western (n=144) and southern (n=34) states of Australia, 24% (n=135) were positive for FQ-resistant *E. coli*, while 22% (n=125) were positive for ESC-resistant *E. coli*. One isolate was confirmed as carbapenem resistant by phenotypic and WGS methods despite 5% of swabs yielding growth on Brilliance CRE plates. The statewide frequency of FQ- and ESC-resistant *E. coli* isolates per swab is presented in Figure 1.

Phenotypic and genotypic characteristics of CIA-resistant E. coli

A total of 284 *E. coli* isolates recovered from selective agars were subjected to antimicrobial susceptibility testing. The *E. coli* isolates exhibited high levels of resistance to ampicillin (86%), trimethoprim/sulfamethoxazole (56%), tetracycline (51%) and streptomycin (48%). Resistance was high to the CIAs ciprofloxacin (64%) and ceftriaxone (62%). Only one isolate was resistant to

imipenem, but no resistance was demonstrated towards meropenem. Low levels of resistance were demonstrated to amoxicillin/clavulanate (21%), cefoxitin (18%), gentamicin (18%) and chloramphenicol (12%). The statewide and overall frequency of CIA-resistant *E. coli* from gulls is presented in Table 1.

Molecular characterization

WGS of 284 *E. coli* isolates revealed a high frequency of carriage of resistance genes bla_{TEM} and $bla_{CTX\text{-}M\text{-}15}$ (41% and 29%, respectively) in the *E. coli* isolates. The other resistance genes detected were $bla_{CMY\text{-}2}$ (13%), $bla_{CTX\text{-}M\text{-}27}$ (12%) and $bla_{CTX\text{-}M\text{-}14}$ (6%). Plasmid-mediated quinolone resistance determinants *qnrB4*, *qnrB6* and *qnrS1* were detected in 16% of the *E. coli* isolates (Table 2).

One *E. coli* ST410 isolate from Victoria recovered from Brilliance CRE was carbapenem resistant and positive for $bla_{OXA\text{-}48}$. Another *E. coli* ST345 isolate from Western Australia (recovered from MacConkey agar infused with 1 mg/L ciprofloxacin) harboured *mcr-1* and was phenotypically resistant to colistin (MIC=12.5 mg/L).

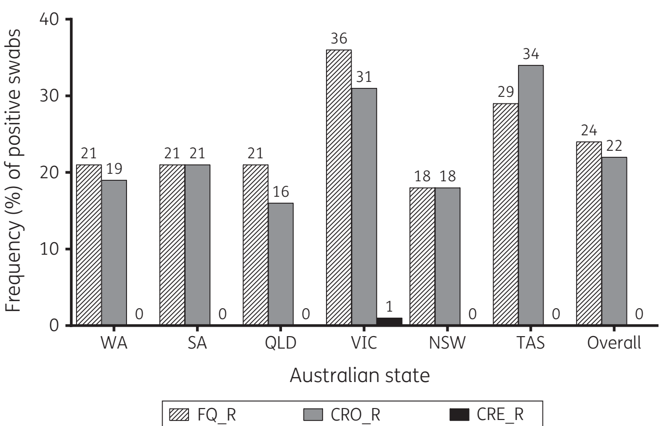

Figure 1. Frequency of detection of CIA-resistant *E. coli* from each swab collected from gulls based on growth selection plate and confirmed by disc diffusion. WA, Western Australia; SA, South Australia; QLD, Queensland; VIC, Victoria; NSW, New South Wales; TAS, Tasmania; FQ_R, FQ resistant; CRO_R, ceftriaxone resistant; CRE_R, carbapenem resistant.

Phylogenetic analysis

MLST of 284 CIA-resistant isolates revealed a heterogeneous population of *E. coli* clones represented by 72 STs (Table S1, available as Supplementary data at *JAC* Online). The major STs were ST131 (17% of isolates), ST10 (8%), ST1193 (6%), ST69 (5%), ST38 (4%), ST2179 (4%), ST744 (4%) and ST963 (4%) (Tables 3 and 4). MLST was indeterminate for six isolates due to low sequence coverage.

E. coli ST131

There are three main clades within ST131, i.e. O16:H5 fim*H41* (clade A), O25:h3 fim*H22* (clade B) and O25:H4 fim*H30* (clade C). Within clade C there are two dominant clades: *H*30-R (C1) and *H*30-Rx (C2).[29] The ST131 isolates (n=47) from Australian gulls predominantly belong to clade C (n=37, 79% of total ST131 isolates). Among the clade C isolates, 23 of the isolates belong to *H*30-R (C1) and 14 belong to *H*30-Rx (C2, positive for $bla_{CTX\text{-}M\text{-}15}$). In addition, nine *H*30-R isolates were positive for $bla_{CTX\text{-}M\text{-}27}$, demonstrating acquisition of ESC resistance. A small proportion of ST131 isolates were clade A, which contained five O16:H5 *fimH41* isolates, two ST131-O16:H5 isolates positive for a *fimH141* allele and three ST131-O25:H4 isolates with a *fimH41* allele (n=10, 21.3% of total ST131 isolates).

FQ resistance in *E. coli* ST131 isolates was attributed to point mutations in the *gyrA*, *gyrB*, *parC* and *parE* subunits within the QRDRs. Mutations in the *gyrA* subunit at nucleotide positions C248T and G259A resulting in Ser-83→Leu and Asp-87→Asn alterations along with changes in nucleotide position G239T (Ser-80→Ile) of the *parC* subunit were observed in 34 (72%) ST131 isolates. The main uropathogenic *E. coli* (UPEC) virulence-associated genes identified in *E. coli* ST131 isolates included toxin-encoding genes *cnf1*, *sat* and *astA*, the siderophore-related gene *iroN*, the adhesin-encoding gene *iha* and the protectin/invasin-encoding gene *iss*.[30]

Table 1. Number of *E. coli* isolates demonstrating phenotypic resistance to CIAs in disc diffusion antimicrobial susceptibility testing across different states

	Western Australia	South Australia	Queensland	Victoria	New South Wales	Tasmania	Overall
Swabs, *n*[a]	144	34	76	114	153	41	562
Positive swabs, *n* (%)[b]	57 (40)	15 (44)	28 (37)	77 (68)	55 (36)	26 (63)	258 (46)
Isolates, *n* (%)[c]	57 (40)	23 (68)	26 (34)	85 (75)	65 (42)	28 (68)	284 (51)
Ampicillin[R], *n* (%)	46 (81)	20 (87)	23 (88)	75 (88)	57 (88)	23 (82)	244 (86)
Ciprofloxacin[R], *n* (%)	37 (65)	13 (57)	17 (65)	55 (65)	45 (69)	17 (61)	184 (65)
Imipenem[R], *n* (%)	0	0	0	1 (1)	0	0	1 (0.4)
Meropenem[R], *n* (%)	0	0	0	0	0	0	0
Ceftriaxone[R], *n* (%)	27 (47)	16 (70)	12 (46)	52 (61)	47 (72)	21 (75)	175 (62)
Cefoxitin[R], *n* (%)	6 (11)	3 (13)	5 (19)	8 (9)	23 (35)	5 (18)	50 (18)
Amoxicillin/clavulanate[R], *n* (%)	6 (11)	6 (26)	9 (35)	8 (9)	21 (32)	9 (32)	59 (21)

[R], resistant.
[a]Total number of swabs collected.
[b]Total number of swabs positive for *E. coli*.
[c]Total number of individual isolates picked from three different selective agars.

Table 2. Numbers and percentages of selectively recovered *E. coli* isolates from gull faecal samples across different states of Australia containing genes encoding CIA resistance

	Western Australia	South Australia	Queensland	Victoria	New South Wales	Tasmania
Swabs, *n*	144	34	76	114	153	41
Isolates, *n*	57	23	26	85	65	28
qnr	7 (12)	2 (9)	8 (31)	15 (18)	4 (6)	7 (25)
bla_{CMY-2}	6 (11)	1 (4)	3 (12)	2 (2)	19 (29)	5 (18)
bla_{CMY-13}	0 (0)	0 (0)	0 (0)	0 (0)	1 (2)	0 (0)
bla_{CMY-42}	0 (0)	0 (0)	0 (0)	2 (2)	0 (0)	0 (0)
bla_{CMY-60}	0 (0)	0 (0)	2 (8)	0	0 (0)	0 (0)
$bla_{CTX-M-11}$	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
$bla_{CTX-M-14}$	4 (7)	1 (4)	1 (4)	4 (5)	6 (9)	1 (4)
$bla_{CTX-M-15}$	10 (18)	8 (35)	5 (19)	35 (41)	13 (20)	11 (39)
$bla_{CTX-M-24}$	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
$bla_{CTX-M-27}$	5 (9)	4 (17)	4 (15)	8 (9)	6 (9)	7 (25)
$bla_{CTX-M-3}$	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
$bla_{CTX-M-55}$	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
bla_{OXA-1}	2 (4)	3 (13)	2 (8)	17 (20)	2 (3)	3 (11)
bla_{OXA-48}	0 (0)	0 (0)	0 (0)	1 (1)	0 (0)	0 (0)
bla_{SHV}	0 (0)	0 (0)	0 (0)	2 (2)	0 (0)	0 (0)
bla_{TEM}	28 (49)	10 (43)	16 (62)	32 (38)	26 (40)	5 (18)
mcr-1	1 (2)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Table 3. *E. coli* STs of public health significance; total numbers and *n* (%) isolated from different selective media

ST	Total number of *E. coli* isolates	MacConkey+ciprofloxacin plates, *n* (%)	Brilliance ESBL plates, *n* (%)	Brilliance CRE plates, *n* (%)
ST131	47	33 (70)	9 (19)	5 (11)
ST10	23	8 (35)	15 (65)	0
ST1193	16	14 (88)	1 (6)	1 (6)
ST69	15	2 (13)	13 (87)	0
ST38	11	3 (27)	8 (73)	0
ST2179	10	4 (40)	0	6 (60)
ST744	10	9 (90)	1 (10)	0
ST963	10	0	9 (90)	1 (10)
ST405	7	4 (57)	3 (43)	0
ST450	7	7 (100)	0	0
ST773	4	4 (100)	0	0

Phylogenetic analysis of *E. coli* ST131 in gulls and publicly available ST131 genomes from humans including Australian isolates (Figure 2) revealed distinct clustering in relation to overall *fimH* types [*H*41, *H*22 and *H*31 (data not shown)]. Similar to previous findings, an FQ-resistant clade (ST131 *H*30-R) and an FQ-resistant and ESBL-producing *H*30-Rx sister clade were observed, that combined 95% of the CIA-resistant gull ST131 isolates (Figure 2). Significant overlap was evident between the Australian gull and human isolates within each of these groups.

E. coli ST10

The second most frequently detected *E. coli* ST (*n*=23, 8%) was the broad host range lineage ST10 reported to cause severe infection in humans, livestock and wild birds.[15] Of the total number of isolates identified, 6 isolates (26%) were ciprofloxacin resistant, 12 isolates (52%) were ceftriaxone resistant and 5 isolates (22%) demonstrated resistance to both ciprofloxacin and ceftriaxone. O9:H9 *fimH54* (6 isolates; 26%) was the dominant serotype among the ST10 isolates. The ESC resistance-encoding genes $bla_{CTX-M-15}$, $bla_{CTX-M-27}$, $bla_{CTX-M-14}$ and $bla_{CTX-M-11}$ were identified in 35%, 13%, 7% and 4% of the isolates, respectively. Sequence analysis detected QRDR FQ resistance-associated mutations in *gyrA*, *gyrB*, *parC* and *parE* in all isolates. The main virulence-associated genes identified were *iss*, *astA*, *pic*, *iha* and *iroN*. The phylogenetic analysis revealed multiple clades of *E. coli* ST10 (Figure S1) with no distinct geographical clustering.

Table 4. Distribution in numbers and percentages of dominant *E. coli* STs across different states

	Western Australia	South Australia	Queensland	Victoria	New South Wales	Tasmania	Overall
Swabs, *n*	144	34	76	114	153	41	562
Isolates, *n*	57	23	26	85	65	28	284
ST, *n* (%)							
ST131	11 (19)	8 (35)	1 (4)	11 (13)	9 (14)	7 (25)	47 (17)
ST10	4 (7)	1 (4)	3 (12)	9 (11)	6 (9)	0	23 (8)
ST1193	7 (12)	1 (4)	0	3 (4)	4 (6)	1 (4)	16 (6)
ST69	1 (2)	0	2 (8)	10 (12)	0	2 (7)	15 (5)
ST38	4 (7)	0	2 (8)	1 (1)	2 (3)	2 (7)	11 (4)
ST2179	0	0	0	0	10 (15)	0	10 (4)
ST744	1 (2)	0	0	5 (6)	4 (6)	0	10 (4)
ST963	2 (4)	0	0	1 (1)	4 (6)	3 (11)	10 (4)
ST405	0	0	0	2 (2)	5 (8)	0	7 (2)
ST450	4 (7)	0	0	2 (2)	1 (2)	0	7 (2)
ST773	0	0	0	4 (5)	0	0	4 (1)

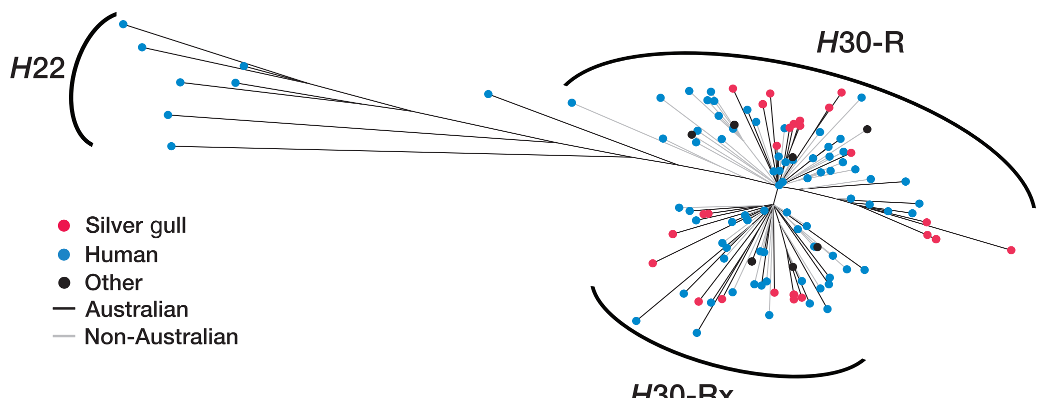

Figure 2. Radial phylogenetic tree of all non-*H41 E. coli* ST131 from gulls and publicly available ST131 genomes from humans including Australian isolates showing distinct clustering of *fimH* types. Two sister groups were evident in the *H30* cluster, an FQ-resistant clade (ST131 *H30*-R) and an FQ-resistant and ESBL-producing *H30*-R nested clade (*H30*-Rx). Intermingling of international and Australian isolates is observed, as is the overlap between isolates of human and gull origin.

E. coli ST1193

Another major *E. coli* ST identified belonged to ST1193, an emerging virulent and resistant lineage[31] (Figure 3). All isolates (n=16) were resistant to ciprofloxacin and susceptible to ceftriaxone, except two isolates, which were positive for $bla_{\text{CTX-M-15}}$ and demonstrated resistance to ceftriaxone. The FQ resistance of all isolates was attributed to the mutations in the QRDRs. The majority of the isolates (n=15) belonged to serotype O75:H5 and the *fimH64* variant. The virulence factors *iha*, *sat* and *vat* were most frequently identified. Phylogenetic analysis of these isolates demonstrated heterogeneous make up of ST1193 clones that belong to three different clusters distributed evenly across all states except Queensland.

E. coli ST69

Susceptibility to ciprofloxacin was demonstrated by 80% of the 15 *E. coli* ST69 isolates, a strain that is considered to be a highly virulent and globally successful UPEC.[32] The serotypes identified included O17/O77:H18 (seven isolates, 47%), O17/O44:H8 (four isolates, 27%), O25:H18 (two isolates, 13%), O15:H1 (a single isolate) and O15:H6 (a single isolate). All isolates were *fimH27* except one isolate from Tasmania with serotype O17/O77:H18 and *H297*. Resistance to ceftriaxone was demonstrated by 93% of the isolates carrying one of the resistance-conferring genes $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-27}}$ or $bla_{\text{CTX-M-14}}$. The common virulence-associated genes were *iha*, *iss*, *sat* and *iroN*. Multiple clades of ST69 were evident in the phylogenetic tree. The majority of the isolates from Victoria grouped together in the phylogeny, while the isolates from the other states were intermingled (Figure S2).

E. coli ST38

The different serotypes identified among the 11 *E. coli* ST38 isolates detected included serotype O86:H18 with *H5* and O1:H15 with *H65*. Resistance to both ciprofloxacin and ceftriaxone was

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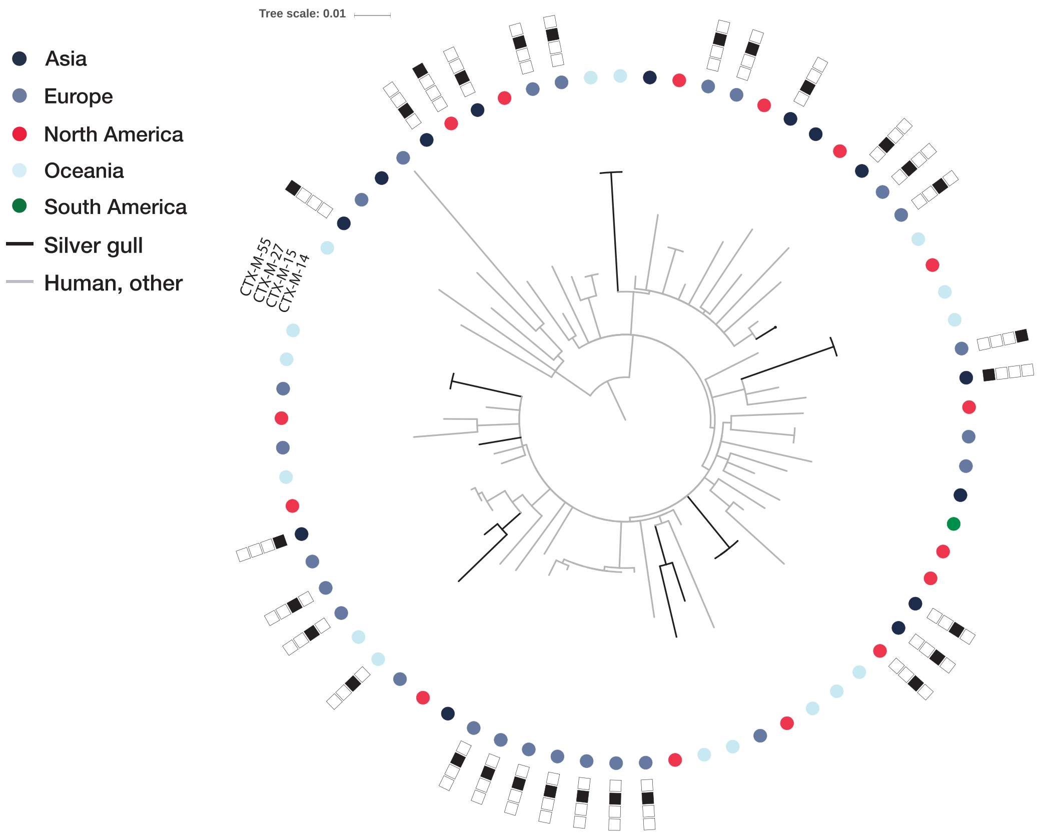

Figure 3. A maximum likelihood phylogeny obtained using SNPs comparing *E. coli* ST1193 from Australian silver gulls with globally collected ST1193, indicating the wide geographical dispersal of *E. coli* ST1193. The inner circle with coloured dots represents the continent of origin. The *E. coli* ST1193 isolates detected in gulls from Australia are scattered throughout the phylogeny.

observed in six isolates. One isolate demonstrated ciprofloxacin resistance, while four isolates demonstrated resistance towards ceftriaxone only. The genes conferring ESC resistance were $bla_{CTX\text{-}M\text{-}15}$, $bla_{CTX\text{-}M\text{-}27}$, $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CMY\text{-}2}$. The major virulence-encoding genes identified were *astA*, *iss*, *iha* and *sat*. Phylogenetic analysis revealed an even distribution of ST38 clones across all states of Australia excluding South Australia (Figure S3).

