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Research paper

Bats as reservoirs of antibiotic resistance determinants: A survey of class 1 integrons in Grey-headed Flying Foxes (*Pteropus poliocephalus*)

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ABSTRACT

Increasing reports of antimicrobial resistance in wildlife highlight the significance of a One Health approach to managing resistance. We investigated the prevalence and diversity of class 1 integrons, a genetic determinant of resistance, in grey-headed flying foxes, a large fruit bat species belonging to the order Chiroptera. Class 1 integrons were detected in both wild flying foxes (5.3%) and captive flying foxes (41.2%) housed in wildlife rehabilitation facilities. Genes encoding resistance to aminoglycosides, trimethoprim and beta-lactams, and Qac efflux pumps were detected. Analysis of conserved integron elements and gene cassette arrays indicate the direction of integron transfer is from humans to flying foxes. The detection of two novel gene cassette arrays (*5′CS-qacH-aacA34-bla*$_{\text{OXA-21}}$*-3′CS* and *5′CS-qacF-3′CS* strongly suggests acquisition of genes from the environmental resistome into class 1 integrons within the flying fox microbiota. The dynamics of class 1 integrons in flying foxes indicates bats have a role in the emergence of novel antibiotic resistance determinants.

1. Introduction

Antimicrobial resistance (AMR) is a global issue that is no longer entirely confined to human health contexts. Increasing reports of AMR in wildlife highlight the need to understand the ecology of resistant bacteria in wildlife settings (Greig et al., 2015). Dissemination of resistance via the microbiota of wild animals is clearly an issue for the management of resistance. Strategies aimed at combatting antimicrobial resistance stress the need for a multidisciplinary and collaborative "One Health" approach, addressing interactions between people, domestic animals, wildlife and the environment (FAO-OIE-WHO, 2010). However, current antimicrobial resistance surveillance programs typically focus on humans and domestic animals, and rarely extend to wildlife.

The rapid spread of AMR has been facilitated by mobile genetic elements, such as plasmids, transposons, and integron gene cassettes. One type of integron, the class 1 integron, is predominantly responsible for spreading antibiotic resistance to most Gram-negative bacteria of clinical importance (Stokes and Gillings, 2011). Class 1 integrons contain a 5′ conserved segment (5′CS) encoding an integrase gene (*intI1*), and a variable number of gene cassettes, which form a gene cassette array. The 3′ conserved segment (3′CS) commonly contains the *qacE* and *sul1* genes (*qacE*Δ*1-sul1*). IntI1 mediates site-specific recombination of free mobile gene cassettes into a primary recombination site (*attI1*). Each gene cassette within the array is separated by secondary recombination sites (*attC*). A promoter (Pc) then drives expression of the inserted gene cassettes, which in clinical pathogens usually encode antibiotic resistance genes (Hall and Collis, 1995). IntI1 also mediates the excision of gene cassettes from the array, producing free gene cassettes. The IntI1 mediated recombination system allows class 1 integrons to capture, remove and shuffle gene cassettes (Hall and Collis, 1995). The association of class 1 integrons with transposons and plasmids allows for transfer of integrons between different bacterial hosts by horizontal gene transfer (Gillings et al., 2008).

Wildlife can acquire antibiotic resistant bacteria from human and domestic animal effluent, wastewater treatment plants, and aquaculture operations (Arnold et al., 2016). Antibiotics released into the environment can apply selective pressure, promoting the fixation of lateral transfer events in environmental bacterial communities and in wildlife microbiota (Allen et al., 2010; Finley et al., 2013). Bacteria that harbor class 1 integrons have been detected in numerous wildlife species, many of which are closely associated with aquatic environments (such as black-headed gull *Larus ridibundus*, lesser flamingo *Phoeniconaias minor*, Australian sea lion *Neophoca cinereal* and Eurasian otter *Lutra lutra*), suggesting water as a primary vector (Alonso et al., 2017a; Delport et al., 2015; Dolejska et al., 2009; Fulham et al., 2018; Sato