Discussion

This study aimed to identify the carriage of CIA-resistant *E. coli* isolated from Australian gulls and describe the underlying resistance determinants, virulence genes and phylogenetic relationships of these resistant isolates. A high level of CIA-resistant *E. coli* carriage was observed among Australian gulls, along with detection of carbapenem and colistin resistance at low frequencies. The rate of CIA-resistant *E. coli* carriage in gulls was surprisingly high in a country where low levels of FQ- and ESC-resistant *E. coli* were observed in a national surveillance programme albeit using non-selective culturing in human sepsis cases (7.3%–11.2% and 6.4%–10.8%, respectively)[33] and in livestock (0%–1% and 0%–5%, respectively).[15,17,20]

A large proportion of the CIA-resistant *E. coli* STs recovered were identified in this study as human-associated clones, suggesting bi-directional transmission between humans and gulls or the existence of an unidentified reservoir with ecological links to both. Additionally, the CIA-resistant *E. coli* among gulls was widely dispersed around the perimeter of the Australian continent. Most notably, *E. coli* ST131 was found to be common. This is a globally disseminated, highly virulent strain in humans that is often associated with FQ resistance and CTX-M-15-type ESBL production.[34] The *E. coli* ST131 isolated from the gull faeces also belonged to the O25:H4, *H*30, CTX-M-15-positive group (Figure 2), which is the most predominant lineage associated with urinary tract infections in humans.[35]

ST10, ST69 and ST405 have previously been identified as UPEC,[36] and the ST405 lineage has been described as a global disseminator of CTX-M-type ESBL, in a similar manner to *E. coli* ST131.[37] Another noteworthy observation is the identification of FQ-resistant ST1193, which has been described as a rapidly emerging extra-intestinal pathogenic *E. coli* ('ExPEC') clone with widespread occurrence in humans in the USA.[31] Also detected in this study was *E. coli* ST744, which has been identified as a human pathogen with the potential to develop resistance in the presence of selection pressure.[38] Table 3 lists the various STs identified from gull faecal samples.

In this study, one $bla_{OXA\text{-}48}$-positive *E. coli* isolate was detected from Melbourne, Victoria. Since its initial identification in Turkey,[39] OXA-48 has emerged as the most prevalent carbapenemase worldwide.[40] The presence of OXA-48 carbapenemase-producing Enterobacteriaceae has extended beyond the hospital setting and

entered the community, livestock, companion animals, wildlife and the environment.[18,41,42] There are reports of detection of NDM-1-producing *Salmonella* in wild birds from Germany,[10] VIM-1-producing *E. coli* in yellow legged gulls from France[11] and IMP-4 *Salmonella* in silver gulls from Australia.[19] Thus far, there are no published reports of detection of OXA-48 in wild birds. Moreover the presence of the bla_{OXA-48} gene in *E. coli* ST410 is concerning, as recently this clone has been identified as one of the internationally emerging 'high-risk' clones.[43] In contrast to the previous study, no IMP-4-producing *E. coli* was detected in gulls in the present study, although sampling was performed at the same locations of New South Wales, Australia (Five Islands, Wollongong). The detection of such human-associated CIA resistance genes in gulls is suggestive of possible interspecies transmission and indicates risk of zoonotic transfer to humans and other animals including livestock.

This study reports, to the best of our knowledge, the first detection of the colistin resistance-encoding gene *mcr-1* in *E. coli* isolates obtained from Australian animals. The isolate identified as *E. coli* ST345 was obtained from a single silver gull from Cottesloe, Western Australia. Following the first detection of *mcr-1* in Europe in 2016 from a gull (*Larus argentatus*),[44] studies from other continents (South America and Asia) have identified migratory wild birds carrying *mcr-1*-positive *E. coli*.[8,45]

The heterogeneous populations of *E. coli* ST131 and other major CIA-resistant *E. coli* clones detected in this study exhibited extensive overlap with human clinical isolates. The source of emergence of CIA-resistant bacteria in wildlife can be attributed to multiple factors. Human waste, wastewater treatment facilities and livestock waste are all considered to be the major hotspots for CIA-resistant *E. coli* as well as for horizontal gene transfer of resistance genes.[46] Furthermore, the close proximity of gulls and humans is considered to be yet another factor resulting in transmission of CIA-resistant bacteria. Although gulls are mostly known to be opportunistic marine feeders, they also readily feed on leftover human food and garbage, which provides an opportunity to acquire bacteria harbouring resistance genes. The gull samples collected in this study were from areas with dense human populations, which may have resulted in gulls acquiring the CIA-resistant *E. coli* at higher frequency than in more remote regions.

The migratory habits of gull species have been cited as the major factor responsible for dissemination of CIA-resistant bacteria.[9] The Australian silver gull's movement pattern is thought to be confined to within 80–1600 km from their hatchery—as such they are designated non-migratory.[47] Nevertheless, the occurrence of silver gulls in human and livestock environments is very common due to the species' successful adaptation as a scavenger. The extent of gull–human co-mingling is high, particularly in coastal urban areas of Australia, thus providing an easy route for exchange of faecal flora. Moreover, gulls are often observed to share roosting locations with other bird species, such as the little tern (*Sternula albifrons*), which has a worldwide distribution and migration pathways extending between Asia and Australia.[48] Additionally, they also inhabit tidal lagoons and wetlands with ducks and wading birds, which may then travel further inland to farming regions. This increases the possibility of transferring these resistant clones to food-producing animals, either via direct contamination of feed and water, or via a 'leapfrog' effect through other aquatic birds, which are not as coastal centric. The potential therefore exists for gulls to be a key element in a web of wildlife capable of acquiring and disseminating CIA-resistant *E. coli* not only between human populations over vast distances but also in livestock and agricultural produce.[22]

Further studies would necessitate identification of CIA-resistant *E. coli* from gull populations in remote locations with sparse human populations to evaluate the possible impact of human activity on transmission of CIA-resistant bacteria and the source of acquisition of these bacteria by gulls.

Conclusions

This study establishes that Australian silver gulls are carriers of virulent and CIA-resistant human-associated *E. coli* clones. The detection of transferable colistin- and carbapenem-resistant *E. coli* clones in an Australian animal also raises questions regarding the sources of these resistant bacteria. The relatedness of isolates among the various STs across states indicates that gulls can act as ecological sponges, indiscriminately accumulating both pathogenic and non-pathogenic CIA-resistant bacteria and potentially disseminating them in a cycle encompassing humans and the broader environment.

Acknowledgements

We would like to thank Mrs Bertha Rusdi for her technical assistance in the laboratory.

Funding

This study was supported by a small research grant from the School of Veterinary and Life Sciences (Murdoch University, Murdoch, Western Australia, Australia). A PhD scholarship and operating fund were awarded to S. M. by The University of Adelaide.

Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S3 are available as Supplementary data at *JAC* Online.

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CHAPTER 3

Implication of foraging and interspecies interactions of birds for carriage of *Escherichia coli* strains resistant to critically important antimicrobials.

Statement of Authorship

Title of Paper	Implications of Foraging and Interspecies Interactions of Birds for Carriage of *Escherichia coli* Strains Resistant to Critically Important Antimicrobials
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Shewli Mukerji, Samantha Gunasekera, James Nicholas Dunlop, Marc Stegger, David Jordan, Tanya Laird, Rebecca Jane Abraham, Mary Barton, Mark O'Dea, Sam Abraham. Implications of Foraging and Interspecies Interactions of Birds for Carriage of *Escherichia coli* Strains Resistant to Critically Important Antimicrobials. Appl Environ Microbiol 86: e01610-20. https://doi.org/10.1128/AEM.01610-20.

Principal Author

Name of Principal Author (Candidate)	Shewli Mukerji		
Contribution to the Paper	Performed experiments and recorded the data. Did the compilation, analysis and interpretation of the data generated. Wrote the manuscript. Responded to comments and suggestion of co-authors and journal editor.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	14th June 2022

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Dr Sam Abraham		
Contribution to the Paper	Supervised and assisted with data interpretation. Provided guidance and inputs in manuscript writing. Is the Corresponding author for this published article. Responded to editor's comment for revision.		
Signature		Date	8th June 2022

Name of Co-Author	Dr Mark O'Dea

Contribution to the Paper	Supervised and assisted with data interpretation. Provided guidance and inputs in manuscript writing. Helped in performing the whole genome sequencing of the selected isolates and the experiments involved in plasmid transfer by conjugation. Corresponding author for this published article. Responded to editor's comment for revision.		
Signature		Date	13/6/22

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Name of Co-Author	Samantha Gunasekera		
Contribution to the Paper	Helped in the examination of antimicrobial resistant genes and mobile genetic elements, whole genome sequencing data analysis. Contributions to the manuscript included writing the genetic analysis section, schematic diagrams to represent the data.		
Signature		Date	13th June 2022

Name of Co-Author	James Nicholas Dunlop		
Contribution to the Paper	Assisted with the most important part for the entire study - sample collection from Penguin Island and provided suggestions in manuscript writing.		
Signature		Date	12th June 2022
Name of Co-Author	Tanya Laird		
Contribution to the Paper	Performed all the laboratory work for whole genome sequencing, library preparation. Helped in collating data needed for creating phylogenetic trees for the manuscript.		
Signature		Date	22/06/2022
Name of Co-Author	Dr Mary Barton		
Contribution to the Paper	Supervised, provided guidance and inputs/comments to improvise the manuscript.		
Signature		Date	21/06/2022
Name of Co-Author	Dr David Jordan		
Contribution to the Paper	Provided guidance and inputs/comments to improvise the manuscript. Helped in statistical analysis of the data.		

Signature		Date	20th June 2022
Name of Co-Author	Dr Rebecca Jane Abraham		
Contribution to the Paper	Helped in performing the MIC for all the selected isolates. Provided guidance and inputs/comments to improvise the manuscript		
Signature		Date	8th June 2022
Name of Co-Author	Marc Stegger		
Contribution to the Paper	Provided guidance and inputs/comments to improvise the manuscript. Helped in comparative analysis of the whole genome sequencing data.		
Signature		Date	22/6/2022

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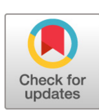

Implications of Foraging and Interspecies Interactions of Birds for Carriage of *Escherichia coli* Strains Resistant to Critically Important Antimicrobials

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ABSTRACT Globally, gulls have been associated with carriage of high levels of *Escherichia coli* strains resistant to critically important antimicrobials (CIAs), a major concern, as these antimicrobials are the sole alternative or one among only a few alternatives available to treat severe life-threatening infections in humans. Previous studies of Australian silver gulls demonstrated high levels of resistance to CIAs, particularly fluoroquinolone and extended-spectrum cephalosporins, among *E. coli* strains (carriage at 24% and 22%, respectively). This study aimed to identify and characterize strains from four distinct bird species inhabiting a common coastal environment, determine the frequency of carriage of CIA-resistant *E. coli* strains, and examine if these resistant clones and their resistance-encoding mobile genetic elements (MGEs) could be transmitted between species. CIA-resistant *E. coli* was detected in silver gulls (53%), little penguins (11%), and feral pigeons (10%), but not in bridled terns. In total, 37 different sequence types (STs) were identified, including clinically significant human-associated lineages, such as ST131, ST95, ST648, ST69, ST540, ST93, ST450, and ST10. Five main mobile genetic elements associated with bla_{CTX-M}-positive *E. coli* strains isolated from three bird species were detected. Examination of clonal lineages and MGEs provided indirect evidence of transfer of resistance between bird species. The carriage of CIA-resistant *E. coli* by gulls and pigeons with proximity to humans, and in some instances food-producing animals, increases the likelihood of further bidirectional dissemination.

IMPORTANCE It has been shown that 20% of Australian silver gulls carry drug-resistant *Escherichia coli* strains of anthropogenic origin associated with severe diseases, such as sepsis and urinary tract infections, in humans. To further characterize the dynamics of drug-resistant *E. coli* in wildlife populations, we investigated the carriage of critically important antimicrobial (CIA) drug-resistant *E. coli* in four bird species in a common environment. Our results indicated that gulls, pigeons, and penguins carried drug-resistant *E. coli* strains, and analysis of mobile genetic elements associated with resistance genes indicated interspecies resistance transfer. Terns, representing a bird species that forages on natural food sources at sea and distant from humans, did not test positive for drug-resistant *E. coli*. This study demonstrates carriage of CIA-resistant bacteria in multiple bird species living in areas commonly

Citation Mukerji S, Gunasekera S, Dunlop JN, Stegger M, Jordan D, Laird T, Abraham RJ, Barton M, O'Dea M, Abraham S. 2020. Implications of foraging and interspecies interactions of birds for carriage of *Escherichia coli* strains resistant to critically important antimicrobials. Appl Environ Microbiol 86:e01610-20. https://doi.org/10.1128/AEM.01610-20.

Editor Isaac Cann, University of Illinois at Urbana-Champaign

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Received 5 July 2020
Accepted 4 August 2020
Accepted manuscript posted online 14 August 2020
Published 1 October 2020

inhabited by humans and provides further evidence for a leapfrog effect of resistance in wildlife, facilitated by feeding habits.

KEYWORDS Antimicrobial resistance, *Escherichia coli*, ST131, CTX-M, gulls, bird, mobile genetic elements, penguins, pigeons

Wildlife carriage of pathogenic bacteria resistant to critically important antimicrobials (CIAs), such as extended-spectrum cephalosporins (ESCs), fluoroquinolones (FQs), carbapenems, and colistin, is of concern for public health and animal health due to the potential to facilitate transmission to humans, other wildlife, and livestock (1–3). Despite insignificant levels of treatment with clinically relevant antimicrobials compared to humans, companion animals, and livestock, antimicrobial resistance (AMR) in wild animals and birds has increasingly been reported worldwide (4–7). Globally, gulls have been implicated in high levels of carriage of CIA-resistant bacteria that are of clinical significance to human health (8–10). Avian populations that adapt to the urban environment may potentially encounter, host, transmit, and even amplify human microorganisms, including potential pathogens. This may result in transmission of these organisms to species outside the urban environment at places where birds congregate, and particularly where colonial breeding or roosting occurs on coastal islands or on wetlands comprising a shared habitat (11).

Coastal islands adjacent to cities often support a range of colonially breeding species. They include gulls, which routinely scavenge in urban areas, and seabirds that forage for aquatic prey in a variety of marine habitats, from coastal shallows to open ocean (12). Coastal breeding islands in an urban context may provide useful One Health observatories to investigate and monitor the transmission of human microorganisms to and between wild bird populations (13). Of particular significance are the potential reservoirs and transmission pathways of bacteria of human origin that are resistant to antimicrobials.

A recent comprehensive study of Australian silver gulls (*Chroicocephalus novaehollandiae*), reported high levels of carriage of FQ- and ESC-resistant *Escherichia coli* strains nationally (24% and 22%, respectively) (14). Genomic characterization revealed that the majority of these CIA-resistant *E. coli* strains originated from humans and belonged to globally disseminated pandemic, multidrug-resistant, extraintestinal, and pathogenic clonal lineages. They included *E. coli* sequence type 131 (ST131), responsible for causing human sepsis and urinary tract infections in Australia and worldwide (15); a rapidly emerging FQ-resistant extraintestinal pathogenic *E. coli* (ExPEC) clone, ST1193 (16); and other clinically relevant STs, including ST10, ST69, and ST38. The study concluded that Australian silver gulls may act as ecological sponges, accumulating and transmitting CIA-resistant pathogenic *E. coli*.

A key unresolved issue is the role of other wild birds in the ecology of *E. coli* strains that are resistant to CIAs, particularly birds that have some form of ecological interaction with silver gulls. To explore this, we studied silver gulls and three other bird species cohabiting on a nearshore breeding island in an urbanized coastal environment. All four species forage from a central location (Penguin Island; 32°17′S, 115°41′E) but occupy different foraging niches. Silver gulls are opportunistic coastal foragers that also scavenge on human food waste and garbage. Feral pigeons (*Columba livia*) in the area forage on grain spillage from the East Rockingham industrial zone and on the seeds of agricultural weeds. In contrast to the gulls and pigeons, little penguins (*Eudyptula minor*) are an aquatic species foraging on schooling baitfish in waters close to the coast. A fourth species, bridled terns (*Onychoprion anaethetus*), are pelagic seabirds that forage on small fish and crustaceans found on the mid- to outer continental shelf but share their breeding habitat on Penguin Island with nesting silver gulls. All four study species nest and/or roost colonially on Penguin Island, a 12.5-ha aeolianite limestone island 600 m off the coast of suburban Rockingham, approximately 50 km south of Perth, Western Australia. The island supports approximately 3,000 pairs of breeding

silver gulls, 500 pairs of little penguins, up to 900 feral pigeons, and 4,000 pairs of bridled terns (12).

The aim of the study was to identify and characterize the CIA-resistant *E. coli* strains isolated from the four different bird species, including determining the phylogenetic relatedness of the isolates and the mobile genetic elements (MGEs) conferring resistance to CIAs. This would allow investigation into AMR mobility through colonizing bacteria in each of the four bird species in an environment with a limited influx of genes from nonavian species. It was hypothesized that discrete MGEs would be associated with resistance genes and that characterization of these would provide indications of gene movement throughout populations. Although it is widely acknowledged that the directionality of movement of AMR is ambiguous and that carriage in wildlife may originate from humans, the study aimed to facilitate better understanding of the routes of further amplification and dissemination of these CIA-resistant *E. coli* strains within the ecological system.

RESULTS

Carriage of CIA-resistant *E. coli*. Among the four bird species, the highest level of carriage of CIA-resistant *E. coli* was detected in silver gulls. Of the 100 gull swabs that were collected, 53 (53%) of the samples carried resistant forms of *E. coli* that demonstrated visible growth on selective agars (MacConkey agar supplemented with ciprofloxacin and Brilliance ESBL agar [ThermoFisher Scientific]). Resistant *E. coli* strains were isolated from 10 pigeon samples (10%) and 6 penguin samples (11%). No bridled terns tested positive for CIA-resistant *E. coli*. These rates of detection of CIA-resistant *E. coli* in swabs from the four different bird species were statistically significant ($P < 0.0001$). Figure 1 presents the frequency of growth of resistant *E. coli* observed for each bird species on selective medium.

Phenotypic and genotypic characteristics of CIA-resistant *E. coli*. Disc diffusion susceptibility testing of all *E. coli* isolates from the gulls that were identified as resistant on the basis of growth on selective media ($n = 94$) demonstrated high levels of resistance to ampicillin (86%), ciprofloxacin (49%), and ceftriaxone (55%) and low levels of resistance to cefoxitin (15%) and amoxicillin-clavulanate (19%). Resistance to ampicillin, ciprofloxacin, and ceftriaxone was common among *E. coli* strains recovered from antimicrobial-supplemented selective agar from feral pigeons ($n = 16$) and little penguins ($n = 9$) (Table 1). However, none of the *E. coli* isolates demonstrated resistance to the carbapenems (imipenem and meropenem). The overall frequencies of resistance based on disc diffusion testing in *E. coli* from selective plating in each of the three bird species are presented in Table 1.

Molecular characterization of sequence types and antimicrobial resistance genes. Among *E. coli* strains isolated from silver gulls ($n = 94$), feral pigeons ($n = 16$), and little penguins ($n = 10$), 37 STs were identified in total. In *E. coli* strains isolated from silver gulls, 30 different STs were detected, while 4 different STs were found in feral pigeons and 3 STs in little penguins. Among the 94 *E. coli* strains from silver gulls, the predominant STs included the following: ST131, 9.6% (9/94); ST69, 6.4% (6/94); ST10, 3.2% (3/94); and ST648, 3.2% (3/94). In feral pigeons, the most predominant were ST131, which was present in 25% (4/16) of samples, and ST 69, ST450, and ST695, which were each present in 12.5% (2/16) of samples. In *E. coli* strains isolated from little penguins, ST1598 and ST95 were the most frequent types and were each present in 20% (2/10) of samples. Dissemination of STs, based on the core genome single-nucleotide polymorphism (SNP) phylogeny of a subset of silver gull isolates and all feral pigeon and little penguin isolates, is demonstrated in Fig. 2.

Examination of antimicrobial resistance genes and MGEs. A range of resistance genes were present, likely representing the diverse origins of the isolates (see Fig. S2 in the supplemental material). Most isolates contained a combination of SNPs within the quinolone resistance-determining regions (QRDRs), accounting for the phenotypic resistance to ciprofloxacin noted in all three bird species. In addition, a small subset of seagull isolates carried the plasmid-mediated quinolone resistance genes *qnrS* ($n = 4$)

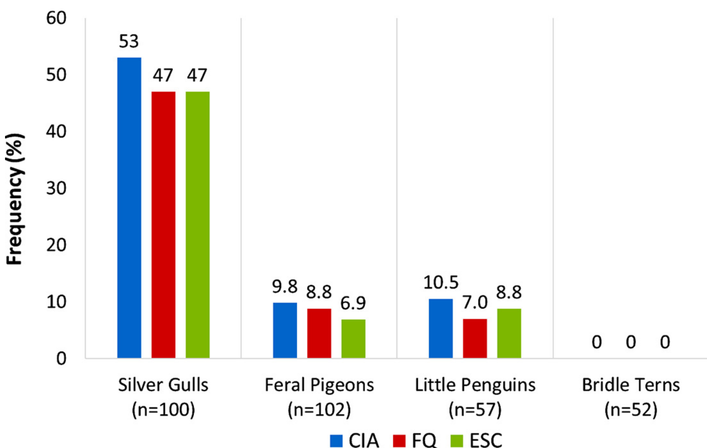

FIG 1 Frequency (percent) of carriage of resistant *E. coli* strains in fecal swabs from the four bird species. CIA, resistant to fluoroquinolones and/or extended-spectrum cephalosporins; FQ, fluoroquinolone resistant; ESC, extended-spectrum cephalosporin resistant. Resistance was measured on selective medium containing each drug and confirmed by antimicrobial susceptibility testing using disc diffusion.

and *qnrB* ($n = 1$). The *qnrS* gene was detected in ST457, ST10, and ST58 isolates, while an ST617 isolate harbored *qnrB*. However, there was no clear pattern in the carriage of resistance genes across sequence types. The most frequently occurring ESC resistance gene identified in *E. coli* isolates across the three bird species was $bla_{CTX-M-15}$, found in 7.4% (7/94) of silver gull origin isolates, 25% (4/16) of feral pigeon origin isolates, and 50% (5/10) of little penguin origin isolates. The $bla_{CTX-M-15}$ gene was found across three different STs in feral pigeons (ST69, ST93, and ST450), three in little penguins (ST95, ST648, and ST1598), and six in silver gulls (ST10, ST127, ST457, ST617, ST648, and ST1598) (Fig. 2).

In order to further characterize the degree of similarity between resistance elements, and thus the indication that horizontal gene transmission was potentially occurring across species, detailed analysis of the CTX-M-type β-lactamase-encoding genes was undertaken, which revealed that there were two main mobile genetic elements associated with $bla_{CTX-M-15}$-positive *E. coli* isolates from all three bird species. A 2,971-bp transposition unit (TU) containing an IS*Ecp1* insertion sequence 48 bp upstream from $bla_{CTX-M-15}$ and a partial *ORF477*Δ downstream (MGE 1) was present in four silver gull origin *E. coli* isolates and three feral pigeon origin *E. coli* isolates, located chromo-

TABLE 1 Resistance profiles of isolates obtained from media selecting for extended-spectrum cephalosporin and fluoroquinolone resistance determined by disc diffusion assay[a]

	Frequency of resistance (% of isolates)			
Antimicrobial	**Silver gull ($n = 94$)**	**Feral pigeon ($n = 16$)**	**Little penguin ($n = 9$)**	***P* value**
Amoxicillin-clavulanate	20.2	0	11.1	0.117
Ampicillin	86.2	87.5	100	0.771
Cefoxitin	16.0	0	0	0.154
Ceftriaxone	55.3	62.5	66.7	0.792
Chloramphenicol	21.3	18.7	22.2	1.000
Ciprofloxacin	47.9	62.5	66.7	0.371
Gentamicin	10.6	6.2	22.2	0.475
Imipenem	0	0	0	NA
Meropenem	0	0	0	NA
Streptomycin	45.7	56.3	22.2	0.274
Tetracycline	52.1	56.2	55.6	0.945
Trimethoprim-sulfamethoxazole	50.0	56.2	44.4	0.892

[a]Data are grouped according to bird species of origin, with associated interspecies carriage *P* value. No resistant isolates from bridled terns were detected.

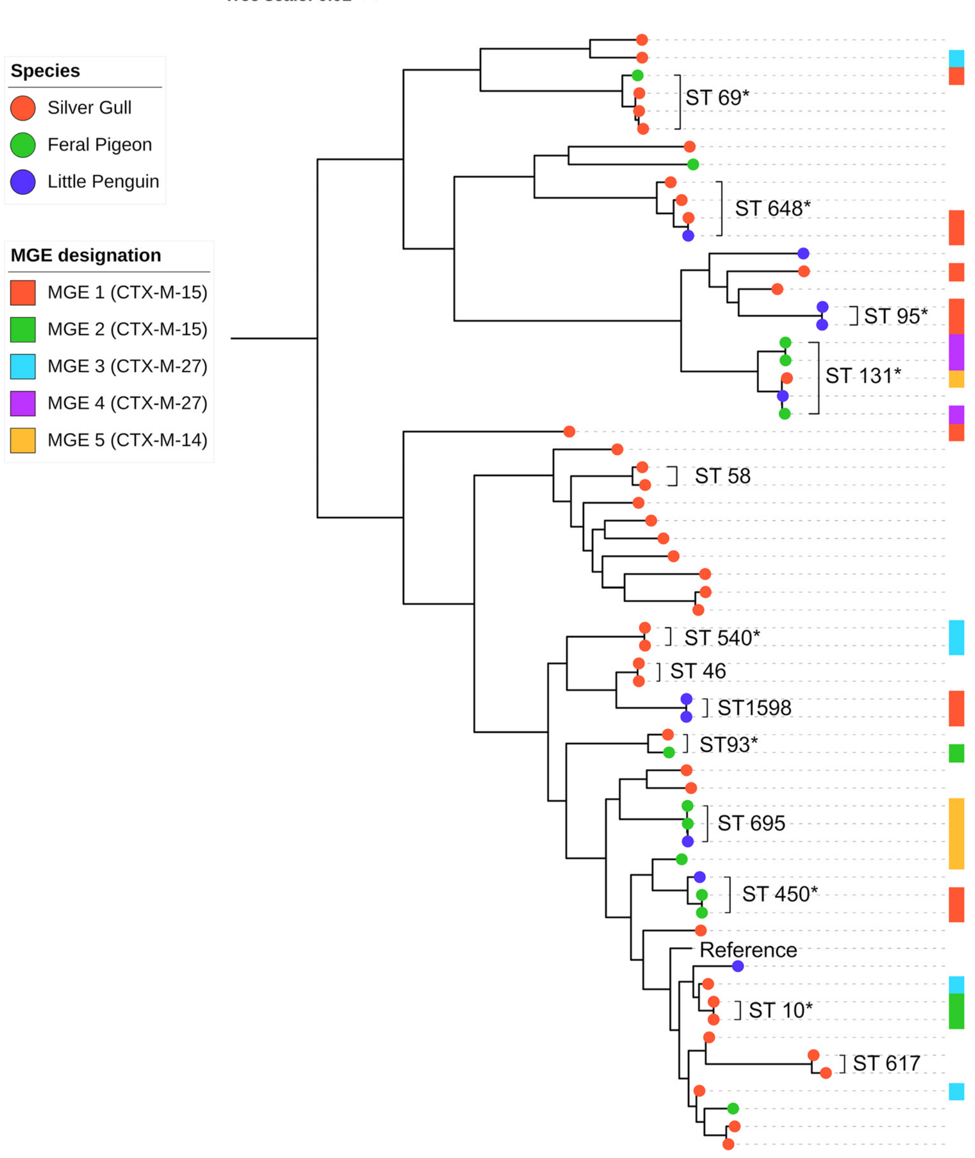

FIG 2 Maximum-likelihood midpoint-rooted phylogenetic tree representing clonal diversity among 61 *E. coli* isolates from silver gulls, feral pigeons, and little penguins on Penguin Island, Western Australia. The colored circles represent different bird species the *E. coli* strain was isolated from. The scale bar corresponds to ∼2,650 SNPs. The asterisks mark clinically significant human-associated CIA-resistant *E. coli* lineages. Mobile genetic element designations (1 to 5) are as follows: IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$-*ORF477*Δ (2,971-bp TU), *bla*$_{\text{CTX-M-15}}$-Tn*2*, IS*26*-*bla*$_{\text{CTX-M-27}}$-IS*903C*, IS*26*-*bla*$_{\text{CTX-M-27}}$-IS*903B*, and IS*Ecp1*-*bla*$_{\text{CTX-M-14}}$/IS*Ecp1*-*bla*$_{\text{CTX-M-14}}$-IS*903C*.

somally in all cases, based on MLplasmids analysis (see below). Five little penguin origin *E. coli* isolates carried the transposition unit, which was chromosomally integrated in three isolates and plasmid mediated in the remaining two (Fig. 3).

Searching for related mobile genetic elements using NCBI nucleotide BLAST revealed high levels of homology (single SNP differences only) between the 2,971-bp transposition unit seen in silver gulls, feral pigeons, and little penguins on Penguin Island and human clinical *bla*$_{\text{CTX-M-15}}$-positive *E. coli* isolates observed internationally, including a uropathogenic human ST131 isolate from Australia (GenBank accession no. CP036245.1).

A Tn*2* transposon that carried the complete *bla*$_{\text{CTX-M-15}}$ gene, a truncated IS*Ecp1* (potentially artificially truncated during contig assembly), and a partial *ORF477*Δ (MGE 2; 4,570 bp long) was found in two silver gull origin *E. coli* isolates and one feral pigeon origin isolate. This mobile genetic element was plasmid mediated in all cases (Fig. 3).

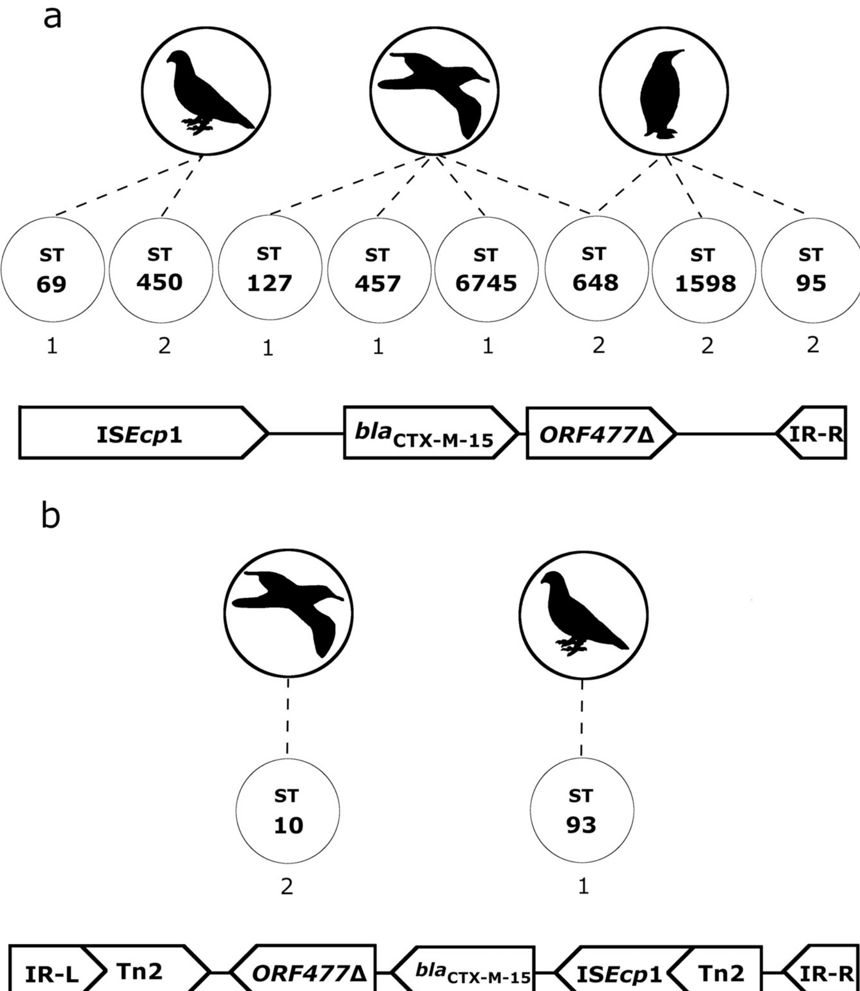

FIG 3 Schematic diagram of commonly observed mobile genetic elements containing $bla_{CTX-M-15}$. (a) MGE 1. (b) MGE 2. The source animals, sequence types of origin, and numbers of isolates are indicated. IR-L, left inverted repeat; IR-R, right inverted repeat.

An NCBI nucleotide BLAST search revealed that two *E. coli* isolates from the United States and one *E. coli* isolate from India carried very similar mobile genetic elements (<5-bp difference); however, the majority of relevant hits were noticeably different (<93% query coverage).

The $bla_{CTX-M-27}$ gene was detected in 5% (5/94) of silver gull origin *E. coli* isolates and 19% (3/16) of feral pigeon origin *E. coli* isolates. All $bla_{CTX-M-27}$-positive feral pigeon origin *E. coli* isolates were ST131. All five silver gull origin *E. coli* isolates harboring $bla_{CTX-M-27}$ contained the ESC resistance gene within the same IS*26*-mediated composite transposon (MGE 3; 1,511 bp long), but this mobile genetic element was present across four STs (ST38, ST540, ST43, and ST3572). Based on analysis using MLplasmids, the $bla_{CTX-M-27}$-associated composite transposon was found to be chromosomally integrated in two $bla_{CTX-M-27}$-positive isolates and plasmid mediated in the remaining three $bla_{CTX-M-27}$-positive isolates. Human clinical and livestock-associated *E. coli* isolates from Japan (2 isolates), Vietnam (1 isolate), and Sweden (1 isolate) have been found to contain identical mobile genetic elements, although the majority of relevant hits had <95% query coverage.

Two out of three feral pigeon origin *E. coli* isolates harboring $bla_{CTX-M-27}$ contained the same IS*26*-mediated composite transposon (MGE 4; 1,513 bp long), which slightly differed from the composite transposon observed in silver gulls (Fig. 4). It was located chromosomally in one isolate and plasmid mediated in the other. The third isolate

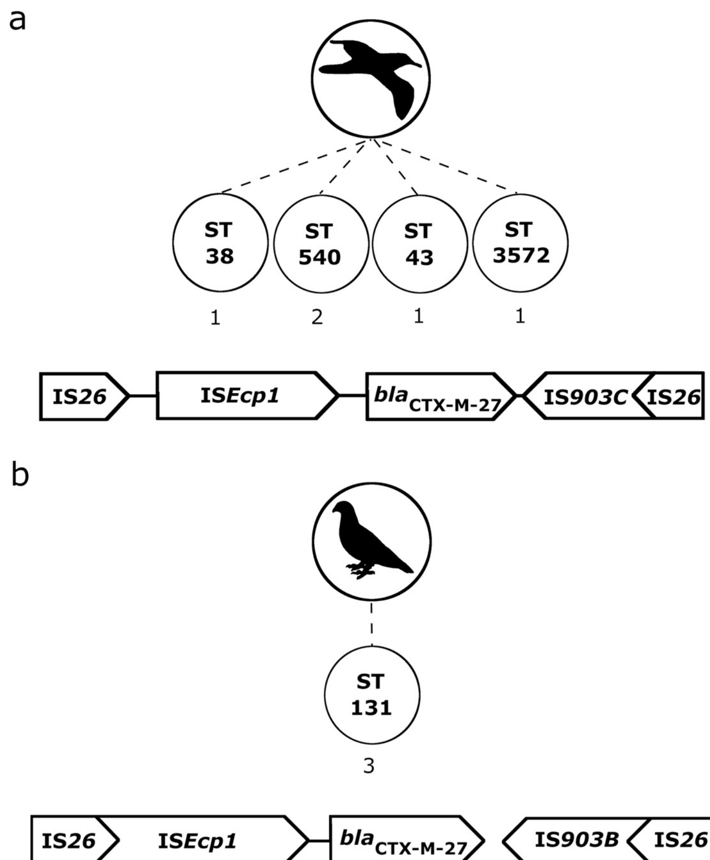

FIG 4 Schematic diagrams of both mobile genetic elements carrying *bla*$_{\text{CTX-M-27}}$, including source animals, sequence types, and total numbers of isolates. (a) MGE 3. (b) MGE 4.

harbored a similar mobile genetic element with minor differences of three and one base deletions in the IS*Ecp1* and IS*903B* sequences, respectively, but was otherwise identical to the composite transposon observed in the other two feral pigeon origin *E. coli* isolates harboring *bla*$_{\text{CTX-M-27}}$. Searches for similar mobile genetic elements using NCBI nucleotide BLAST found 20 isolates across North America, South America, Europe, and Asia containing identical mobile genetic elements.

The *bla*$_{\text{CTX-M-14}}$ gene was present in 3.2% (3/94) of silver gull origin *E. coli* isolates and was chromosomally integrated in all cases. Two of these isolates contained a complete IS*Ecp1* sequence located 42 bp upstream from *bla*$_{\text{CTX-M-14}}$, while one isolate contained only 204 bp of the 3′ end of IS*Ecp1* (potentially due to contig assembly), also located 42 bp upstream from *bla*$_{\text{CTX-M-14}}$. The *bla*$_{\text{CTX-M-14}}$ gene was also present in 12.5% (2/16) of *E. coli* isolates from feral pigeons and 10% (1/10) of little penguin origin *E. coli* isolates. Both *bla*$_{\text{CTX-M-14}}$-positive feral pigeon origin *E. coli* isolates and the little penguin origin *E. coli* isolate were ST695 (Fig. 5). All three isolates harbored the same mobile genetic element conferring resistance to ESCs (MGE 5), with the mobile genetic element comprising the entire sequence of IS*Ecp1* at the 5′ end and then *bla*$_{\text{CTX-M-14}}$ 42 bp downstream. Searches for similar mobile genetic elements using NCBI BLAST found that over 25 isolates across Asia, Europe, North America, and Asia contained a highly conserved element (1-bp difference). Among the isolates included in this study, the number of interisolate SNPs observed in all five mobile genetic elements was low. SNPs were present only in MGE 3 and MGE 5 and consisted of a single nucleotide difference in each, indicating that shared MGEs were highly similar among both the different *E. coli* lineages and the bird species.

Less prominent ESC resistance genes found in silver gulls included *bla*$_{\text{CTX-M-3}}$ and *bla*$_{\text{CMY-2}}$. One *E. coli* isolate from a silver gull harbored *bla*$_{\text{CTX-M-3}}$; however, the

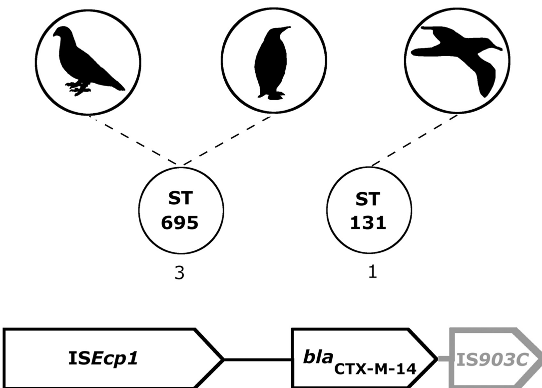

FIG 5 Schematic diagram of mobile genetic element carrying *bla*$_{CTX-M-14}$, including sequence type information, numbers of isolates, and source animals. The region of the mobile genetic element shown in gray was present only in ST695 isolates.

associated mobile genetic element was not detected in any other isolates across the three bird species containing *bla*$_{CTX-M}$ genes. Four silver gulls carried *bla*$_{CMY-2}$; however, the ESC resistance gene was not detected in feral pigeons or little penguins.

Plasmid transfer by conjugation. The conjugation experiment undertaken to evaluate the potential for horizontal transfer of resistance by conjugation under laboratory conditions revealed that 43.8% of the selected representative isolates ($n = 16$) were capable of transferring ESC resistance to donor *E. coli* J53, with a conjugation efficiency ranging from 10^{-2} to 10^{-6} (Table 2). Phenotypic antimicrobial susceptibility testing of transconjugants confirmed resistance to ceftriaxone. Conjugation led to the successful transfer of ESC resistance genes, such as *bla*$_{CMY-2}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, and *bla*$_{CTX-M-27}$. Conjugation also led to the transfer of resistance to other classes of antimicrobials, such as second-generation cephalosporins (cefoxitin), tetracyclines, and penicillins (ampicillin) (Table 2). In addition, an elevated MIC for fluoroquinolones (ciprofloxacin) was observed in two of the transconjugants that acquired plasmids from donor isolates that carried a plasmid-mediated quinolone resistance gene, *qnrS* (143CIP and 157ESB). The ciprofloxacin MIC (0.25 mg/liter) for the two transconjugants was categorized by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as non-wild type based on the epidemiological cutoff value (ECOFF) breakpoint compared to donor strain J53 (MIC < 0.015 mg/liter) (Table 2).

DISCUSSION

The key finding of this study was that in a discrete coastal environment where populations of four bird species exist in close cohabitation, three of them (silver gulls, feral pigeons, and little penguins) harbored *E. coli* strains resistant to CIAs while the remaining species (bridled terns) did not. The principal ecological species factor

TABLE 2 Donor and transconjugant characteristics of ESC-resistant *E. coli* isolates subjected to conjugation under laboratory conditions

Isolate ID	Characteristics			
	Donor		Transconjugant	
	Species of origin	AMR gene(s)	Transfer efficiency	AMR phenotype[a]
143CIP	Little penguin	*bla*$_{CTX-M-15}$, *qnrS*, *bla*$_{TEM-1}$, *tetA*	1.16×10^{-2}	AMP, CRO, CFT, CIP[b], TET
130ESB	Silver gull	*bla*$_{CMY-2}$, *bla*$_{TEM-33}$	1.11×10^{-2}	AMP, CRO, CFT, FOX
157ESB	Silver gull	*bla*$_{CTXM-15}$, *qnrS*	1.00×10^{-6}	AMP, CRO, CFT, CIP[b]
194ESB	Silver gull	*bla*$_{CTX-M-27}$, *strA*, *strB*, *sul2*	4.16×10^{-3}	AMP, CRO, CFT
219ESB	Feral pigeon	*bla*$_{CTX-M-15}$	2.48×10^{-3}	AMP, CRO, CFT
233ESB	Little penguin	*bla*$_{CTX-M-14}$	1.08×10^{-2}	AMP, CRO, CFT
288ESB	Feral pigeon	*bla*$_{CTX-M-14}$	7.51×10^{-4}	AMP, CRO, CFT

[a]AMP, ampicillin; CRO, ceftriaxone; CFT, ceftiofur; FOX, cefoxitin; CIP, ciprofloxacin; TET, tetracycline.
[b]Phenotypic resistance was categorized based on non-wild-type classification by ECOFFs.

explaining this difference is the distinctly different feeding behavior of bridled terns, which consists of foraging on natural food sources at sea distant from human populations.