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et al., 2009). How free-living terrestrial wildlife species (such as European mouflon *Ovis orientalis musimon*, wild boar *Sus scrofa*, black kite *Milvus migrans*, or monitor lizards *Varanus* spp.) acquire antibiotic resistant bacteria is less clear (Alonso et al., 2017a; Alonso et al., 2017b; Fischer et al., 2013; Waturangi et al., 2003). Class 1 integrons detected in aquatic and terrestrial wildlife are frequently identical to those identified in human or domestic animal microbiota (Partridge et al., 2009; Stokes and Gillings, 2011). Given the number of class 1 integrons released by humans and their domestic animals each day, the dominant direction of transfer is clearly towards wild species, and into the environment (Gillings, 2018; Zhu et al., 2017).

Proximity to humans and domestic animals strongly influences the prevalence of class 1 integrons in wildlife (Skurnik et al., 2006). The occurrence of AMR in captive wildlife is considerably greater than their free-living counterparts (Delport et al., 2015; Kinjo et al., 1992; Power et al., 2013). Understanding the potential flux of resistance genes and integrons through various environmental compartments and wild species requires a study organism with a wide-ranging distribution that intersects with urban environments. One animal that fulfils these requirements is the grey-headed flying fox (*Pteropus poliocephalus*), a large species of fruit bat endemic to Australia. The grey-headed flying fox is found in coastal regions of Eastern Australia, extending from Ingham in Queensland to Adelaide in South Australia (Eby, 2008). The species is highly mobile with bats regularly travelling > 50 km each night to reach foraging sites (Eby, 1991), or moving hundreds of kilometres between camps (Eby, 1991; Roberts et al., 2012). Each year, thousands of injured and orphaned grey-headed flying foxes are rescued, rehabilitated and released by wildlife care groups, providing opportunity for exposure to human associated microorganisms, including antibiotic resistant bacteria.

To understand the potential for class 1 integrons to spread from human dominated ecosystems into wild animals we examined the prevalence and diversity of integrons in grey-headed flying foxes. We screened two free-living wild colonies (referred to hereinafter as "wild") and compared these to flying foxes temporarily housed in captivity at rehabilitation facilities (referred to hereinafter as "captive").

2. Methods

2.1. Grey-headed flying fox samples

Fecal samples were collected from individual grey-headed flying foxes in Adelaide Botanic Park, South Australia (2017; *n* = 46), with approval from The University of Adelaide Animal Ethics Committee (No. S-2015-028). Grey-headed flying fox fecal DNA extractions from a previous study (Schiller et al., 2016) were used in this study, with samples from Ku-ring-gai Flying Fox Reserve, Sydney, New South Wales (*n* = 30) and two captive sites Wambina Flying Fox Sanctuary, Matcham, New South Wales (*n* = 77) and Tolga Bat Hospital, Atherton, Queensland (*n* = 25).

2.2. DNA extraction and PCR protocols

DNA was extracted from feces (100–150 mg) using the Bioline ISOLATE Fecal DNA Kit following the manufacturer's instructions. Fecal DNA underwent PCR amplification of the 16S rRNA gene using universal eubacterial primers f27 and r1492 (Table 1) (Lane, 1991) and GoTaq® Green Master Mix (Promega, Madison, WI, USA) (Waldron and Gillings, 2015) to confirm that the DNA was amplifiable. Samples with 16S positive amplicons were screened for the class 1 integron-integrase gene (*intI1*) using HS463a and HS464 (Table 1) (Waldron and Gillings, 2015) with PCR amplification performed using GoTaq® Colourless Master Mix (Promega, Madison, WI, USA), and cycling conditions of 94 °C 3 min; 35 cycles 94 °C 30 s, 60 °C 30 s, 72 °C 1 min 30 s; 72 °C 5 min (Waldron and Gillings, 2015).

IntI1 positive samples were then amplified using primers HS458 and HS459 (Table 1) (Holmes et al., 2003), using cycling conditions of 94 °C 3 min; 35 cycles 94 °C 30 s, 55 °C 30 s, 72 °C 1 min 30 s; 72 °C 5 min (Waldron and Gillings, 2015). HS458 and HS459 target the conserved *attI1* and 3′ *qacE*Δ*1* regions. This amplifies the entire gene cassette array, and amplicons vary in size depending on the number and type of gene cassettes present in the array. Samples can contain multiple cassette arrays of differing sizes, indicating the carriage of integrons containing different genes in the cassette array. HS458/HS459 amplicons of low intensity were excluded from further analysis.

2.3. DNA purification, cloning and sequencing

Gene cassette array amplicons were purified using the MinElute PCR Purification Kit (QIAGEN, Hilden, Germany), and those with single bands were sequenced directly using primers HS458 and HS459. For large amplicons (≥1200 bp) the internal sequencing primer HS320 (Table 1) (Holmes et al., 2003) was used to generate overlapping fragments. For one very large amplicon (≥2200 bp) three additional internal sequencing primers (FF509F, FF710R and FF1340R) (Table 1) were designed to obtain full length sequences.

PCR products containing multiple gene cassette arrays underwent DNA cloning using the TOPO® TA Cloning® Kit with Vector pCR™4-TOPO® and One Shot® TOP10 *E. coli* cells (Invitrogen, Carlsbad, CA, USA). Between eight and sixteen colonies of transformed *E. coli* were selected from each cloned sample and DNA from cell lysate (95 °C 5 min and centrifugation; RCF = 12,000, 5 min, 22 °C) was screened using HS458 and HS459 PCR as above. Amplicons of variable sizes were selected for sequencing. Alternatively, amplicon bands were excised from an agarose gel and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), then sequenced as above.

DNA sequencing was performed at the Australian Genome Research Facility (Sydney, NSW, Australia), and at The Ramaciotti Centre for Genomics (Sydney, NSW, Australia) using Big Dye Terminator chemistry version 3.1 and ABI 3730/3730xl Capillary Sequencers (Applied Biosystems, Foster City, CA, USA). Sequences were manually checked for quality, sequences assembled using Geneious R11 software (Biomatters Limited, Auckland, New Zealand) and analysed for the presence of antibiotic resistance genes using Integrall (http://integrall.bio.ua.pt/?search#). Annotation was performed manually using the Blastn suite and Blastx (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Class 1 integron gene cassette arrays were confirmed by the presence of the 3′ conserved region containing *qacE*Δ*1*. For arrays containing multiple gene cassettes, *attC* recombination sites located between cassettes were identified using the highly conserved core sequence GTTRRRY and complementary inverse core sequence RYYYAAC (Escudero et al., 2015). Representative sequences generated from this study have been lodged in GenBank under accession numbers MH556868-MH556885 (Supplementary material 1).

3. Results

3.1. PCR detection of class 1 integrons

Fecal DNA from 178 grey-headed flying fox samples (wild = 76 and captive = 102) was PCR screened for *intI1* and results revealed 66 of 178 (37.1%) fecal samples were *intI1* positive (12/76 wild and 54/102 captive). Gene cassette arrays were successfully amplified from 51 of the 66 *intI1* positive samples (5/76 wild and 46/102 captive) (Fig. 1 and Supplementary Table). Gene cassette arrays were not detected in 15 of the 66 *intI1* positive samples. Failure of primers HS458 and HS459 to amplify the gene cassette region of these class 1 integrons can be due to truncation or genetic variation of the 3′CS region (Antunes et al., 2007; Dawes et al., 2010; Reid et al., 2017). Based on the sizes of cassette array amplicons, 34 samples contained a single type of array, 14 samples contained two different arrays, and three samples contained at

Table 1
Primers used for amplification of 16S rRNA, class 1 integron components, and sequencing.

Primer	Sequence 5′–3′	Target	Reference
f27	AGAGTTTGATCMTGGCTCAG	16S rRNA	(Lane, 1991)
r1492	TACGGYTACCTTGTTACGACTT	16S rRNA	(Lane, 1991)
HS463a	CTGGATTTCGATCACGGCACG	*intI1*	(Waldron and Gillings, 2015)
HS464	ACATGCGTGTAAATCATCGTCG	*intI1*	(Waldron and Gillings, 2015)
HS458	GTTTGATGTTATGGAGCAGCAACG	*attI1*	(Holmes et al., 2003)
HS459	GCAAAAAGGCAGCAATTATGAGCC	*qacE*Δ*1*	(Holmes et al., 2003)
HS320	AGTAAAGCCCTCGCTAG	*qacE*Δ*1*	(Holmes et al., 2003)
FF509F	CAGGTGTTAGAAGGCCCAGG	*attC*	This publication
FF710R	CGCGGATGGATAACTCGACA	*aacA34*	This publication
FF1340R	AATTCCGCATTGCTGATCGC	*bla*$_{\text{OXA-21}}$	This publication

least three distinct arrays (Fig. 1). These conclusions were confirmed by DNA sequencing.