This study further validates the findings from an earlier Australian study that described high levels of CIA-resistant *E. coli* carriage in gulls. Antimicrobial susceptibility testing confirmed that 36% (34/94) of *E. coli* isolates from gulls were resistant to ciprofloxacin, 43% (40/94) were resistant to ceftriaxone, and 12% (11/94) of isolates were resistant to both. These findings are similar to those of the previous survey. The frequencies of resistance to FQ and ESC among the *E. coli* isolates from feral pigeons were 25% (4/16) of isolates resistant to FQ or ESC and 38% (6/16) resistant to both antimicrobials. Resistance to either FQ or ESC antimicrobials was demonstrated by 33% (3/9) of the *E. coli* isolates from little penguins, while another 33% (3/9) of isolates were resistant to both FQ and ESC. Resistance to FQs was likely mediated by SNP accumulations in the QRDRs, as outlined in Fig. S2. The main resistance gene associated with ESC resistance isolated from all the bird species was $bla_{CTX\text{-}M\text{-}15}$, followed by $bla_{CTX\text{-}M\text{-}27}$ and $bla_{CTX\text{-}M\text{-}14}$. Other, less prevalent ESC resistance genes included $bla_{CTX\text{-}M\text{-}3}$ and $bla_{CMY\text{-}2}$. Other genes associated with resistance to less important antimicrobial classes were scattered among isolates, with no clear pattern based on species of origin or phylotype, and may have played some role in coselection for β-lactamase-harboring isolates.

Bioinformatics analysis revealed that each ESC resistance gene always occurred within the context of a mobile genetic element, with all CTX-M-type and CMY-type β-lactamase genes found downstream from an IS*Ecp1* insertion sequence and CTX-M-type β-lactamase genes associated with IS*26* insertion sequences. While not a direct indication of interspecies transmission, examination of these elements could be used as a proxy for mapping the movement of resistance, indicating that even without direct clonal transmission, horizontal transmission of the elements carrying the resistance markers may be responsible for the profiles seen across bird species.

Two mobile genetic elements carrying $bla_{CTX\text{-}M\text{-}15}$ were identified among silver gulls, feral pigeons, and little penguins and appear to be promiscuous based on their ability to be incorporated into multiple *E. coli* lineages. The most prevalent of these mobile genetic elements was found in all three bird species, while the other was found in two out of the three bird species. While ST648 harboring MGE 1 was common to gulls and penguins, the remaining MGEs were not clonally shared between species, further indicating that horizontal transmission of MGEs may be responsible for resistance profiles, rather than direct transfer and colonization of clones already harboring these elements. One mobile genetic element carrying $bla_{CTX\text{-}M\text{-}27}$ was found in all $bla_{CTX\text{-}M\text{-}27}$-positive silver gull origin *E. coli* isolates, while two different mobile genetic elements also carrying $bla_{CTX\text{-}M\text{-}27}$ were found in feral pigeon origin *E. coli* isolates. Two similar mobile genetic elements carrying $bla_{CTX\text{-}M\text{-}14}$ were found in the $bla_{CTX\text{-}M\text{-}14}$-positive silver gull origin *E. coli* isolates. A third distinct but structurally similar mobile genetic element carrying $bla_{CTX\text{-}M\text{-}14}$ was common to all $bla_{CTX\text{-}M\text{-}14}$-positive feral pigeons and little penguins. These findings provide further evidence suggesting transmission of AMR between the three bird species may be occurring through the horizontal gene transfer of mobile genetic elements in an interclonal and interspecies pathway. Further evidence of interspecies transmission of the CIA-resistant *E. coli* strains is the sharing of human-associated ExPEC clones, like ST131, ST69, ST95, and ST10, among the three affected bird species (17). This finding is consistent with those of an earlier study in which a high prevalence of ST131, ST69, and ST10 strains was observed among *E. coli* isolates from Australian silver gulls (14).

SNP-based analysis of each mobile genetic element of interest determined that very limited sequence diversity was observed, further supporting the hypothesis that common genetic elements are circulating throughout the ecosystem. However, comparison of each mobile genetic element of interest detected in the avian population on Penguin Island to similar mobile genetic elements found that SNPs were not common within these mobile genetic elements, regardless of geographical origin, with MGEs 1,

4, and 5 demonstrating high levels of similarity to previously recorded elements (≥99% query coverage and ≥99% similarity). The presence of these (or highly similar) circulating elements in international isolates (predominantly human clinical and livestock-associated *E. coli* isolates) precludes the ability to state that these elements circulate only in the Penguin Island birds. However, of the four bird species studied, only the bridled tern is migratory, and no resistant isolates were detected in the species, indicating circulation of these elements throughout the ecosystem is a more likely occurrence than migratory bird species introducing them from overseas. In contrast to MGEs 1, 4, and 5, MGEs 2 and 3 showed greater diversity than international elements, with similarity over 93 to 95% query coverage, giving further weight to the theory that these mobile elements are being transferred between selected bird species in the studied ecosystem.

Various investigations worldwide implicate gulls as reservoirs of high levels of CIA-resistant *E. coli*, which is primarily attributed to their scavenging behavior. This study found that carriage of CIA-resistant bacteria was significantly greater in gulls than in the other three species, making them a keystone species in AMR transmission throughout the environment. Although gulls are naturally opportunistic shoreline foragers around cities and towns, they have adapted to feeding primarily from landfill sites, sewage treatment plants, and urban wetlands, which elevates their risk of exposure to the CIA-resistant bacteria (18). In this case, the disposal of human refuse contaminated with human fecal flora (sanitary products) at landfill sites is hypothesized to be the most likely source of initial transference from human to bird populations, although other explanations, such as overflow of sewage effluents into coastal environments during periods of high rainfall, are also possible. Proximity to human activities has been shown to have a substantial impact on the intestinal flora of gulls and other wildlife (19–21).

Studies have demonstrated that *E. coli*, once released into the external environment through fecal deposition on the soil, sediments near marine coastal areas and can successfully replicate and survive for prolonged periods outside the host body in the secondary environment (22). The absence of resistant *E. coli* in bridled terns suggests that transmission may be occurring via contaminated water. The little penguins make landfall through the poorly mixed shallows around Penguin Island, where gulls concentrate, wash, and defecate, and these birds may be ingesting CIA-resistant *E. coli* bacteria from the shallow water at their landfalls or from contaminated colony soil. Coastal freshwater resources in artificial and natural wetlands used by both gulls and pigeons are the most likely location for transmission to feral pigeons.

The findings from this study have significant ecological implications, with the potential leapfrog movement of resistant *E. coli* mobile elements within a semicontained environment likely to be related to the feeding habits of different species and the ecological niche that supports them. In addition, it is reasonable to propose that more complex environments, such as those on the major avian flyways, would precipitate further mixing of species and transfer of resistance components originating from multiple countries, followed by dissemination. While this study did not investigate offshore dissemination of resistance, pigeons and gulls also have proximity to humans, livestock, and companion animals, which further increases the risk of transmission. It is noteworthy that the transfer of CIA-resistant *E. coli* from the pigeons and gulls is not confined or limited to the near-coastal areas, as these birds are frequent visitors to urban and human residential areas. Fecal droppings of pigeons and gulls carrying CIA-resistant *E. coli* strains can contaminate the surface water, as well as agricultural lands, and could be further transmitted to livestock or food-producing animals, humans, and other bird species.

There is no indication of health issues affecting seabirds harboring resistant bacteria; however, the study location at Penguin Island is a popular tourist destination and has a high number of visitors for coastal recreational activities. The fact that a commonly visited destination is contaminated with clinically significant, human-associated pathogenic, and CIA-resistant *E. coli* strains warrants further investigation to determine the

sources of contamination that the gulls encounter and to further clarify the process of amplification and transmission of these CIA-resistant *E. coli* strains within the adjacent bird colonies. In addition, larger-scale studies to determine the effectiveness of surveying MGEs as a historical roadmap of interspecies bacterial transmission will also assist in ongoing ecosystem-wide studies. Future studies should include large-scale resistome studies of indigenous microflora of the bird species and environmental samples, including freshwater, to evaluate the carriage and potential transfer of AMR genes.

MATERIALS AND METHODS

Sample collection. All sampling procedures were carried out under the approval of the Murdoch University Animal Ethics Committee (permit no. R3060/18). A total of 311 cloacal swabs were taken from the four bird species nesting or roosting on Penguin Island. The swabs were collected over a 6-month period (May to October 2018). The gulls and terns were captured using walk-in nest traps, while pigeons and penguins were captured at night, using headlamps and long-handled nets for the pigeons. Sampling was performed indoors wherever possible, and the birds were released into their colony areas within 5 min. Samples included cloacal swabs from 100 silver gulls, 57 little penguins, 102 feral pigeons, and 52 bridled terns. The swabs were collected into charcoal medium (Copan) and transported to the Murdoch University Antimicrobial Resistance and Infectious Diseases Laboratory in Perth, Western Australia, within 24 h of collection. To resuscitate the bacteria, the swabs were incubated for 4 h in 3 ml of buffered peptone water (Oxoid, ThermoFisher Scientific).

Bacterial isolation. Following nonselective enrichment in buffered peptone water, *E. coli* isolates resistant to FQ and ESC were identified by streaking the swabs onto two selective agar plates: MacConkey agar supplemented with 1 mg/liter ciprofloxacin (ThermoFisher Scientific) and Brilliance ESBL agar (ThermoFisher Scientific). Presumptive *E. coli* isolates (one colony per plate) identified on the selective agar after overnight incubation at 37°C were subcultured onto sheep blood agar (Micromedia, Australia), and pure colonies were obtained. Species identification was performed by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (Microflex; Bruker). Confirmed *E. coli* isolates were selected for further characterization. A flow diagram illustrating the sequence of experiments used in isolating and characterizing *E. coli* isolates resistant to critically important antimicrobials from the birds is shown in Fig. S1 in the supplemental material.

Phenotypic antimicrobial susceptibility testing. Antimicrobial disc diffusion testing using Clinical and Laboratory Standards Institute (CLSI) guidelines and recommended breakpoints for interpreting phenotypic AMR (23) was performed on all identified *E. coli* isolates ($n = 126$). The 12 antimicrobials used were ampicillin, ciprofloxacin, imipenem, meropenem, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, ceftriaxone, streptomycin, cefoxitin, amoxicillin-clavulanate, and chloramphenicol. *E. coli* ATCC 25922 was used as a control according to CLSI guidelines during antimicrobial susceptibility testing.

Phylogenetic analysis using PCR. DNA extraction was performed as described previously using 6% Chelex (Bio-Rad) (24). A multiplex sequence-type-specific PCR (ST PCR) was performed to identify clinically significant human-associated *E. coli* ST131, ST69, and ST95 (25). Electrophoresis was performed using 2% agarose gels containing 0.01% SYBRsafe dye (Invitrogen).

A multiplex PCR-based assay described previously (26) was performed to determine the phylogenetic groups of the resistant *E. coli* isolates. Following phylogenetic grouping, isolates belonging to each group were further delineated using a random amplified polymorphic DNA (RAPD) assay (27), and whole-genome sequencing (WGS) was performed on a subset with unique profiles.

Molecular characterization using whole-genome sequencing. Whole-genome sequencing was performed on all CIA-resistant *E. coli* isolates from little penguins and feral pigeons and on a subset of isolates from silver gulls ($n = 41$; 43.6%) selected based on RAPD, ST PCR, and AMR profiles. Sequencing was performed as previously described on an Illumina NextSeq 500 platform using a V2 midoutput 2-by-150 flow cell (24). FASTQ files underwent adapter trimming using BaseSpace (Illumina), and quality assessment was performed using FastQC v0.11.7 (28). FASTQ files that failed quality control were trimmed with Trimmomatic v0.38 (29) using the SLIDINGWINDOW setting, and reads where average quality dipped below a phred score of 25 across a 5-bp window were trimmed. *De novo* assembly was performed with SPAdes genome assembler v3.12.0 (30) using default settings. Contig files were uploaded to the Center for Genomic Epidemiology (http://www.genomicepidemiology.org) and parsed through the Bacterial Analysis Pipeline (31) to detect AMR genes and determine isolate multilocus sequence typing (MLST). SNPs within the QRDRs were detected using ABRicate (https://github.com/tseemann/abricate). Heatmap in R studio was used to map AMR gene presence and absence. Contigs containing AMR genes were uploaded to Galileo AMR (32; https://www.arcbio.com/amr) to annotate AMR-associated mobile genetic elements. Mobile genetic elements conferring resistance to ESCs were extracted and used for reference to mapping of the FASTQ read data using Bowtie2 v2.3.4.1 (33), and consensus sequences were collected using SAMtools v1.9 (34). Contigs were also uploaded to MLplasmids v1.0.0 (35) to infer whether each contig containing a mobile genetic element conferring resistance to ESCs formed part of the chromosome or plasmid. A phylogenetic tree was constructed to investigate the clonal similarity among CIA-resistant *E. coli* isolates from Penguin Island, first by producing a core SNP alignment using Snippy v4.1.0 (available from https://github.com/tseemann/snippy), using the *E. coli* K12 MG1655 chromosome (GenBank accession number NC_000913.3) as a reference (36). The core SNP alignment was then used for maximum-likelihood phylogenetic-tree construction with RAxML v8.2.11

(37) after regions of recombination were removed using ClonalFrameML v1.11 (37, 38). The final phylogenetic tree was annotated using iTOL v4.3.3 (39).

Key mobile genetic elements were aligned using MUSCLE and investigated for intraelement variations (SNPs) between isolates before being aligned against BLAST hits using the NCBI nr/nt nucleotide database to determine their presence and variation in international *E. coli* isolates.

Plasmid transfer by conjugation. Transfer of the resistance plasmid was performed by bacterial conjugation with the sodium azide-resistant *E. coli* J53 recipient strain, using a representative subset of ESC-resistant *E. coli* isolates ($n = 16$) carrying various β-lactamase gene variants of bla_{CTXM} and bla_{CMY-2} from all three bird species. Conjugation was performed using overnight culture of donor and recipient *E. coli* isolates grown in Luria broth (LB) (Thermo Fisher) at 37°C. Strains were mixed at a ratio of 1:2 (donor/recipient) and incubated for 4 h. Transconjugants were selected by serially diluting the donor-recipient mixture and immediately inoculating 5 μl (in triplicate) of serial dilutions (10^{-1} to 10^{-6}) onto MacConkey agar containing sodium azide (150 mg/liter) and/or ceftriaxone (8 mg/liter). The transconjugants were subcultured again onto MacConkey agar containing sodium azide (150 mg/liter) and ceftriaxone (8 mg/liter) and blood agar and subjected to MIC testing as previously described (24). The isolates were tested against the following 13 antimicrobials: amoxicillin-clavulanate, ampicillin, cefoxitin, ceftiofur, chloramphenicol, ciprofloxacin, colistin, ceftriaxone, florfenicol, gentamicin, streptomycin, tetracycline, and trimethoprim-sulfamethoxazole. MICs were interpreted using clinical breakpoints recommended by CLSI (23), and ECOFFs were interpreted using EUCAST guidelines (https://www.eucast.org/organization/). Conjugation efficiency was calculated by dividing the total colony count of transconjugants by the colony counts of donor cells.

Statistical analysis. Comparison of proportions of isolates and samples expressing resistance were made using contingency table analysis and Fisher's exact test in Stata SE v16.0 (StataCorp LLC, College Station, TX, USA).

Data availability. All sequence data were deposited in the NCBI database under accession number PRJNA613145.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

ACKNOWLEDGMENT

We thank the Citizen Science Program for collecting the bird samples.

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CHAPTER 4

Proximity to humans may explain the acquisition of antimicrobial resistant clinically significant *Escherichia coli* and *Klebsiella pneumonia* in Silver Gulls.

Statement of Authorship

Title of Paper	Proximity to human settlement is directly related to carriage of critically important antimicrobial-resistant *Escherichia coli* and *Klebsiella pneumoniae* in Silver Gulls
Publication Status	☑ Published ☐ Accepted for Publication ☐ Submitted for Publication ☐ Unpublished and Unsubmitted w ork w ritten in manuscript style
Publication Details	Mukerji, Shewli ; Sahibzada, Shafi ; Abraham, Rebecca ; Stegger, Marc ; Jordan, David ; Hampson, David J. ; O'Dea, Mark ; Lee, Terence ; Abraham, Sam. Proximity to human settlement is directly related to carriage of critically important antimicrobial-resistant *Escherichia coli* and *Klebsiella pneumoniae* in Silver Gulls Veterinary microbiology, 2023, Vol.280, p.109702-109702

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Name of Principal Author (Candidate)	Shewli Mukerji		
Contribution to the Paper	Performed experiments and recorded the data. Did the compilation, analysis and interpretation of the data generated. Wrote the manuscript. Responded to comments and suggestion of co-authors and journal editor.		
Overall percentage (%)	75%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	25th May 2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);
ii. permission is granted for the candidate in include the publication in the thesis; and
iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Contribution to the Paper	Supervised and assisted with data interpretation. Provided guidance and inputs in manuscript writing. Is the Corresponding author for this published article. Responded to editor's comment for revision.		
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Contribution to the Paper	Supervised and assisted with data interpretation. Provided guidance and inputs in manuscript writing. Helped in performing the whole genome sequencing of the selected isolates and the experiments involved in plasmid transfer by conjugation. Corresponding author for this published article. Responded to editor's comment for revision.		
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Contribution to the Paper	Provided guidance and inputs/comments to improvise the manuscript. Helped in comparative analysis of the whole genome sequencing data.		
Signature		Date	25th May 2023

Veterinary Microbiology 280 (2023) 109702

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Proximity to human settlement is directly related to carriage of critically important antimicrobial-resistant *Escherichia coli* and *Klebsiella pneumoniae* in Silver Gulls

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ARTICLE INFO

Keywords:
E. coli
K. pneumoniae
Proximity
Human population
Critically important antimicrobial
Resistance
Silver Gulls

ABSTRACT

Human population and activities play an important role in dissemination of antimicrobial resistant bacteria. This study investigated the relationship between carriage rates of critically important antimicrobial-resistant (CIA-R) *Escherichia coli* and *Klebsiella pneumoniae* by Silver Gulls and their proximity to human populations. Faecal swabs (n = 229) were collected from Silver Gulls across 10 southern coastline locations in Western Australia (WA) traversing 650 kms. The sampling locations included main town centres and remote areas. Fluoroquinolone and extended-spectrum cephalosporin-resistant *E. coli* and *K. pneumoniae* were isolated and tested for antimicrobial sensitivity. Genome sequencing was performed on n = 40 subset out of 98 *E. coli* and n = 14 subset out of 27 *K. pneumoniae* isolates to validate phenotypic resistance profiles and determine the molecular characteristics of strains. CIA-R *E. coli* and *K. pneumoniae* were detected in 69 (30.1 %) and 20 (8.73 %) of the faecal swabs respectively. Two large urban locations tested positive for CIA-R *E. coli* (frequency ranging from 34.3 % to 84.3 %), and/or for CIA-R *K. pneumoniae* (frequency ranging from 12.5 % to 50.0 %). A small number of CIA-R *E. coli* (3/31, 9.7 %) were identified at a small tourist town, but no CIA-R bacteria were recovered from gulls at remote sites. Commonly detected *E. coli* sequence types (STs) included ST131 (12.5 %) and ST1193 (10.0 %). Five *K. pneumoniae* STs were detected which included ST4568, ST6, ST485, ST967 and ST307. Resistance genes including $bla_{\text{CTX-M-3}}$, $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-27}}$ were identified in both bacterial species. High-level colonisation of CIA-R *E. coli* and *K. pneumoniae* in Silver Gulls in and around urban areas compared to remote locations substantiates that anthropogenic activities are strongly associated with acquisition of resistant bacteria by gulls.