3.2. Gene cassette array diversity in fecal samples

DNA sequencing of gene cassette arrays identified twelve different types of gene cassette array from the 51 *intI1* and HS458/HS459 positive samples (Fig. 1). Known genes that confer resistance to antibiotics were identified in nine of these arrays (Table 2). In total, gene cassette arrays encoding antibiotic resistance genes (ARGs) were detected in 5.3% (4/76) of wild samples and in 41.2% (42/102) of samples from captive flying foxes (Table 2). The majority (52/55) of arrays that carried ARGs contained a single gene cassette, and the remaining arrays (3/55) contained two or three gene cassettes (Table 2). Of the 17

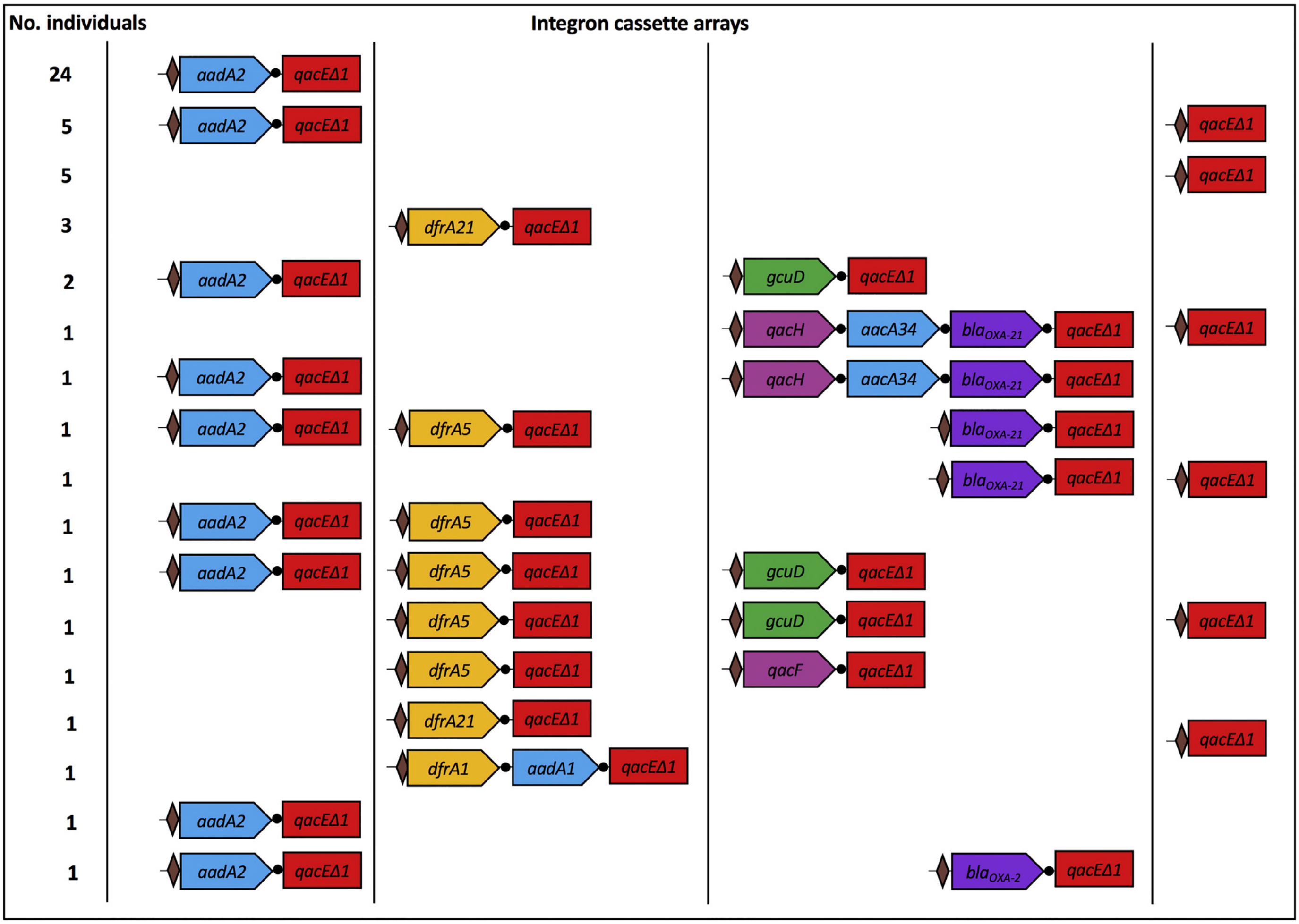

Fig. 1. Schematic map of class 1 integron gene cassette arrays identified in grey-headed flying foxes
Numbers of individual samples with each combination of arrays are listed in the left hand side. Gene cassettes are represented by broad arrows, also showing the direction of transcription. Brown diamonds represent the primary integron recombination site, *attI1* into which gene cassettes are generally inserted upon acquisition; black circles represent the gene cassette recombination site, *attC*. Gene symbols are as follows: *aacA* genes encode aminoglycoside (6′) acetyltransferases and confer resistance to aminoglycoside antibiotics; *aadA* genes encode aminoglycoside (3″) adenylyltransferases that confer resistance to aminoglycoside antibiotics; *bla*$_{\text{OXA}}$ genes are members of the OXA beta-lactamase family and confer resistance to narrow-spectrum beta-lactam antibiotics; *dfrA* genes encode dihydrofolate reductases that confer resistance to trimethoprim; *gcuD* is a cassette of unknown function; *qac* genes encode efflux pumps that confer resistance to quaternary ammonium compounds; *qacE*Δ*1* is also a member of the Qac efflux pump family, and is the 3′ terminus of all the gene cassette arrays depicted here.

Table 2
Cassette arrays encoding resistance genes identified in class 1 integrons recovered from wild and captive grey-headed flying fox fecal samples.

Sampling location	No. of samples with gene cassette arrays	Gene cassette arrays identified	No. of times gene cassette arrays were detected
Wild Sites			
Adelaide Botanic Park	3/46 (6.5%)	*aadA2*	2
		qacH-aacA34-bla$_{\text{OXA-21}}$	2
Ku-ring-gai Flying Fox Reserve	1/30 (3.3%)	*dfrA5*	1
		qacF	1
Total	4/76 (5.3%)	Total	6
Captive Sites			
Wambina Flying Fox Sanctuary	35/77 (46.8%)	*aadA1*	2
		aadA2	28
		bla$_{\text{OXA-2}}$	1
		bla$_{\text{OXA-21}}$	2
		dfrA5	5
		dfrA21	4
Tolga Bat Hospital	7/25 (28.0%)	*aadA2*	6
		dfrA1-aadA1	1
Total	42/102 (42.1%)	Total	49

instances where cassette arrays did not encode ARGs, the genes were either of unknown function, *gcuD* ($n = 3$) or contained no gene cassettes ($n = 14$) (Fig. 1). In these instances, DNA sequences consisted of the *attI* recombination site directly adjoining the 3′ conserved sequence of *qacEΔ1*.

3.3. Antibiotic resistance gene diversity

Ten different ARGs were identified by sequencing the amplified cassette arrays. Six of these genes (*aacA34*, *aadA2*, *bla*$_{\text{OXA-21}}$, *dfrA5*, *qacF* and *qacH*) were detected in wild flying fox samples, while seven different ARGs (*aadA1*, *aadA2*, *bla*$_{\text{OXA-2}}$, *bla*$_{\text{OXA-21}}$, *dfrA1*, *dfrA5*, and *dfrA21*) were detected in captive flying fox samples (Fig. 2).