1. Introduction

Globally, seagulls are thought to play a significant role in carriage and dissemination of pathogenic bacteria expressing resistance to critically important antimicrobials (CIA) (Franklin et al., 2020; Ahlstrom et al., 2018; Dolejska et al., 2016; Stedt et al., 2015). In Australia, Silver Gulls (*Chroicocephalus novaehollandiae*) are common native fauna of coastal environments with a tendency to congregate as large flocks in urban areas owing to the successful adaptation of scavenger-based foraging. An Australia-wide survey of this species demonstrated that urban seagulls have high levels of faecal carriage of fluoroquinolone and extended-spectrum cephalosporin-resistant (CIA-R) *E. coli*, with prevalence rates of 24 % and 22 % respectively (Mukerji et al., 2019). A study by Dolejska et al., 2016 also demonstrated high levels of transmission of Carbapenemase producing bacteria into wild birds due to anthropogenic activities. The sequence types (STs) identified were human-associated extra-intestinal pathogenic *E. coli* ST131 (clades O25:H4 *H*30-R and *H*30-Rx) that is reported globally, ST1193, and other clinically significant strains belonging to ST10, ST69, ST38, ST95 and ST450. These are known, in humans, to cause severe life-threatening infections such as septicaemia, gastroenteritis, urinary tract infection, neonatal meningitis and hospital acquired pneumonia (Mukerji et al., 2019). Another

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https://doi.org/10.1016/j.vetmic.2023.109702
Received 13 February 2022; Received in revised form 17 January 2023; Accepted 16 February 2023
Available online 17 February 2023
0378-1135/© 2023 Published by Elsevier B.V.

relatively recent study by Wyrsch et al. (2022a, 2022b) also detected more than 170 antibiotic -resistant lineages from 96 STs. 25 STs identified in the study harboured carbapenemase gene bla_{IMP-4} (Wyrsch et al., 2022a, 2022b). The potential role of seagulls as a wildlife vector for the spread of antimicrobial resistance (AMR) was further substantiated by an investigation into the transmission of resistant *E. coli* strains and associated mobile genetic elements (MGE) between different species of wild birds (including seagulls) that shared a coastal habitat adjacent to an urban environment (Mukerji et al., 2020). The study found a significantly higher frequency of CIA-R *E. coli* in seagulls (53 %) compared to penguins (11 %) and pigeons (10 %), with no CIA-R *E. coli* found in Bridled Terns. Another study detected plasmids carrying bla_{IMP-4} genes in *E. coli* ST216 that were related and identified in different members of Enterobacteriaceae from humans, companion animals and wildlife (Tarabai et al., 2021).

The widespread and elevated levels of carriage of CIA-R pathogenic *E. coli* of anthropogenic origins among seagulls in the absence of any direct exposure to antimicrobials raises concerns for public health since both the site of selection and source of exposure is uncertain (Atterby et al., 2016). Sewage, landfill refuse facilities and effluent from hospitals and nursing homes are hypothesised to be involved as exposure sources owing to the seagull's propensity to scavenge across varying urban environments (Smith and Carilile, 1993). While proximity to human activities is considered to be the primary reason for the transference of CIA-R bacteria to seagulls (Ramey and Ahlstrom, 2020; Oteo et al., 2018; Dolejska et al., 2016), there are only few studies such as by Wyrsch et al. (2022a, 2022b) that provide direct evidence in the form of comparison of rates of colonisation between birds found in areas of low and high density of human habitation.

One limitation of previous studies is that they focused solely on detection and evaluation of CIA-R *E. coli* carriage in wild birds and seagulls in particular. There are not many studies that clarify if seagulls also carry other bacterial species expressing clinically important forms of resistance, with the exception of one cross-sectional study that reported the carriage of antimicrobial resistant *E. coli and Salmonella* from Australian seagulls (Dolejska et al., 2016). Identification of other clinically significant species might act as a useful signal for ecological linkage between humans and seagulls. *Klebsiella pneumoniae* is another member of the Enterobacteriaceae family and is one of the ESKAPE pathogens (comprising *Enterococcus faecium*, *Staphylococcus aureus*, *K. pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) (Boucher et al., 2009). *K. pneumoniae* is an opportunistic pathogen causing sepsis, urinary tract infections, cystitis, surgical wound infections, and septicaemia in humans, causing high mortality rates and extended hospitalization (Effah et al., 2020). Like *E. coli*, *K. pneumoniae* demonstrates a high propensity to acquire resistance to critically important antimicrobials, making it difficult to treat. Moreover, there is good evidence that resistance determinants readily transfer amongst the *Enterobacteriaceae* family, including *Klebsiella* and *E. coli* (Huang et al., 2018). *K. pneumoniae* is in fact regarded as adept at acquiring resistance to CIAs, including carbapenems and is viewed as a prominent carrier and disseminator of resistance genes to clinically significant human pathogens from various environmental sources (Wyres and Holt, 2018).

In this study we hypothesise that the carriage of CIA-resistance, via *E. coli* and *K. pneumoniae*, in gulls is related to the density of the local human population, and that the level of carriage decreases with reduced proximity from anthropogenic activity. The validity of this hypothesis was tested by assessing a trend between the human population density and carriage of CIA-R *E. coli* and *K. pneumoniae* in seagulls.

2. Materials and methods

2.1. Sample collection

Samples were collected by swabbing freshly voided Silver Gull faecal droppings which then were transported in Ames Charcoal Media (Copan) to the Antimicrobial Resistance and Infectious Diseases Laboratory at Murdoch University for processing within four to five days of collection. Sampling was done at one time point (between January – December 2018) based on the availability of freshly voided faecal samples. A total of 229 samples were collected from 10 different southern coastline locations in Western Australia (WA). The sampling locations including main town centres and remote locations as shown in Table 1. Amongst the selected sampling locations, the Albany area (i.e. Albany township and adjacent Emu Point Beach) had the largest human population (36,583 as of 2016) (https://quickstats.censusdata.abs.gov.au/census_services/getproduct/census/2016/quickstat/C50246) and is WA's sixth largest town. The Esperance area (i.e. Esperance town centre and nearby Bandy Creek) was identified as densely populated location, with a population size of 14,236 (2016 census) (https://quickstats.censusdata.abs.gov.au/census_services/getproduct/census/2016/quickstat/C50246). The township of Denmark has a relatively low base population size (5845 as of 2016), although this increases by several times during the tourist season. All the other sites, comprising Conspicuous Cliff, Betty's Beach, Cheynes Beach, Lucky Bay, and Cape Arid have no permanent residents, although people visit for tourism. They were classified as remote regions due to their lack of permanent residents and distance from the nearest town centres (>10 km). The sampling sites and population density are shown on the map of WA (Fig. 1). This study was approved by Murdoch University Animal Ethics Office (Animal Ethics Cadaver/ Tissue Notification Permit No. 872).

2.2. Isolation

The isolation procedure included initial enrichment of swabs in three mL of buffered peptone water (ThermoFisher) for four hours. The enriched samples were streaked onto two selective agar plates to isolate resistant *E. coli* and *K. pneumoniae* and were incubated for 16–20 hrs at 37 °C. These plates were respectively screened for presumptive ciprofloxacin-resistant (MacConkey agar infused with 1 ug/mL ciprofloxacin, ThermoFisher) and ceftriaxone-resistant (Brilliance ESBL, ThermoFisher) Enterobacteriacae colonies. Presumptive CIA-R *E. coli* and *K. pneumoniae* (one colony per species per plate) were further subcultured on Sheep Blood Agar plates (ThermoFisher) and species identity confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Microflex, Bruker).

2.3. Antimicrobial sensitivity testing

Minimum inhibitory concentrations (MIC) for antimicrobials were determined for the 29 *K. pneumoniae* and 98 CIA-R *E. coli* isolates recovered from the selective plates. Broth micro-dilution was performed using the Robotic Antimicrobial Susceptibility Platform (RASP) (Truswell et al., 2021) as per Clinical Laboratory Standards Institute (CLSI) guidelines, with recommended breakpoints for interpreting phenotypic antimicrobial resistance being applied (CLSI, n.d.). In addition to ciprofloxacin and ceftriaxone, the panel included antimicrobials of low and high importance for public health including ampicillin, gentamicin, sulfamethoxazole/ trimethoprim, and tetracycline. The control culture *E. coli* ATCC 25922 was used as per CLSI guidelines (CLSI, n.d.). The MIC data were analysed using the EUCAST epidemiological cut-off value (ECOFF) wildtype breakpoints as indicators of resistance.

Samples were confirmed as positive for CIA-R *E. coli* or *Klebsiella* if they yielded growth of an *E. coli* or *Klebsiella* on the screening plates (based on species confirmation by MALDI-TOF MS) and demonstrated phenotypic resistance based on ECOFF to a either ciprofloxacin or ceftriaxone in the broth microdilution assays.

2.4. Whole genome sequencing

Whole genome sequencing (WGS) was performed on a subset of

Table 1
Sampling location details with number of swabs collected from each location.

Sampling Location	Region	Number of Swabs	Location Description	Distance from Main City/Townships	Human Population (2016)
Albany Town Centre	Urban	32	Port City	418 km south east of Perth	36,583
Albany Emu Point Beach	Urban	32	Suburb and tourist destination	8.5 km from Albany	316
Esperance Town Centre	Urban	61	Town	720 km from Perth, 482 km from Albany	14,236
Esperance Bandy Creek	Urban	10	North eastern suburb of Esperance	6 km from Esperance business district	304
Denmark	Semi-urban	31	Tourist township	423 km south-south-east of Perth	5845
Betty's Beach	Remote	11	Tourist destination	50 km east of Albany	0
Cape Arid	Remote	8	Tourist destination	731 km south east of Perth, 120 km east of Esperance	0
Cheynes Beach	Remote	35	Tourist destination	65 km east of Albany and 470 km south of Perth	0
Conspicuous Cliff	Remote	4	Tourist destination	13 km east of township of Walpole	0
Lucky Bay	Remote	5	Tourist destination	63.6 km east of Esperance	0

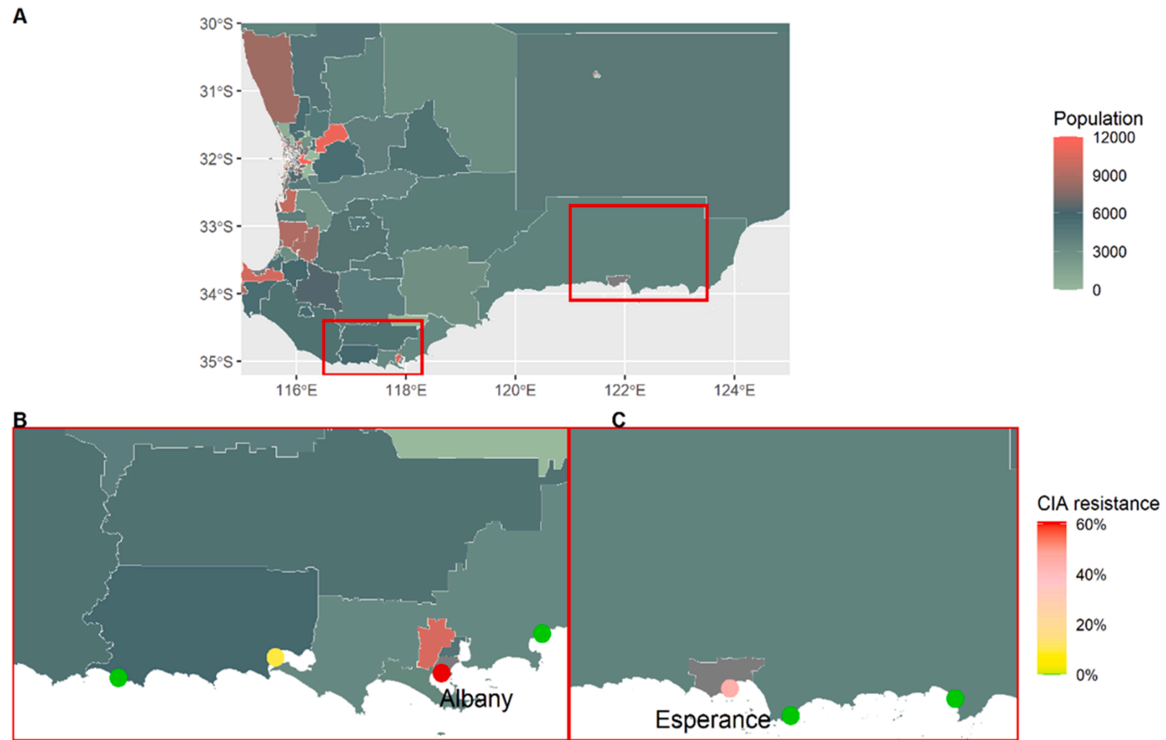

Fig. 1. Proportion of CIA resistance found in seagull faecal droppings collected from different sampling location in Western Australia (WA). A is a choropleth map showing the population density for WA, with the red box showing the area where sampling was performed. The inset map "B" is zoomed in on the sampling areas, with the proportion of CIA resistance shown in points (green=0 to red=100 %), while choropleths show the human population density. Resistance can be seen in the populated areas of Albany and Esperance.

confirmed CIA-R *K. pneumoniae* (n = 14) and CIA-R *E. coli* (n = 40) isolates based on susceptibility profiles. DNA extraction was performed from isolate cultures grown overnight on Sheep Blood Agar by using the MagMax DNA Multi-Sample extraction kit (ThermoFisher Scientific) according to manufacturer's protocol. Sequencing libraries were prepared using the Celero DNA-seq library preparation kit (Tecan) according to the manufacturer's protocol. Sequencing was performed on the Illumina NextSeq 550 platform using a Mid-Output 300 cycles Kit v2.5.

De novo assembly of the sequence data was performed using SPAdes v3.14.0 (Bankevich et al., 2012). Resistance and virulence genes were identified using ABRicate v1.0.1 (Seemann., 2021) with ResFinder (Zankari et al., 2012) and VFDB (Chen et al., 2016), based on the *de novo* assembled draft genomes. Identified resistance and virulence genes were considered present if they were at greater than 95 % coverage and identity. The structure of contigs harbouring resistance genes identified in both species were investigated further in Geneious Prime v2021. Multi-locus sequence types (ST) for each isolate were identified using the MLST tool (version 2.19.0) described by Torsten Seeman using the PubMLST data base (Seemann., 2019).

3. Results

3.1. Detection of CIA-R E. coli and K. pneumoniae

A total of 229 swabs were collected from selected sites. A total of 98 *E. coli* isolates and 27 *K. pneumoniae* isolates were recovered from the selective agar plates, but not all were confirmed to be CIA-R (i.e. resistant to ciprofloxacin and/or ceftriaxone) following microbroth MIC testing. Among the 229 swabs collected, 30.1 % (n = 69) were

confirmed positive for CIA-R *E. coli* and 8.73 % (n = 20) for CIA-R *K. pneumoniae.* Only 7.42 % (n = 17) of the swabs yielded both CIA-R *E. coli* and *K. pneumoniae.* None of the samples from remote areas yielded CIA-R *E. coli.* Seagull samples from the urban locations were positive for CIA-R *E. coli* at frequencies ranging from 34.3 to 84.3 % (Table 2). A relatively low rate of CIA-R *E. coli* (3/31; 9.7 %) was detected in the small semi-urban tourist town of Denmark (Table 2). CIA-R *K. pneumoniae* was detected only in the urban areas of Esperance and Albany, with frequencies ranging from 12.5 % to 50.0 % (Table 2). The overall proportion of resistance against CIAs was clearly higher in regions with higher human population density (Figs. 1 and 2).

3.2. Phenotypic and genotypic characteristics

3.2.1. E. coli isolates

Minimum inhibitory concentration (MIC) testing against a panel of six antimicrobials was performed on 98 *E. coli* isolates recovered from the selective plates. Resistance to ceftriaxone and ciprofloxacin was confirmed in 51 % and 84.7 % of the isolates respectively. High levels of resistance to ampicillin (98.0 %), tetracycline (64.3 %) and sulfamethoxazole/trimethoprim (51 %) were found, with 17.3 % of isolates demonstrating resistance towards gentamicin (Fig. 3).

Whole genome sequencing on a representative subset of CIA-R *E. coli* (n = 40) isolates revealed 28 different sequence types (STs) (Table 3). The most frequently detected were ST131 (12.5 %) and ST1193 (10.0 %) (Table 3). Beta-lactam resistance genes were found in all but one of the sequenced *E. coli* isolates, with bla_{TEM-1} being the most commonly found (50.0 %), followed by bla_{EC-5} (27.5 %), $bla_{CTX-M-15}$ (22.5 %) and $bla_{CTX-M-27}$ (12.5 %). Tetracycline resistance-associated genes were found among 64.1 % of the isolates, with *tet*(A) found most frequently (27.5 %) followed by *tet*(B) (25.0 %), and *tet*(D) and *tet*(M) (both at 5 %). A single isolate carried bla_{CMY-42}. Plasmid Mediated-Quinolone Resistance (PMQR) genes *qnr*S1 and *qnr*B4 were detected in 22.5 % and 7.5 % of the *E. coli* isolates, respectively. However, only two isolates with PMQR were associated with phenotypic resistance against ciprofloxacin.

Using PlasmidFinder, a total of 24 different plasmids were predicted among 39 of the 40 *E. coli* isolates. The most commonly identified plasmids were IncFIB(AP001918) (50.0 %), Col(BS512) (32.5 %), IncFIA (32.5 %) IncFII(pRSB107)_pRSB107 (22.5 %) and IncFII (17.5 %). ST131 carried the greatest number of different plasmids (30.0 %) when compared to other STs such as ST1193 (22.5 %), ST450 (20.0 %), and ST127 (17.5 %).

3.2.2. K. pneumoniae isolates

K. pneumoniae isolates (n = 27) exhibited high frequencies of resistance to ampicillin (100 %), sulfamethoxazole/trimethoprim (100 %), tetracycline (81.5 %), ceftriaxone (88.9 %) and ciprofloxacin (85.2 %) (Fig. 4). Only 18.5 % of *K. pneumoniae* isolates were resistant to gentamicin.

Genome sequencing of a subset of 14 CIA-R *K. pneumonia* isolates identified 5 different STs, comprising ST4568 (64.3 %), ST6 (14.3 %) and ST485, ST967 and ST307 (all at 7.1 %). Of the beta-lactamase genes, bla_{TEM-1} was the most frequently detected (92.8 %), followed by $bla_{CTX-M-3}$ (71.4 %), $bla_{SHV-187}$ (71.4 %) and $bla_{CTX-M-15}$ (21.4 %). Fosfomycin resistance genes (*fosA*) were also found in all isolates. Tetracycline resistance genes including *tet*(A), *tet*(B), *tet*(D), *tet*(M) were found in 78.5 % of the selected isolates. Macrolide resistance genes (*mph*) were identified among 71.4 % of isolates. PMQR genes were detected in 13 isolates, including *qnr*S1 (78.5 %) and *qnr*B1 (14.2 %). Only two isolates carried two different plasmids (ColRNAI, IncFIB(K)_Kpn3).

4. Discussion

The aim of the study was to investigate the relationship between the carriage of CIA-R bacteria by gulls and the proximity to human population and anthropogenic activities in the sampling areas. There are a number of critically important antimicrobials (WHO, 2018), but this study focused on resistance to two of the most important classes – quinolones (exemplified by ciprofloxacin) and 3rd and 4th generation cephalosporins (exemplified by ceftriaxone). The findings indicate a strong association between nearby human population and carriage of CIA-R bacteria by Australian silver gulls. The frequency of CIA-R *E. coli* carriage was high in the samples collected from the populated areas of Albany Town Centre and nearby Emu Point, and in Esperance Town Centre and nearby Bandy Creek (ranging from 34.3 % to 84.3 %) compared to remote locations in this study. *K. pneumoniae* CIA-R frequencies at these sites varied from 0 % to 50 % (Figs. 1 and 2). In contrast, in the small semi-urban tourist township of Denmark the carriage rate of CIA-R *E. coli* was much lower (9.7 %), and no CIA-R *K. pneumoniae* was identified. Most importantly, CIA-R bacteria were not recovered from any of the remote sites that did not have a permanent human population (Figs. 1 and 2). Accordingly, there was a clear correlation between the presence and density of human population and the occurrence of CIA-R *E. coli* and *K. pneumoniae* carriage by local gulls (Figs. 1 and 2). Fewer samples were obtained at the remote sites compared to the urban sites, and this reflected a much lower density of gull populations at the remote sites – meaning that it was more difficult to obtain larger numbers of fresh representative samples. The higher populations of gulls at the urban sites is likely to reflect more abundant food sources, but also increases the likelihood of transmission of CIA-R bacteria between the birds and into the local environment where humans may be exposed (Murray and Carrick, 1964). It is somewhat reassuring that the gulls in the remote sites away from human populations did not carry detectable CIA-R bacteria; however, they were only screened for *Enterobacteriaceae* possessing two classes of CIA, and it is possible that strains carrying resistance to other CIAs or first line

Table 2
Percentage of *E. coli* and *K. pneumoniae* isolated from seagull faecal droppings collected from different sampling location in Western Australia. The table shows total number of swabs collected from targeted regions and total confirmed CIA resistance in both *E. coli* and *K. pneumoniae* against ciprofloxacin (CIP), ceftriaxone (ESBL), and ciprofloxacin and/or ceftriaxone (CIA).