The vast majority of cassette arrays contained a single gene cassette (Fig. 1). Two wild flying foxes from Adelaide Botanic Park carried a cassette array with three gene cassettes, consisting of *5′CS-qacH-aacA34-bla*$_{\text{OXA-21}}$*-3′CS*. A single individual from the Tolga Bat Hospital carried an array with two gene cassettes, *5′CS-dfrA1-aadA1-3′CS*. (Table 2). For the full details of all gene cassettes see Supplementary material 1.

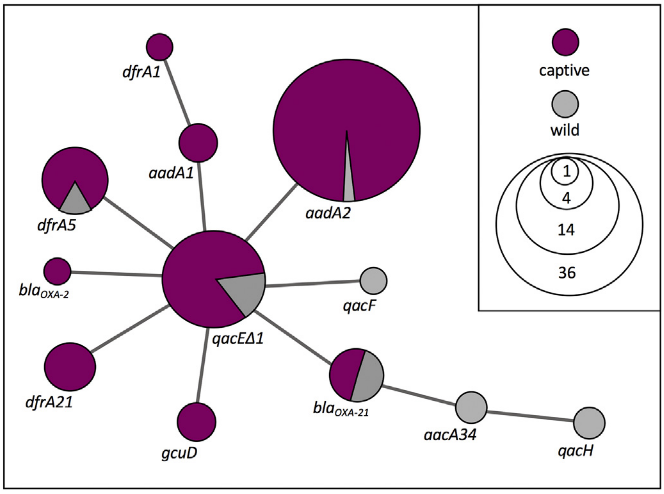

Fig. 2. Network diagram of class 1 integron gene cassette arrays identified in wild and captive grey-headed flying foxes.
The *qacEΔ1* conserved region present in all arrays is positioned in the center. Radiating circles show the sequential acquisition of gene cassettes by class 1 integrons.

The relative abundance of the types of gene cassettes recovered from grey-headed flying foxes varied (Fig. 2) with the *5′CS-aadA2-3′CS* gene cassette being very common. Wild flying foxes carried some of the more common arrays (*5′CS-aadA2-3′CS* and *5′CS-dfrA5-3′CS*) roughly in the same proportions as the captive animals, but in addition they differed by the possession of arrays that were not recorded from captive flying foxes. These included two novel arrays, *5′CS-qacH-aacA34-bla*$_{\text{OXA-21}}$*-3′CS* and *5′CS-qacF-3′CS*, which have never been previously reported (Fig. 2); Nine GenBank entries are identical to the *aacA34* gene cassette, but in each case the *aacA34* cassette is found within different cassette arrays from those found here in bats. The novel arrays containing *aacA34* were present in two flying foxes (FF781 and FF783), and in both cases a novel *qacH* cassette was also found. These novel *qacH* cassettes were distinct from any previously reported *qacH* cassette and differed from their nearest homolog in GenBank (accession MG738685.1) by 3 of 509 nucleotides (FF781) or 5 of 509 nucleotides (FF783). The translated *qacH* protein was identical for one *qacH* cassette (FF781) and differed by one differed by amino-acid residue for the second *qacH* cassette (FF783), compared to their nearest homolog in GenBank. Blast searches of the second novel array identified a *qac* cassette, here designated *qacF*, resulted in no identical cassettes detected in GenBank. The closest homologs to *qacF* were variously annotated as *qacF* or *qacH*. The novel *qacF* cassette differed from its nearest homolog in GenBank (accession CP024875.1) by 3 of 511 nucleotides and the translated *qacF* protein differed by one amino-acid residue.

Because of the screening method we implemented, each cassette array that we recovered terminated in the 3′ conserved segment, represented in this case by the truncated *qacEΔ1* element. Sequence alignments of all the *qacEΔ1* elements exhibited nucleotide identities > 99%.