Location	Region	Swabs (n)	Number (n) of swabs positive (%)					
			*E. coli*_CIP (n = 64)	*E. coli*_ESBL (n = 44)	*E. coli*_CIA (n = 69)	*K. pneumoniae*_CIP (n = 17)	*K. pneumoniae*_ESBL (n = 19)	*K. pneumoniae*_CIA (n = 20)
Albany Town Centre	Urban	32	26 (81.2)	22 (68.7)	27 (84.3)	2 (6.2)	4 (12.5)	4 (12.5)
Albany Emu Point Beach	Urban	32	11 (34.3)	2 (6.2)	11 (34.3)	0	0	0 (0)
Esperance Town Centre	Urban	61	18 (19.6)	16 (22.9)	22 (36.1)	10 (11.4)	10 (11.4)	11 (18.0)
Esperance Bandy Creek	Urban	10	6 (60)	4 (40)	6 (60)	5 (50)	5 (50)	5 (50)
Denmark	Semi-Urban	31	3 (9.7)	0	3 (9.7)	0	0	0
Betty's Beach	Remote	11	0	0	0	0	0	0
Cape Arid	Remote	8	0	0	0	0	0	0
Cheynes Beach	Remote	35	0	0	0	0	0	0
Conspicuous Cliff	Remote	4	0	0	0	0	0	0
Lucky Bay	Remote	5	0	0	0	0	0	0

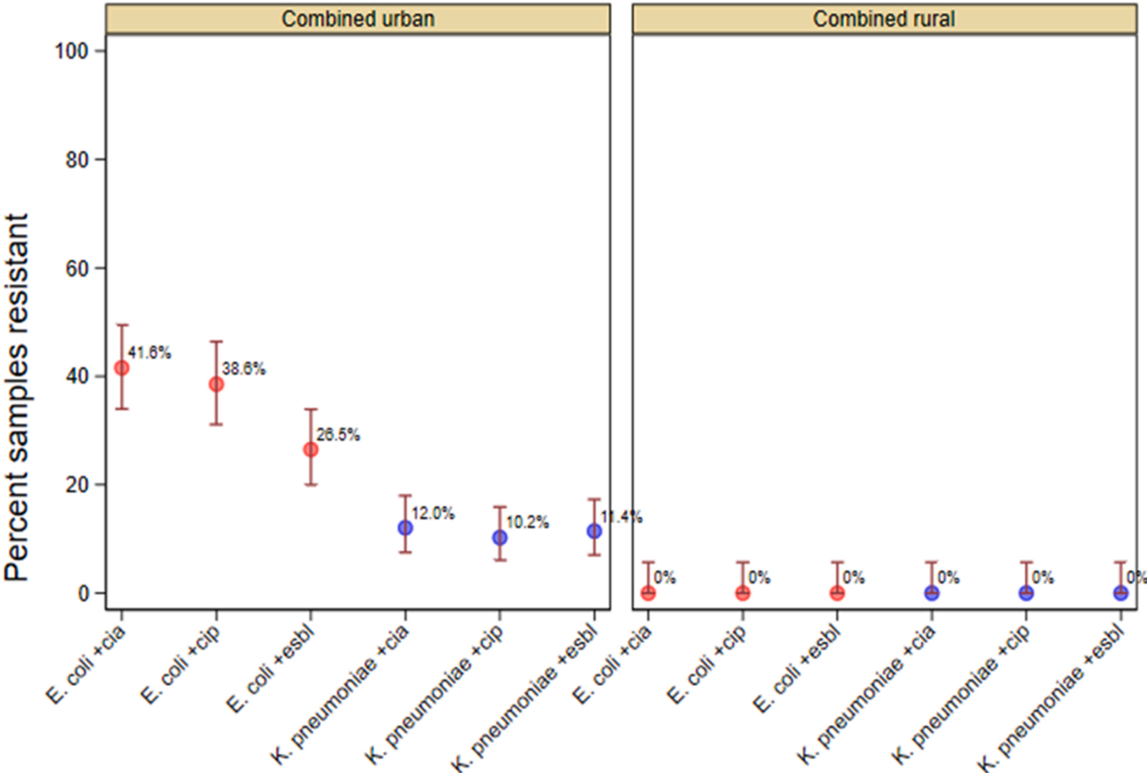

Fig. 2. Percent of seagull faecal swab positive (±95 % confidence intervals) for *E. coli* (red marker) and *K. pneumoniae* (blue markers) expressing resistance to ciprofloxacin (+CIA), extended spectrum beta lactams (+ESBL) and critically important antimicrobials (+CIA, either +CIP or + ESBL), with data combined for all urban (including semi-urban) and all remote locations.

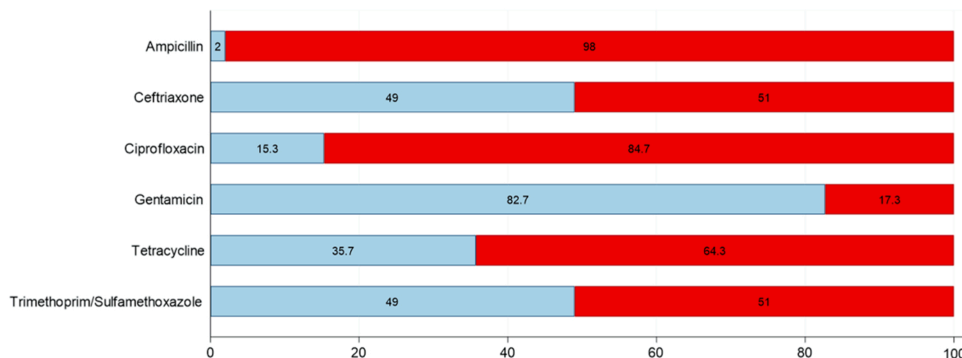

Fig. 3. Overall resistance of *E. coli* isolates recovered from seagulls on CIA-resistance screening agar plates from two urban and a semi-urban location in Western Australia. Interpretation is based on EUCAST ECOFF wildtype breakpoints. No CIA-R isolates were found in remote locations. A single isolate per sample was included in the analysis. Key: blue – percent wildtype, red - percent non-wildtype (resistant).

antibiotics went undetected.

Apart from ciprofloxacin and ceftriaxone, the other antimicrobials selected for MIC testing included ones that are also categorised as critically and highly important by the World Health Organisation (WHO). These antimicrobials belong to the major drug classes and included Ampicillin (aminopenicillins), Gentamicin (aminoglycosides), Tetracycline (chlortetracycline) and sulfamethoxazole/trimethoprim (sulfonamides, dihydrofolate reductase inhibitors and combination) (WHO, 2018). Rates of resistance to these antimicrobials were also high in the isolates recovered from urban areas, but their occurrence in remote areas could not be tested because no non-CIA-R isolates were available as a result of the screening method used.

The findings provide new details on the ecological distribution of CIA-R human pathogens in urban wildlife and consequently the increased risk for in-contact human populations or for colonisation of food-producing animals by exposure to gulls scavenging around livestock production enterprises. Studies have indicated that there is movement of CIA-resistant bacteria between humans, gulls and animals (Medvecky et al., 2022; Nesporova et al., 2020; Tarabai et al., 2021). Seagulls are frequent visitors to the food-animal enterprises in some remote areas, and so in these circumstances transmission of CIA-R *E. coli* or *K. pneumoniae* to animals via contamination of pasture, food troughs and/or watering points is a real prospect. In both cases there is scope for newly acquired CIA resistance from the environment to be amplified by use of antimicrobials for treatment of human or food-animal disease. There also is potential for detection of such organisms during surveillance or diagnostic investigation to be inappropriately attributed to emergence of CIA resistance in the corresponding host due to poor antimicrobial stewardship.

While the current study evaluates a section of coastline comprising

Table 3
Number of *E. coli* isolates identified according to sequence type in MLST, with their associated resistance genes and plasmids.

MLST	No of Isolates	Resistance Genes	Plasmids
10	1	*aadA*1, *aadA2*, bla_{TEM-1}, *cmlA*1, *dfrA*12, *qnrS*1, *sul*3, *tet*(M), bla_{EC}	IncR, IncX1
38	2	*aadA*1, bla_{TEM-1}, *sul*2, *aph* (3'')*-Ib*,*aph*(6)*-Id*, *catA*1, bla_{EC-8}, *dfrA*1, *sat2_gen*, *tet* (D)	IncFIB(AP001918), IncFII (pRSB107)_pRSB107
48	1	*aadA*2, bla_{TEM-1}, *dfrA*12, *floR*, *qnrS*1, *sul*1, *sul*2, *aph* (3'')*-Ib*, *aph*(3')*-Ia*, *aph*(6)*-Id*, *tet*(A), bla_{EC-15}	p0111
58	1	bla_{EC-18}, *qnrS*1	IncY
68	2	*sul*1 (1), *sul*2, *aph*(3'')*-Ib*, *aph*(6)*-Id*, bla_{EC}, *mph*(A) (1), *tet*(A), $bla_{CTX-M-27}$	IncFII
127	2	bla_{TEM-1}, *sul*2 (1), $bla_{CTX-M-15}$, bla_{EC-5}	IncFII,Col(BS512), IncFIA, IncFII(29)_pUTI89 (1)
131	5	bla_{TEM-1} (3), *sul*1 (1), *sul2* (1), *aadA*5 (1), *aph*(3'')*-Ib* (1), *aph*(6)*-Id* (1), *dfrA*17 (1), *mph*(A) (1), *tet*(B) (1), *tet*(A) (1), $bla_{CTX-M-15}$ (1), bla_{EC-5}, $bla_{CTX-M-27}$ (2)	IncFIB(AP001918) (3), IncFIA (3), IncFII(29) _pUTI89 (2), IncFII (pRSB107)_pRSB107 (3), Col (MP18) (1)
155	2	bla_{EC-18}, bla_{TEM-1} (1), *qnrS*1 (1), *tet*(A) (1), $bla_{CTX-M-15}$ (1)	IncFIB(AP001918) (1), IncFIB(K)_Kpn3 (1), IncY (1), IncI1_Alpha (1), IncX3 (1)
189	1	*floR*, *qnrS*1, *sul*2, bla_{EC}, *tet* (A), bla_{LAP-2}	IncFIB(AP001918)
196	1	*aadA*1, bla_{EC-18}, *qnrS*1, *tet* (A), *dfrA*14, *arr-2*, bla_{OXA-10}, *cmlA*5, *fosA7*0.5	IncFIB(AP001918), Col (BS512), IncY, IncFII(pSE11) _pSE11
200	1	bla_{EC-18}, *sul*1, *sul*2, *aph*(3'')*-Ib*, *dfrA*17,*mph*(A),bla_{DHA-1}, $bla_{TEM-235}$,*qnrB*4	IncFII,IncB/O/K/Z_2
224	1	bla_{EC-18}, *sul*2, *aph*(3'')*-Ib*, *aph*(3')*-Ia*, *aph*(6)*-Id*,*mph* (A), *tet*(A), *aac*(3)*-IId*,*dfrA7*	Col (BS512), p0111
405	1	bla_{TEM-1}, bla_{EC-8}, *aac*(3)*-IId*	Col (BS512), IncFII(29) _pUTI89
410	1	bla_{TEM-1}, *sul*2, *aadA*5, *aph* (3'')*-Ib*, *aph*(6)*-Id*, *dfrA*17, *mph*(A),*tet*(B), *aac*(6')*-Ib-D*181*Y*, bla_{OXA-1}, bla_{EC-15}, *aac*(3)*-IId*, bla_{CMY-42}, *catB*3	IncFIB(AP001918), IncFIA, IncFII(pAMA1167-NDM-5) _pAMA1167-NDM-5
450	2	bla_{TEM-1}, *catA*1, $bla_{CTX-M-15}$, bla_{EC-15}	IncFIB(AP001918), Col (BS512), IncFIA, IncFII (pRSB107)_pRSB107
453	1	bla_{TEM-1}, *tet*(B), $bla_{CTX-M-15}$, bla_{EC-13}	IncFII, IncFIA, IncX4
624	1	*sul*1, *sul*2, *aph*(3'')*-Ib*,*aph* (6)*-Id*, *dfrA*17, *tet*(B), bla_{DHA-1}, *qnrB*4, bla_{EC-19}	IncFIB(AP001918)
744	1	bla_{TEM-1}, *sul*1, *sul*2, *aadA*5, *aph*(3'')*-Ib*, *aph*(3')*-Ia*, *aph* (6)-Id, bla_{EC}, *catA*1, *dfrA*17, *mph*(A), *tet*(B)	NA
770	1	NA	ColpVC, IncFIB(AP001918)
963	1	*sul*1, *sul*2, *aph*(3'')*-Ib*, *aph* (6)*-Id*, *dfrA*17, *tet*(B), bla_{DHA-1}, *qnrB*4, bla_{EC-8}	IncFIB(AP001918)
998	1	bla_{TEM-1}, *tet*(B), bla_{EC-19}	IncFIB(AP001918)
1193	4	bla_{TEM-1} (1), *sul*2 (3), *aph* (3'')*-Ib* (3), *aph*(6)*-Id* (3), *dfrA*17 (3), *mph*(A) (1), *tet* (B) (3), *aac*(3)*-Iie* (2), *aac* (6')*-Ib-D*181*Y* (2), $bla_{CTX-M-15}$ (2), bla_{EC-5} (4), bla_{OXA-1} (2)	ColpVC (2), Col (BS512), IncFIA (3)
1431	1	bla_{EC-13}	IncFIB(AP001918)
2607	1	bla_{EC-18}, bla_{TEM-1}, *qnrS*1, *tet* (A)	IncFIB(AP001918), IncFIC (FII), IncX1_4
4213	1	bla_{EC-18}	IncFIB(AP001918), IncFII (pRSB107)_pRSB107
4493	1	*aadA*1, *aadA*2, bla_{EC-18}, bla_{TEM-1}, *cmlA*1, *dfrA*12, *dfrA*5, *floR*, *qnrS*1, *sul*1, *sul*2, *sul*3, *tet*(M)	IncFIB(AP001918), IncFIA (HI1)_HI1, IncFII, IncHI1A, IncHI1B(R27)_R27
7401	1	*sul*1, *sul*2, *aadA*5, *aph*(3'')*-Ib*,*aph*(6)*-Id*, *dfrA*17, *mph* (A), *tet*(A), bla_{EC-8}, $bla_{CTX-M-27}$	ColpVC, IncFIB (AP001918), Col (BS512), IncFIA, IncX4, IncFII(pRSB107)pRSB107
8889	1	*aadA*2, *dfrA*12, *qnrS*1, *sul*3, *tet*(A), bla_{EC-15}	Col (BS512), IncFIB(K)_Kpn3

approximately 650 km of seagull habitat, this is only small in relation to the entire coastline of Australia, and so there is a need to establish if the relationship between humans and CIA-R carriage in seagulls is more generally applicable. A barrier to working across such a vast expanse has hitherto been the capacity of laboratories to process meaningful numbers of samples and assessing sufficient isolates in adequate details. With the evolution of RASP technology, demonstrated in this study, it is clear that high throughput processing of colonies by laboratory robotics can succeed without sacrifice of measurement quality, thus making it possible now to undertake a continental-scale comparison of the CIA-R bacteria carriage rates in urban seagulls and humans.

This study further substantiates that avian species like gulls are not only limited to accumulating, amplifying, and disseminating CIA-resistant *E. coli*, but they can also disseminate other key Gram-negative bacteria that are pathogenic for humans, such as *K. pneumoniae* which is known for being highly virulent with a propensity to cause invasive infections (Li et al., 2014). The latter organism is highly adept at rapidly accumulating multi-drug resistance genes including those responsible for producing *K. pneumoniae* carbapenemase (KPC), New Delhi metallo-β-lactamase-1 (NDM-1) and other β-lactamase enzymes that make treatment of infections highly challenging due to lack of alternative options (Huang et al., 2018). Out of the five different *K. pneumoniae* STs identified in this study, ST485 and ST307 previously have been detected in wastewater treatment plants (WWTP) and clinical samples in other parts of the world (Surleac et al., 2020). *K. pneumoniae* ST307 has been described as a globally emerging lineage carrying multiple resistance plasmids transmissible to other *K. pneumoniae* STs, as well as to different bacterial species (Heiden et al., 2020; Lowe et al., 2019). ST307 isolates in this study harboured the clinically significant human-associated resistance gene $bla_{CTX-M-15}$. *K. pneumoniae* ST4568 was identified in this study, and although there is limited data on this organism's impact on humans, it was found to possess resistance genes such as $bla_{CTX-M-15}$ and $bla_{CTX-M-3}$.

The current study recorded similar observations as in a previous Australia-wide investigation of the carriage of CIA-R *E. coli* amongst seagulls. These included finding a high prevalence of *E. coli* ST131, a virulent human strain associated with fluoroquinolone FQ resistance and $bla_{CTX-M-15}$ type ESBL production, followed by rapidly emerging extraintestinal pathogenic *E. coli* (ExPEC) ST1193 and the presence of human associated resistance genes in these isolates (Mukerji et al., 2019). There are several other studies supporting the premise that anthropogenic contributions into the environment greatly influence the prevalence and carriage of antibiotic resistant bacteria by seagulls that inhabit the environment (Atterby et al., 2016; Ahlstrom et al., 2018; Atterby et al., 2017; Baez et al., 2015; Russo et al., 2021; Cummins et al., 2020), and that this is potentiated by the foraging habits of seagulls.

5. Conclusions

This study, conducted across a stretch of the southwestern Australian coastline, has corroborated an earlier, smaller scale study that indicated

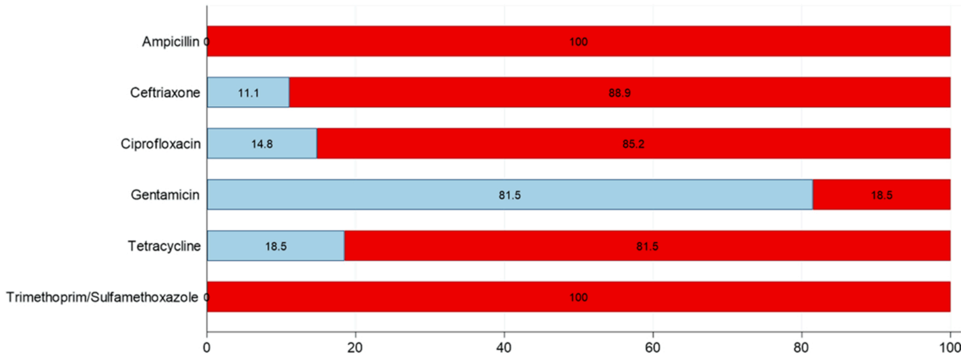

Fig. 4. Overall resistance of *K. pneumoniae* isolates from seagulls on CIA-resistance screening agar plates from urban locations in Western Australia. Interpretation is based on EUCAST ECOFF wildtype breakpoints. Non-wildtype isolates were only found at Albany town centre and at Esperance town centre and nearby Bandy creek. A single isolate per bird was included in the analysis. Key: blue – percent wildtype, red - percent non-wildtype (resistant).

that seagulls act as very efficient vectors in carrying and disseminating antimicrobial resistant bacteria, including clinically significant strains. Where the potential for interactions between humans and seagulls is greatest, colonisation with hazardous organisms occurs more frequently. Considering the ecological mobility of Silver Gulls and similar avian species, the findings point to a need to obtain a broader understanding of the pathways through which the resistant bacteria or genes enter these hosts and their subsequent fate in the environment following shedding in faecal material. Studies are required to understand and evaluate the need for measures that disrupt the pathway of contamination by reducing the accessibility of these species to 'exposure hot spots' such as human waste, hospital effluent and landfill sites.

Funding

This project was supported by the University of Adelaide and Murdoch University.

Declaration of Competing Interest

None to declare.

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CHAPTER 5

Infrequent antimicrobial resistance and limited sharing of commensal *Escherichia coli* strains found in Silver Gulls, Feral pigeons and Little Penguins inhabiting a common environment.

Infrequent antimicrobial resistance and limited sharing of commensal *Escherichia coli* strains found in Silver Gulls, Feral pigeons and Little Penguins inhabiting a common environment.

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Keywords: *Escherichia coli*; birds; diversity; antimicrobial resistance; microbial ecology

Abstract

Objectives: Numerous studies have implicated silver gulls as reservoirs of critically important antimicrobial (CIA) resistant *E. coli* and other clinically important bacteria. A previous study has demonstrated that gulls can easily acquire, amplify, and disseminate these CIA resistant *E. coli* to other cohabiting bird species. The aim of this study was to investigate whether in the same way as resistant strains, commensal *E. coli* strains are shared between bird species during their interactions while inhabiting a common environment.

Methods: Cloacal swabs were collected from 259 birds that included silver gulls, feral pigeons and little penguins. Out of the total confirmed *E. coli* isolates identified from CHROMagar^{TM} ECC agar, eight isolates from each of 30 Silver Gulls and 30 feral pigeons, and four to eight isolates from 15 little penguins were selected for further study of their antimicrobial resistance profiles and molecular characteristics.

Results: Out of the 595 *E. coli* isolates investigated, 69.4% of them were susceptible to all antimicrobials tested. A total of 12 different AMR profiles were identified. Ampicillin resistance was most prevalent (23%) among the *E. coli* isolates from all three bird species. Only one *E. coli* isolate from a gull was categorized as multi drug resistant (MDR), with an AMR score of 13. The predominant resistance genes detected included bla_{EC} (24.3%) and $bla_{\text{TEM-1}}$(12.4%), which are known to confer phenotypic resistance against β-lactams, followed by genes conferring resistance to cephalosporin including $bla_{\text{EC-18}}$ (22.6%), $bla_{\text{EC-5}}$ (21.5%) and $bla_{\text{EC-15}}$ (12.4%). Unique RAPD profiles ranging from one to eight per bird were identified amongst *E. coli* isolated from all three bird species.

Conclusions: This study found limited strain sharing of commensal *E. coli* strains between the three bird species inhabiting a common local environment. Only low levels of CIA resistance were detected among the *E. coli* isolates, indicating that the potential risk of transmission of these strains to humans by gulls and other feral birds may have been overestimated and use of selective media containing the antibiotics of concern would be important for selection and better representation of the resistant commensal *E. coli* strains.