4. Discussion

Class 1 integrons were detected in almost one third of grey-headed flying foxes sampled in this study. Their prevalence was considerably higher in captive flying foxes (41.2%) than in wild flying foxes (5.3%). These findings are consistent with previous studies that report a high prevalence of class 1 integrons in captive wildlife (Ahmed et al., 2007; Power et al., 2013), and that proximity to human influences is a key predictor of the abundance of class 1 integrons in animal populations (Skurnik et al., 2006). Within the Australian context, class 1 integrons have been detected at low levels in non-captive Australian wildlife (Delport et al., 2015; Fulham et al., 2018; Power et al., 2013), and antimicrobial resistant bacteria have been detected at low levels in Australian livestock and companion animals (Abraham et al., 2015; Barlow et al., 2015; Saputra et al., 2017).

The initial source of the integrons detected in grey-headed flying foxes is most likely to be humans or domestic animals. There are a number of reasons to come to this conclusion. First, the sequence of the *qacEΔ1* element that terminated every cassette array was identical, or nearly so. The sequence identity of *qacEΔ1* reflects the recent common origin of the clinical variant of the class 1 integron, which is thought to have arisen as a single copy, in a single cell, sometime in the early 20th Century, before it spread to most Gram negative pathogens (Ghaly et al., 2017). The presence of the *qacEΔ1* element in the integron platform is known to be characteristic of class 1 integrons from clinical or agricultural compartments. Second, the gene cassettes detected in captive flying foxes are typical of those from clinical contexts (Partridge et al., 2009). Third, the frequency of class 1 integrons in captive flying foxes far exceeds the frequency in their wild relatives, suggesting captivity as a driving factor. Finally, estimates suggest that as many as 10^{23} copies of the clinical class 1 integron are released from humans and their domestic animals every day (Zhu et al., 2017), firmly establishing that the direction of transmission is likely to be from humans into captive wildlife.

The most parsimonious explanation for our observations is that grey-headed flying foxes brought into captivity are exposed to bacteria carrying clinical class 1 integrons via water, food or handling, and that these bacteria then colonize their microbiota. This colonization must also occur multiple times, since captive flying foxes can carry up to three distinct integron cassette arrays that are not simple derivatives of each other. In many cases, individual flying foxes carried identical arrays or collections of arrays which could represent multiple, independent acquisitions, or result from transmission between individuals. We cannot distinguish between these possibilities, and potentially both circumstances could be occurring, although flying fox behaviour suggests that transmission of bacteria between individuals is highly likely (Mühldorfer, 2013). Flying foxes undergoing rehabilitation are housed in large aviaries until they are fit for release back into the wild. Under these conditions, individuals roost in very close proximity, and are held within a confined space, potentially promoting the transfer of gut microbiota between animals (Mühldorfer, 2013). It is unknown if the class 1 integrons remain confined to the colonising bacteria or if they have been transferred to endemic bacterial species in the flying fox microbiota.

Sick or injured grey-headed flying foxes entering wildlife care facilities are frequently administered antibiotics of veterinary importance, suggesting that selection for resistance could also play a role in the spread of integrons. Integron gene cassettes detected in grey-headed flying foxes can confer resistance to three different antibiotic classes, aminoglycosides (streptomycin and spectinomycin), narrow-spectrum beta-lactams (oxacillin, cloxacillin, and methicillin), and trimethoprim. These antibiotics are included on the List of Antimicrobial Agents of Veterinary Importance developed by the World Organization for Animal Health (OIE) (OIE, 2015) in conjunction with the World Health Organization (WHO) list of Critically Important Antimicrobials for Human Medicine (WHO, 2017). Additionally, *qac* genes confer resistance to quaternary ammonium compounds and other disinfectants commonly used in hospitals, veterinary practice and animal husbandry (Jaglic and Cervinkova, 2012). Currently there are no reported resistance substrate profiles for the closest homologs to the *qacF* and *qacH* proteins detected in the wild flying foxes.

Every year, thousands of grey-headed flying foxes enter care and rehabilitation facilities, where there appears to be a high probability of acquiring integrons that confer antibiotic resistance. The majority of captive grey-headed flying foxes are then released back into wild colonies, where they have the potential to disseminate these bacteria and associated antibiotic resistance determinants into wild flying foxes. Here we have shown that a small, but significant number of wild flying foxes carry clinical class 1 integrons. Given the tendency of flying foxes to roost in very dense colonies, it is possible these integrons were acquired from released animals that had re-joined wild colonies.