Introduction

Escherichia coli is a common resident of the gastrointestinal tract (GIT) of mammals and birds (1). It is an opportunistic pathogen and its ability to cause disease in humans and animals is dependent upon the carriage of virulence genes that enable colonisation within a specific host (2). Different strains of *E. coli* belong to eight main phylogroups, of which A, B1, B2 and D are the most prevalent (3,4).

Wild bird species host a diverse gut microbiota including different strains of *E. coli* (5). The ability to fly allows birds to gain access to a wider range of environments and interactions than terrestrial mammals, with foraging habits, migratory behaviour, nestling/roosting habitats, and breeding habits having an impact on their gut microbiota - particularly when multiple bird species share the same environment (6). Several studies have established that gulls are frequent carriers of *E. coli* strains that are resistant to antimicrobials that are of clinical importance (7, 8). These resistant strains may be deposited into the environment via faecal droppings and subsequently disseminated to other bird species which share the same environment (8). Through this transmission route, different bird species that occupy different ecological niches but congregate in a common environment may share the same resistant *E. coli* strains.

Furthermore, once deposited via faecal droppings, *E. coli* can survive for prolonged periods in secondary environments like soil or sediments in coastal areas (9). Being genetically flexible and adaptable, *E. coli* can acquire antimicrobial resistant determinants from these secondary environments by horizontal gene transfer (HGT) and mobile genetic elements (MGE) (9). Although *E. coli* has been extensively studied, most investigations have focused on understanding the genetic characteristics of pathogenic *E. coli* (6). There is limited data on the genomic characteristics of commensal *E. coli* that colonise the GIT of wild birds, as most of these strains have little clinical importance to human health. Studies have established that although there are several transmission routes for pathogenic *E. coli,* the primary route is via the faecal-oral pathway with further dissemination being facilitated by environmental contact (contaminated water, soil and food) (10). Similarly, proximity of species and direct contact with the host can contribute to the dissemination process (10).

In avian species, key factors that influence the frequency of resistant *E. coli* carriage are proximity to human populations, foraging habits, and body weight (11 - 14). Furthermore, evidence supports variability in commensal *E. coli* strains colonising different sections of the GIT within a single host (15). This is attributed to the genetic flexibility of *E. coli* strains whereby different strains are equipped with specific genes that allow them to adhere to specific cell receptors that occur in different regions of the GIT (15). Although avian species like gulls, pigeons and penguins are not directly exposed to antimicrobials, selection pressure still exists on the intestinal microbiota due to the presence of antimicrobial contaminants in the environment (16). These contaminants exert selection pressure on survival of resistant pathogenic and commensal *E. coli* which leads to acquisition of further resistance determinants (17).

In our previous study using selective agar plates incorporating antimicrobials, we demonstrated that critically important antimicrobial (CIA) resistance genes are shared between birds that co-habitat the same geographical area (7, 8). The genetic diversity and the ability to propagate in a similar way to pathogenic *E. coli* make commensal *E. coli* a better alternative to elucidate the transmission routes and the intensity of strain sharing. This study examined the hypothesis that the commensal *E. coli* strains carried by avian species inhabiting a common environment are shared. Although *E. coli* strains are known for host specific colonisation there are certain strains that have a broad host range (7) and thus these can be expected to colonise more than one avian species. A secondary aim of this study was to determine the antimicrobial resistance profiles and molecular characteristics of the commensal *E. coli* strains isolated from the three bird species – silver gulls, feral pigeons and little penguin sharing a common semi-urban environment, and to evaluate the diversity of antimicrobial resistant commensal *E. coli* between and within bird species. These commensal strains are more likely to be deposited in the environment in significant loads due to a higher cfu/gm than the less prevalent CIA resistant clones.

Materials and Methods

Sample Collection

Sampling was performed following the procedure described previously (8), with the approval of the Murdoch University Animal Ethics Committee (permit no R3060/18). Cloacal swabs were collected from 259 birds, consisting of 100 Silver Gulls, 57 Little Penguins and 102 feral pigeons nesting or roosting in Penguin Island (32°18'S, 115 41'E) just off the coast of Western Australia 53.0 km south of Perth (8). Samples from a subset of 30 birds from each species were used in the current study.

Isolation

Upon arrival at the Murdoch University Antimicrobial Resistance and Infectious Diseases Laboratory in Perth, swabs were incubated for 4 hours in 3 mL of buffered peptone water (OXOID, ThermoFisher Scientific) to resuscitate the bacteria. After incubation, each culture underwent 10-fold serial dilution to 10^{-6}, and three dilutions (10^{-4}, 10^{-5}, 10^{-6}) were streaked onto CHROMagar™ ECC agar (Chromoagar) (Edwards Group, Australia) using a Robotic Antimicrobial Susceptibility Platform (RASP) (18). All agar plates were incubated for 16 to 20 hours at 37º C. Presumptive identification of *E. coli* on agar was performed based on colony colour according to the manufacturer's instructions. Eight *E. coli* colonies from each plate were isolated using RASP (18). Each isolate was inoculated into a well of a 96 deep well plate with 1000 uL Luria-Bertani (LB) broth (OXOID, ThermoFisher Scientific) and incubated for between 16 to 20 hours at 37º C. After incubation, 2 µL of each culture suspension was spot- inoculated onto LB (OXOID, ThermoFisher Scientific) agar square plates and incubated for another 16 to 20 hours at 37º C. Growth on each spot was subjected to species identification by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Microflex, Bruker). The remaining culture suspension was stored at -80 º C for further antimicrobial susceptibility testing and genomic sequencing.

Out of the total confirmed *E. coli* isolates identified, 595 isolates from 30 birds from each species were selected for further characterisation. These comprised of 240 *E. coli* isolates each recovered from gulls and feral pigeons and 115 isolates from little penguins.

Antimicrobial sensitivity testing

Minimum inhibitory concentrations (MIC) for antimicrobials in confirmed *E. coli* isolates were determined using the microbroth dilution technique on RASP (18) following the Clinical and Laboratory Standards Institute (CLSI) guidelines (19). The antimicrobials for MIC testing were selected to cover multiple antimicrobial classes and included ampicillin, ciprofloxacin, ceftriaxone, gentamicin, trimethoprim/sulfamethoxazole and tetracycline. *E. coli* ATCC 25922 was used as a control as recommended in CLSI guidelines for all MIC testing. The MIC data were interpreted using epidemiological cut-off (ECOFF) values according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (https://www.eucast.org/).

In this study, isolates classified as wild type are referred as susceptible while those classified as non-wild type are referred as resistant. Analysis and graphing of data were conducted using Stata (v15.1).

AMR index scheme

Based on public health significance, the Australian Strategic and Technical Advisory Group (ASTAG) have divided the antimicrobials into three categories depending on their level of importance – high, medium and low (20). The AMR index is based on the cumulative sum of the antimicrobial weightings towards which the isolate demonstrates resistance (Table 1) (21).

Table 1. AMR index scheme based on weight of each antimicrobial and ASTAG Australian importance rating.

Antimicrobial	Australian Importance Rating	Weight
Ampicillin	Low	1
Ceftriaxone	High	3
Ciprofloxacin	High	4
Gentamicin	Medium	2
Trimethoprim-sulfamethoxazole	Medium	2
Tetracycline	Low	1

Random Amplified Polymorphic DNA Analysis (RAPD)

DNA extraction was performed on the 595 isolates using 6% Chelex (Bio-Rad), as described previously (22). The extracted DNA was subjected to a multiplex PCR-based assay as described by Clermont et al., 2013 (23) to determine the phylogenetic group of the commensal *E. coli* isolates followed by further delineation of isolates belonging to each group by performing RAPD assays (24).

Whole Genome Sequencing (WGS) and analysis

WGS was performed on a subset of isolates with unique RAPD profiles as previously described (16). A total of 169 isolates (gulls 90; feral pigeons 44; little penguins 35) were subjected to WGS. Sequencing files were assembled using Spades (v3.14.0) (https://github.com/ablab/spades/tree/v3.14.0) with multilocus sequence types (MLSTs) determined using MLST (v2.15) (https://github.com/tseemann/mlst).

New sequence types (STs) were assigned through EnteroBase (https://enterobase.warwick.ac.uk/).

Abricate was used to determine the presence of antimicrobial resistance genes using default settings (https://github.com/tseemann/abricate).

Abricate was additionally used to detect virulence genes using the *E. coli* virulence finder database with coverage and identity of >95% and >99%, respectively.

Results

Antimicrobial susceptibility

Antimicrobial susceptibility testing was performed on 595 *E. coli* colonies. This included eight *E. coli* isolates from each 30 gulls and 30 feral pigeons, but only between four and eight isolates from 15 little penguins due to low recovery rates on selective agar. Among the *E. coli* isolates, ampicillin resistance were the most common, with 23% of *E. coli* from across the three bird species demonstrating resistance to ampicillin (Figure 1). A total of 12 different AMR profiles were identified. A total of 69.4% of isolates were susceptible to all antimicrobials tested and therefore designated an AMR index score 0.

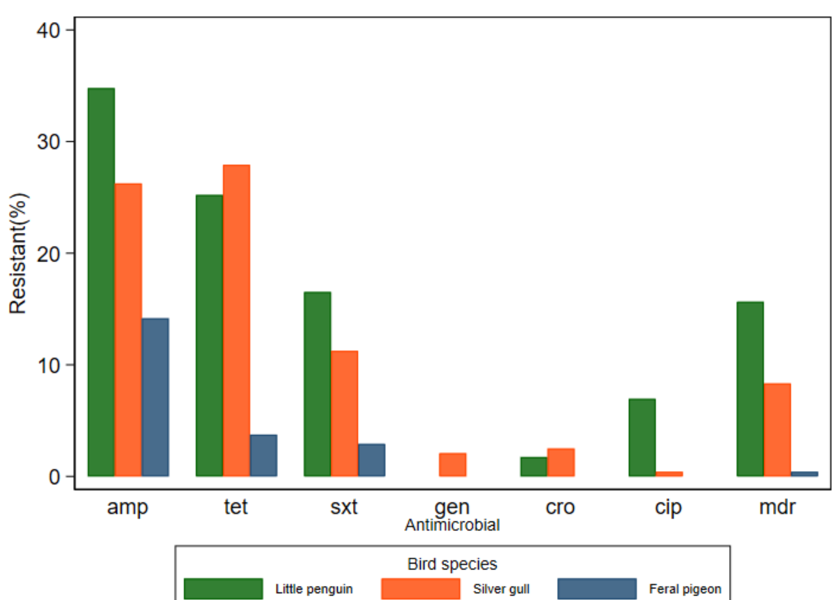

Figure 1. Variation in the percent of isolates resistant towards each antimicrobial across bird species. Abbreviation of antimicrobials: amp = ampicillin, tet = tetracycline, sxt = trimethoprim/sulfamethoxazole, gen = gentamicin, cro = ceftriaxone, cip = ciprofloxacin, and mdr = multi-drug resistant

Individual bird species – AMR diversity

Among *E. coli* isolated from gulls (n=240) the overall prevalence of resistance to tetracycline, ampicillin and trimethoprim/sulfamethoxazole were high at 27.9%, 26.3% and 11.3% respectively. Resistance to ceftriaxone and gentamicin was detected in 2.5% and 2.1% isolates respectively. Only a single isolate was found to be resistant to ciprofloxacin.

The AMR index used to evaluate the carriage of overall resistance in individual isolates revealed that 61.7% of the *E. coli* isolates from gulls had an AMR index score of 0 (Figure 1). Among the gull *E. coli* isolates, only one was assigned the highest AMR index score of 13, demonstrating resistance to more than three antimicrobial classes and therefore categorized as multi drug resistant (MDR). One isolate was assigned an AMR index score of 9 and three isolates were identified with a score of 8 (Figure 2).

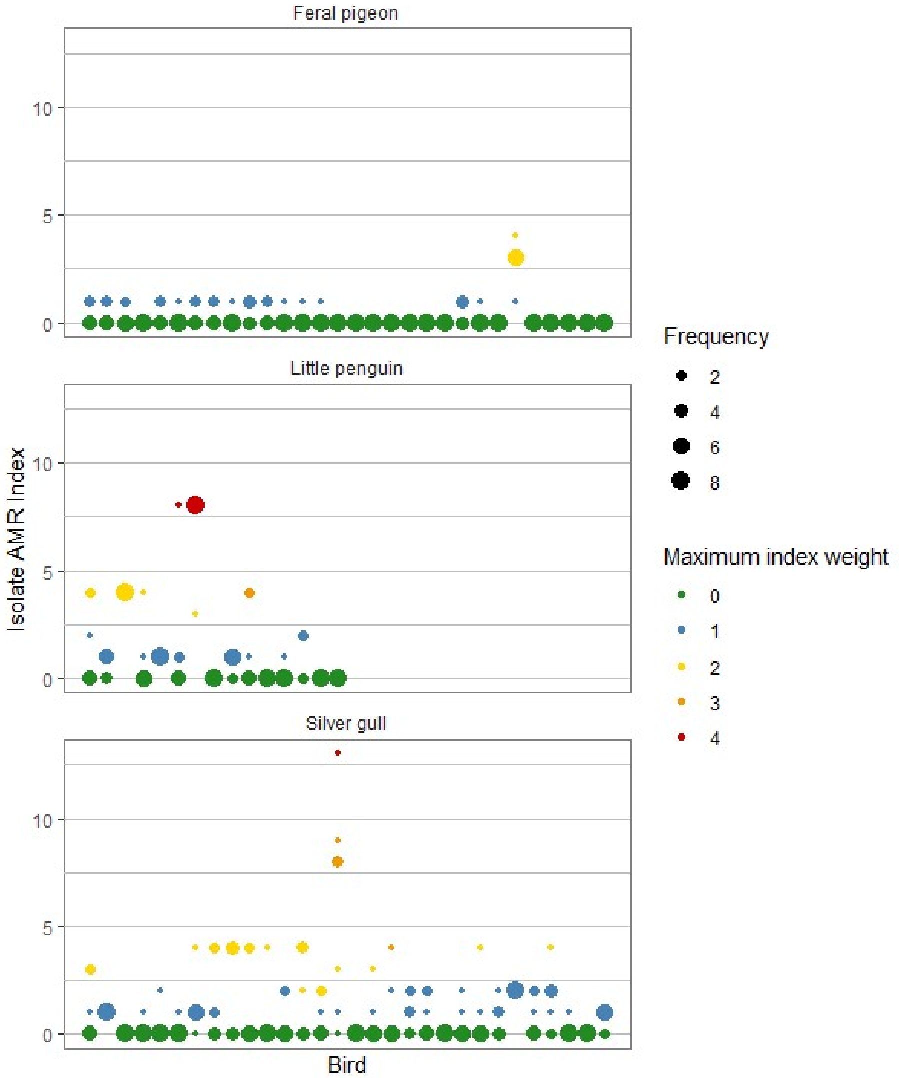

Figure 2. Variation in the antimicrobial index for isolates within individual birds distributed amongst three bird species. Bird number is across the x-axis and isolate AMR index is on the y-axis. Displayed also is the number of isolates, and the index weighting of the highest antimicrobial resistance detected in each isolate.

Amongst the 240 *E. coli* isolates from feral pigeons the highest AMR index score detected was 4. Overall, 82.5% of isolates were susceptible to all antimicrobials, having an AMR index score of 0. Four different AMR profiles were identified in these isolates. The prevalence of resistance was in the order ampicillin (14.2%), tetracycline (3.8%) and trimethoprim/sulfamethoxazole (2.9%). None of the isolates demonstrated resistance to ciprofloxacin, gentamicin, or ceftriaxone.

An AMR index score of zero was ascribed to 58.3% of the115 *E. coli* isolates from little penguins. The highest AMR score was 8. Some isolates were resistant to ampicillin (34.8%), tetracycline (8.7%), trimethoprim/sulfamethoxazole (7.8%), ciprofloxacin (6.9%) or ceftriaxone (1.7%). Gentamicin resistance was not observed in any of the isolates.

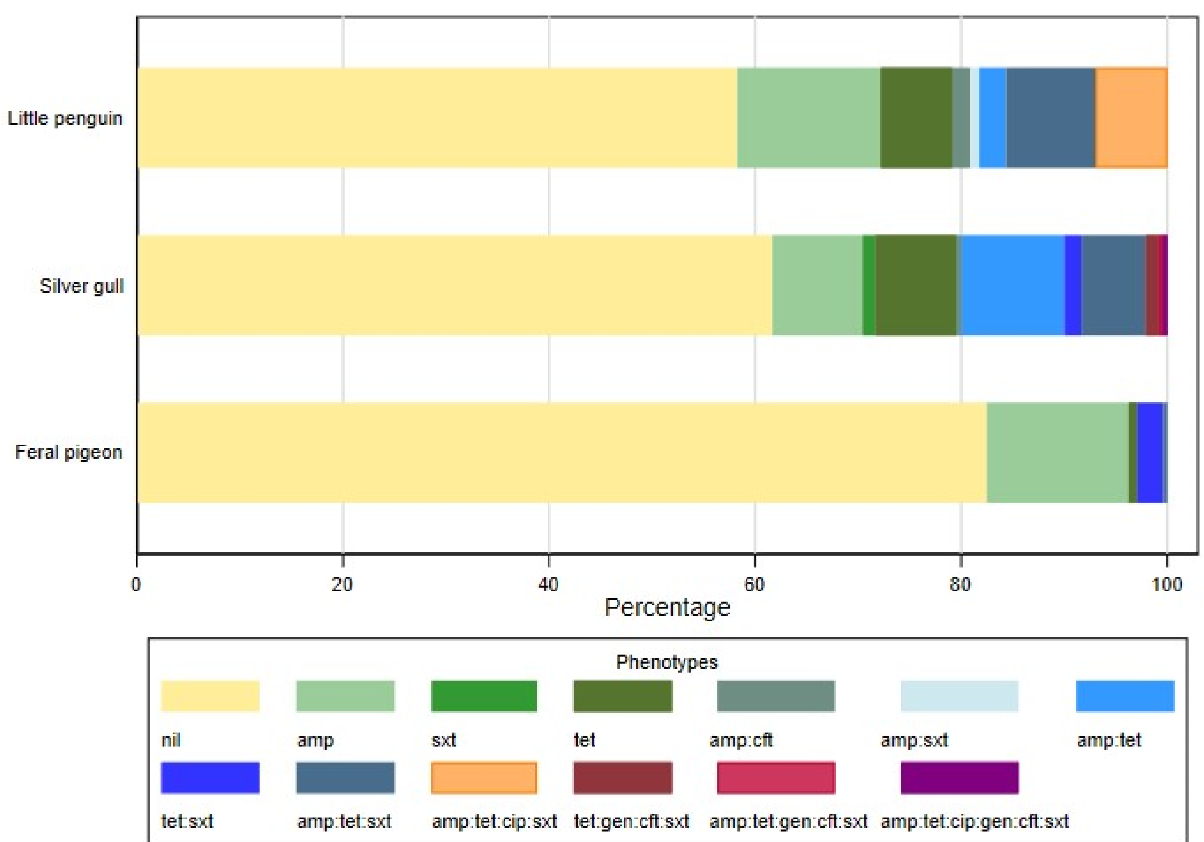

Figure 3. Antimicrobial resistance phenotypes of isolates (n=595) within and across three bird species. Abbreviation of antimicrobials: cip = ciprofloxacin, cft = ceftriaxone, tet = tetracycline, amp = ampicillin, gen = gentamicin, sxt = trimethoprim/sulfamethoxazole

The most prevalent AMR profile common in all three bird species were amp and amp:tet:sxt. The AMR profiles common only between gulls and little penguins comprised of amp:cft and amp:tet. (Figure 3).

The most common resistance genes detected in the *E. coli* isolates included bla_{EC} (24.3%) and bla_{TEM-1}(12.4%) known to confer phenotypic resistance against β-lactams, followed by genes conferring resistance to cephalosporin including bla_{EC-18} (22.6%), bla_{EC-5} (21.5%) and bla_{EC-15} (12.4%). Table 2 shows the significant resistance genes detected in the commensal *E. coli* isolates.

Table 2: The frequency of the resistance conferring genes identified and the number of isolates positive in each bird species.

Resistance Genes	**Antimicrobial**	**No of isolates (%)**	**Number of isolates detected in each bird species**		
			Silver Gulls	**Feral pigeons**	**Little Penguins**
aadA1	Streptomycin	4 (2.3)	-	-	4
aadA2	Streptomycin	2 (1.1)	-	-	2
aadA5	Streptomycin	2 (1.1)	-	2	-
aph(3")-Ib	Streptomycin	12 (6.8)	4	1	7
aph(6")-Id	Streptomycin	12 (6.8)	4	1	7
$bla_{CTX-M-15}$	Cephalosporin	1 (0.6)	-	1	-
bla_{EC-15}	Cephalosporin	22 (12.4)	20	2	-
bla_{EC-18}	Cephalosporin	40 (22.6)	10	24	6
bla_{EC-19}	Cephalosporin	5 (2.8)	5	-	-
bla_{EC-5}	Cephalosporin	38 (21.5)	21	4	13
bla_{EC-8}	Cephalosporin	19 (10.7)	8	10	1
bla_{EC}	β-lactam	43 (24.3)	24	9	10
bla_{TEM-1}	β-lactam	22 (12.4)	9	1	12
$bla_{TEM-235}$	β-lactam	1 (0.6)	1	-	-
catA1	Chloramphenicol	8 (4.5)	3	-	5
cmlA	Chloramphenicol	2 (1.1)	-	-	2
dfrA1	Trimethoprim	2 (1.1)	-	-	2
dfrA14	Trimethoprim	1 (0.6)	-	1	-
dfrA17	Trimethoprim	5 (2.8)	1	2	2
dfrA5	Trimethoprim	5 (2.8)	3	-	2
sul1	Sulfonamide	3 (1.7)	1	-	2
sul2	Sulfonamide	14 (7.9)	6	3	5
sul3	Sulfonamide	2 (1.1)	-	-	2
qnrS1	Quinolone	2 (1.1)	-	-	2
tet(A)	Tetracycline	18 (10.2)	13	2	3
tet(B)	Tetracycline	8 (4.5)	-	-	2

A single isolate from a feral pigeon harboured 11 resistance genes including *aph(3")-Ib*, *aph(6")-Id*, $bla_{CTX-M-15}$, bla_{EC-8}, bla_{shv145}, bla_{TEM-1}, *dfrA14*, *fosA*, $oqxA_{10}$, $oqxB_{17}$ and *sul2*. Two other isolates carrying 11 resistance genes was recovered from two different little penguins. The resistance genes

harboured by these isolates included *aadA1*, *aadA2*, *aph(3")-Ib*, *aph(6")-Id*, bla_{EC}, bla_{TEM-1}, *cmlA1*, *qnrB19*, *qnrS1*, *sul3* and *tetA*.

Genomic characterisation

Preliminary evaluation of genetic diversity amongst the 595 isolates was performed using RAPD prior to selected isolates being subjected to WGS. Unique RAPD profiles ranging from one to eight per bird were identified in all three bird species, with highest number of eight unique profiles being detected in one little penguin. Except for one identical RAPD profile shared between *E. coli* isolated from feral pigeons and gulls all others were unique and did not overlap with other species. Five RAPD profiles were shared between the *E. coli* isolated from gulls and little penguins. Overall, the unique RAPD profiles identified in *E. coli* isolated from each bird species consisted of 49 for gulls, 52 for feral pigeons and 11 for little penguins (Figure 4).

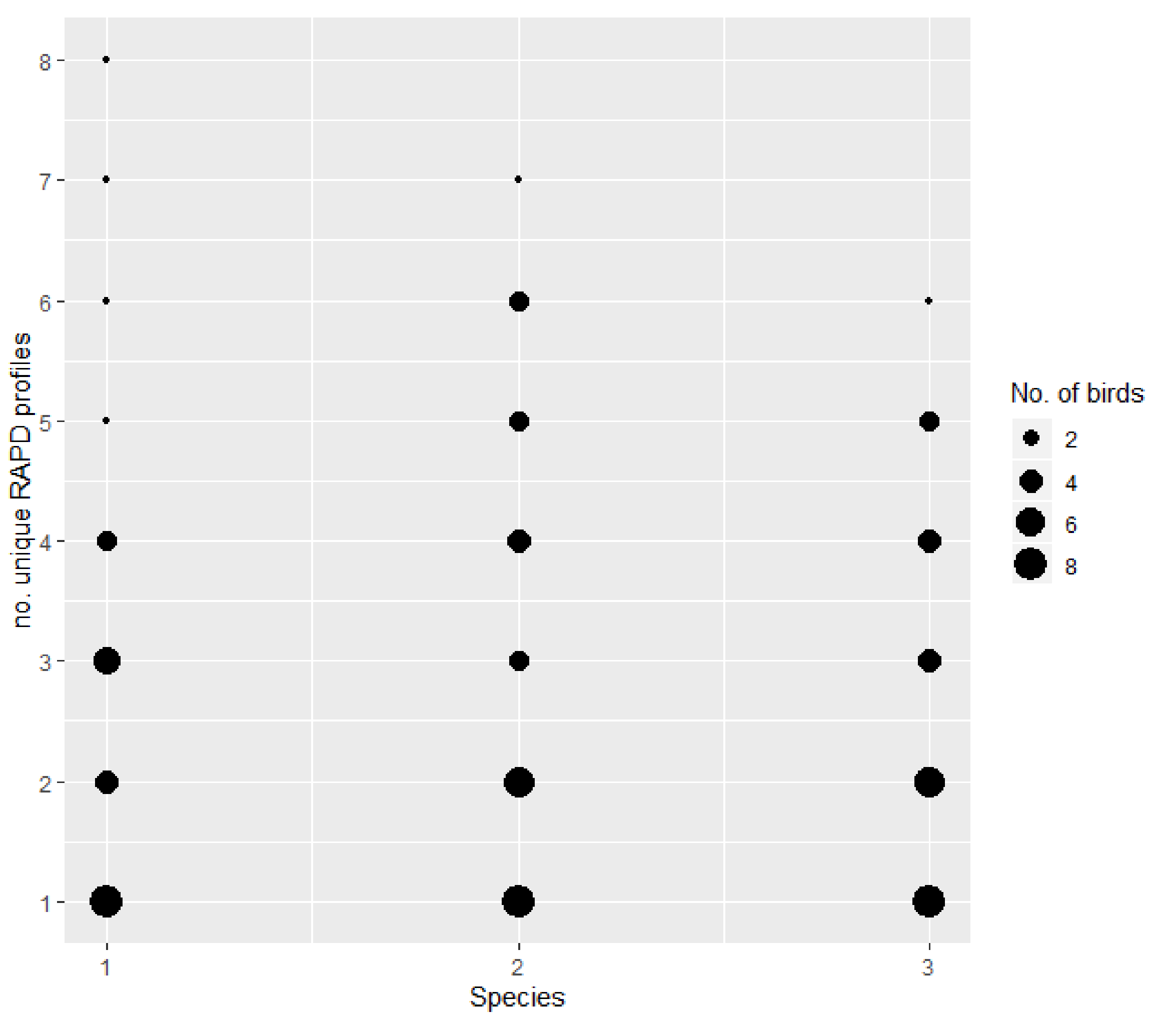

Figure 4. The number of unique RAPD profiles in each bird across samples from three bird species. 1 = Silver gulls, 2 = feral pigeons and 3 = little penguins

Table 3: Unique and overlapping sequence types identified in the *E. coli* isolates from the three bird species.

Sequence Types (ST)	Number of isolates Silver gulls (n)	Number of isolates feral pigeons (n)	Number of isolates little penguins (n)
10	3	-	-
12	-	-	1
34	1	-	-
38	1*	1*	-
48	1	-	-
56	-	1	-
58		1	-
68	1	-	-
73	-	-	2
80	1	-	-
95	-	-	3
108	-	1	-
117	1	-	-
127	1	-	-
131	1±	1±	4±
155	1±	2±	1±
165	2	-	-
196	-	1	-
201	1	-	-
206	1	-	-
208	1	-	-
216	2	-	-
219	1	-	-
227	1	-	-
372	1	-	-
399	-	-	3
457	3	-	-
491	3	-	-
515	1	-	-
538	3	-	-
540	1	-	-
542	-	1	-

607	1	-	-
695	1	-	-
710	1	-	-
718	-	1	-
746	-	-	2
770	6	-	-
906	-	-	1
914	1	-	-
937	-	2	-
1031	2	-	-
1122	-	-	2
1161	4*	1*	-
1286	3	-	-
1316	-	1	-
1326	-	-	1
1432	-	-	2
1588	-	1	-
1611	1±	4±	1±
1858	-	1	-
1972	-	-	1
2161	1	-	-
2280	-	1ǂ	5ǂ
2526	1	-	-
2611	1	-	-
2705	-	-	1
2949	-	2	-
3018	1	-	-
3476	-	1	-
3508	-	1	-
3519	-	1	-
4038	-	2	-
4045	1	-	-
4429	1	-	-
5281	1	-	-
5662	-	1	-
5891	-	4	-
6025	-	-	1
9287	2	-	-
11899	-	3	-
11904	-	1	-
11905	-	2	-
11906	-	1	-
11907	-	1	-
11908	-	2	-
11909	-	1	-
11910	1	-	-
11911	1	-	-
11912	1	-	-

*Indicates the ST was detected in Silver gulls and feral pigeon

± Indicates the ST was detected in all three bird species

ǂ ST detected in feral pigeon and little penguins

Isolates exhibiting unique RAPD profiles from each bird species were subjected to WGS (n=169). Genomic characterisation identified 81 STs from the three bird species. There were 38 unique STs amongst isolates from gulls, 25 in feral pigeons, and 12 in little penguins (Table 3). The most prevalent STs were ST131 and ST1611 (3.6% each), detected in all three bird species, ST2280 (3.6%) was detected only in feral pigeons and little penguins, ST10 (1.8%) was detected only in gulls, and ST1122 (1.2%) was identified in *E. coli* isolates from little penguins. ST155 was detected in gulls, feral pigeons and little penguins while ST457 and ST1286 were detected only amongst the gull isolates. ST5891 was identified in *E. coli* isolates from feral pigeon. ST95 and ST399 each were detected only in isolates from little penguins while ST38 and ST1161 were isolated from gulls and feral pigeons.

Discussion

This study analysed diversity and antimicrobial susceptibility amongst 595 *E coli* isolates recovered from 30 birds from each of three species that shared a common environment on Penguin Island, a small island just off the coast of Western Australia, situated adjacent to an urban environment on the coast. This number of birds and isolates were considered sufficient to give a reasonable estimate of the *E. coli* ecology amongst these species at the location studied. It is acknowledged that the selection of only eight isolates per bird does not reflect the extent of overall diversity, as the most dominant strains will be over-represented amongst those colonies selected. Despite this, contrary to our initial hypothesis, the findings of this study indicate that there was limited strain sharing of commensal *E. coli* strains between the three bird species that shared a common local environment. Overall, there were 49 unique RAPD profiles identified amongst the gull isolates, 52 for feral pigeons and 11 for little penguins. Following WGS, amongst the 81 STs that were identified, only three STs, ST131, ST155 and ST1611 were common between all the three bird species included, while ST38, ST58 and

ST1161 were only shared between gulls and feral pigeons. No strain sharing was observed between feral pigeons and little penguins. The little penguin faecal swabs yielded lower numbers of *E. coli* isolates than did the other two species, and most of the strains were different from those from the other bird species. These differences may be attributed to different foraging habits of these bird species, and the results suggest that the threat of cross-species transmission of potentially pathogenic and/or resistant *E. coli* strains between avian species is less than might have been thought.

Some of the sequence types that were detected, such as ST12, ST73, ST131, ST399 and ST746 previously have been detected in human hospital outbreaks, in wastewater treatment plants, and in faeces of other avian species, and they have harboured critically important antimicrobial resistance genes (25 - 28). Sequence types that are clinically significant for human health that were detected amongst the isolates from feral pigeons included ST38, ST58, ST108, and ST155. *E. coli* ST38 is recognised as an emerging uropathogenic *E. coli* (UPEC) and has been found to carry enteroaggregative *E. coli* (EAEC) virulence determinants (29). *E. coli* ST58 isolated from feral pigeons previously has been isolated from humans, the environment, food animals and wildlife including avian species (30). In another study *E. coli* ST58 carrying the *mcr-1* gene was isolated from chickens, turkeys, cattle, and the environment, while ST108 was isolated from chickens (31). ST155 belonging to phylogroup B1isolated from wastewater previously has been associated with zoonotic transmission of extended spectrum β lactamase genes to humans (32, 33).

In our previous study investigating the implications of foraging and interspecies interaction of the same three bird species from Penguin Island, 53%, 11% and 10% of the *E. coli* isolated from gulls, little penguins and feral pigeons respectively were identified as being CIA resistant (8). These resistant strains included clinically significant human-associated lineages, such as ST131, ST95, ST648, ST69, ST540, ST93, ST450 and ST10. These samples were initially cultured on MacConkey agar supplemented with ciprofloxacin and Brilliance ESBL agar to select for fluoroquinolone and extended spectrum cephalosporin resistant strains, which resulted in statistically significant ($P< 0.0001$) high rates of detection in all three bird species as compared to current study (8). In contrast, low levels of CIA resistance were detected among the *E. coli* isolates from all three bird species in the

current study. At first sight this seems unusual, since previous studies have identified resistant *E. coli* isolates in the faeces of many gulls and other urban birds (7, 8, 34 – 36). The likely cause of this discrepancy is that those other studies used enrichment with buffer peptone water and selective agar incorporated with CIAs to recover resistant strains. The current study did not use enrichment, and it is assumed that the strains that were recovered from MacConkey agar were more representative of the overall *E. coli* population in the GIT of the birds. It is also assumed that had enrichment with CIAs been used in the current study the apparent extent of carriage of resistant strains would have been increased at the expense of a more accurate assessment of the situation pertaining to the overall *E. coli* population. The low levels of CIA-R *E. coli* detected in this study is likely due to the absence of direct selection pressure from CIAs or other antimicrobials (both from use and exposure) resulting in low CFU of CIA-R *E. coli* carriage among these urban bird populations.

Despite being clinically significant from a human health perspective, only one out of the 595 isolates demonstrated resistance to ciprofloxacin and was identified as ST131. None of the isolates demonstrated resistance to ceftriaxone. The other four ST131 isolates identified in this study had an AMR index score 0, and one isolate had an AMR index score 2. Most of the other *E. coli* STs identified had an AMR index score of 4, 3, 1 or 0. These findings suggest that the avian species studied do not carry a large reservoir of resistant *E. coli* strains that may be transmitted between species and potentially to human beings. Resistant strains may be carried, but they do not represent the dominant strains present in most cases, thus reducing the risk of environmental contamination and exposure of other species to significantly resistant strains.

Conclusions

By analysing faecal samples this study found that there was limited sharing of dominant *E. coli* strains between silver gulls, feral pigeons and little penguins that co-inhabited a small island just off the coast of Western Australia, adjacent to an urban environment on the coast. Furthermore, amongst the dominant *E. coli* strains examined, only limited resistance to antimicrobial agents was identified. The method used to isolate the bacteria did not use enrichment on agar containing antimicrobials, and consequently the isolates that were obtained were believed to be a better representation of the normal

E. coli intestinal microbiota than those previously studied. Potential transmission of resistant strains to humans resulting from the carriage of resistant *E. coli* strains by gulls and other feral birds may have been overestimated in previous studies, since resistant strains may not commonly be dominant amongst the total *E. coli* population in these birds.

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Chapter 6 General Discussion and Conclusions

The emergence of critically important antimicrobial resistance (CIA-R) in Gram-negative bacteria from humans, animals and wildlife is a health emergency. This limits treatment options for life-threatening infections in both humans and animals. In addition, the concerns are heightened due to the potential transmission of these highly resistant bacteria among humans and animals that share the same ecological niche. Understanding the prevalence, genomic context of emerging CIA-R bacteria, the frequency of resistant bacteria in various environmental sources, including avian species, is crucial to controlling their dissemination. Wild birds sharing proximity to humans have been identified as potential reservoirs for the amplification and transmission of these resistant bacteria.

In Chapter 2 the prevalence of critically important antimicrobial (CIA) resistant *E. coli* in Australian silver gulls across urban locations in various states was examined, focusing on anthropogenic sources. High levels of resistance to CIA, extended spectrum cephalosporin (21.7%), and fluoroquinolone (23.8%) were observed in silver gulls (Chapter 2, Figure 1), with carbapenem and colistin resistance observed at lower frequencies. Notably, one isolate in Victoria harboured carbapenem resistance *bla*$_{\text{OXA-48}}$, and one in Western Australia exhibited colistin resistance *mcr-1*. Whole genome sequencing of the *E. coli* isolates identified these as predominantly clinically significant human-associated extra-intestinal pathogenic *E. coli* (ExPEC) clones. A significant overlap was detected with human clinical isolates (Chapter 2 Figure 2 and 3), indicating a potential bidirectional transmission or an undetermined reservoir connecting both. The proximity of gulls to humans, their foraging habits (including feeding on human leftovers), and access to human waste, wastewater, and livestock waste were recognized as key factors implicating gulls as major carriers of CIA-resistant *E. coli*. The identification of human-associated sequence types in gulls, such as ST131, ST10, ST1193, ST69, and ST38, further establishes the possibility of transmission between humans and gulls or the existence of a shared unidentified reservoir that is accessible to both.

The findings of Chapter 2 were a prelude and prompted further investigation into the implications of foraging and interspecies interaction among birds carrying CIA-resistant *E. coli* strains. Chapter 3 focused on four bird species with distinct foraging habits in a common urbanized coastal habitat. The study further substantiated and confirmed high frequencies of CIA-resistant *E. coli* in silver gulls (53%), little penguins (11%), and feral pigeons (10%), while no resistance was found in bridled terns due to their different foraging habits, mainly comprising of food from natural sources at sea and away from human populations (Chapter 3, Figure 1). Predominant resistance genes in extended spectrum cephalosporin-resistant strains in the *E. coli* strains isolated from the bird species included $bla_{\text{CTX-M-15}}$, followed by $bla_{\text{CTX-M-27}}$ and $bla_{\text{CTX-M-14}}$ (Chapter 3, Figure 2). Major transmission hotspots for little penguins were shallow waters where gulls concentrate, wash, and defecate, while coastal freshwater resources in wetlands that are shared by the gulls and feral pigeons were considered as major source of transmission to feral pigeons. Analysis of mobile genetic elements (MGE) suggested potential interspecies resistance transfer (Chapter 3, Figure 3). As these birds share proximity with humans, are frequent visitors in urban areas, and also have access to water and agricultural lands, they not only heighten transmission risks within the environment but also have the potential to acquire new resistance components from migrating bird species across countries.

The colonisation rates of CIA resistant *E. coli* between birds inhabiting areas with low and high human population density was compared in chapter 4. The possibility of carriage of bacterial species expressing clinically important forms of resistance other than just *E. coli* was also investigated in this study. The finding of this study indicated a strong association between proximity to human population and carriage of CIA resistant bacteria by gulls. The samples collected from remote sites without any permanent human settlement did not yield any CIA resistant bacteria. The frequency of CIA resistant *E. coli* was high, frequency ranging from 34.3%-84.3% in the gulls from large urban locations whereas in certain small tourist town only 9.7% CIA resistant *E. coli* were detected (Chapter 4, Figure 1). Furthermore, besides CIA resistant *E. coli*, another highly virulent bacteria known for causing invasive infections and armed with the ability to rapidly acquire multi-drug resistance genes, *K. pneumoniae* was also isolated from gulls at a frequency ranging from 12.5% - 50.0% in large urban

locations in this study. The recurrence of the findings once again establishes gulls as an undoubtedly efficient vectors in the carriage of CIA resistant bacteria and indicates that the carriage rates of the human associated clinically significant species is directly proportionate to the level of interactions between gulls and human population. The study also concluded there is a need for broader and better understanding of the pathways of transmission of these bacteria and their subsequent fate in the environment.

In the final chapter, the focus was to assess commensal *E. coli* strain sharing among different bird species in a shared environment. Faecal swab samples revealed limited sharing of dominant commensal *E. coli* strains between silver gulls, feral pigeons, and little penguins, with 69.4% of isolates susceptible to all tested antimicrobials. Ampicillin resistance was predominant at 23% across all three bird species (Chapter 5, Figure 1). The most prevalent AMR profile among the three species was amp:tet:sxt, while amp:cft and amp:tet were specific to gulls and little penguins (Chapter 5, Figure 3). Prevalent resistance genes detected were bla_{EC} (24.3%), $bla_{EC\text{-}18}$ (22.6%), $bla_{EC\text{-}5}$ (21.5%), $bla_{TEM\text{-}1}$ (12.4%), and $bla_{EC\text{-}15}$ (12.4%) (Chapter 5, Table 2). The unique RAPD profiles identified in *E. coli* isolated from each bird species consisted of 49 for gulls, 52 for feral pigeons and 11 for little penguins (Chapter 5, Figure 4). The number of unique STs detected in gulls, feral pigeons and little penguins were 38, 25 and 12 respectively. The most frequently detected STs included ST131, ST1611, ST2280, ST10 and ST1122 (Chapter 5, Table 3). In the dominant *E. coli* strains examined in this study overall resistance to antimicrobial agents was limited. This is attributed to not using selective enrichment on agar media incorporating target antimicrobials.

Recommendations:

Based on the findings of this study the main recommendations from a public health perspective would include.

1. Practising basic hygiene such as hand hygiene especially when in recreational areas, parks, waterbodies, urban areas, and agricultural land that are also frequently visited by avian species carrying CIA resistant bacteria.

2. Monitor carriage and transmission rates of CIA-resistant bacteria by implementing regular surveillance focusing on specific bird species that are known to congregate closure to areas with anthropogenic activities and have adapted to scavenging on human leftover food and wastes.
3. Identify and limit the access to the CIA resistant bacteria exposure source are equally important steps that would help confine the spread of these bacteria into the environment and other species.
4. Use of selective agar media incorporated with antimicrobials is highly recommended to eliminate any bias or over representation of the dominant population.