Clinical class 1 integrons retain their ability to acquire and express gene cassettes whether they are resident in clinical contexts or in the gut microbiota of wild animals (Gillings et al., 2009b). Integrons in wild animals are likely to be exposed to different and diverse selective forces from those acting upon clinical pathogens, and this raises the possibility that they could acquire novel gene cassettes. Two grey-headed flying foxes from the wild colony in Adelaide carried integrons with a rare gene cassette, *aacA34*, that occurred within a novel array (*5′CS-qacH-aacA34-bla*$_{\text{OXA-21}}$*-3′CS*). This rare gene cassette, *aacA34,* encodes an aminoglycoside (6′) acetyltransferase which potentially confers resistance to aminoglycosides, however, currently there are no reported antimicrobial resistance substrate profiles for the *aacA34* protein. All previous records of *aacA34* in GenBank and the literature, were from human clinical pathogens isolated in Thailand (Kansakar et al., 2011) or Korea (GenBank accession EF490315.1). The *aacA34* cassette has never been reported in Australia, and it is uncertain whether the cassette reported here represents the capture of a previously unrecorded resistance cassette from a human source, or a de novo, independent acquisition from the environmental resistome.

The two wild grey-headed flying foxes that carried the *aacA34* cassette also carried a novel gene cassette, *qacH*, that encoded an efflux protein from the Qac family. The top GenBank sequence matches to this *qacH* cassette were recovered from *Pseudomonas baetica* (a fish pathogen), environmental DNA samples or uncultured organisms, and it is known that environmental organisms that carry class 1 integrons dynamically exchange diverse *qac* cassettes (Gillings et al., 2009a). The *qacH* cassette is the most proximal in the array, and is thus the most recently acquired and most strongly expressed cassette in the array (Collis and Hall, 1995). Its novelty, and its restriction to wild individuals, strongly suggests that it was acquired from the environmental resistome, via insertion into the class 1 integron while resident in a wild flying fox.

A second example of such a potential wild acquisition was observed in an individual from the Ku-ring-gai Flying Fox Reserve. This case also involved a *qac* cassette, *qacF*, and the closest matches were to *qac* cassettes recovered from environmental samples, or to *qac* cassettes located on the chromosomes of *Betaproteobacteria* (*Laribacter hongkongensis* CP022115.1 and *Acidovorax* spp. T1 CP021648.1). In both these cases, the *qac* homologs were associated with chromosomal integrons, rather than the transposon or plasmid-borne clinical variants.

5. Conclusion

This study suggests that wild grey-headed flying foxes coming into contact with humans acquire class 1 integrons while in care, and their subsequent release provides an opportunity for transmission of integrons into wild conspecifics. These acquired integrons retain their ability to sample and express gene cassettes from the general environment, as evidenced by the detection of novel cassette arrays in wild flying fox populations.

The emergence of class 1 integrons in wildlife parallels the emergence of infectious diseases (EIDs). There is clear potential for spill-over and spill-back of antibiotic resistant bacteria from reservoir populations (Daszak et al., 2000). Drivers of EIDs such as translocation of wildlife, introduction of domestic animals, urban encroachment, and ex situ contact (Daszak et al., 2000), are all pathways through which antibiotic resistant bacteria can be transmitted from humans or domestic animals into wildlife. Additionally, a range of zoonotic enteric bacteria such as *Escherichia coli*, *Salmonella* spp., and *Shigella* spp. have been isolated from bat species including flying foxes (Adesiyun et al., 2009; Banskar et al., 2016; Heard et al., 1997; Henry et al., 2018; Nowak et al., 2017), thus there is potential for these bacteria to acquire class 1 integrons while resident in the flying fox microbiota. The dynamics of class 1 integron carriage and the detection of novel gene cassette arrays in grey-headed flying foxes presents opportunities for spill-back events, transmitting novel resistance determinants into human populations and domestic animals. Our documentation of the connections between human dominated ecosystems and wild bat colonies suggest that more attention should be given to understanding the dynamics and interactions of bat species with the environmental resistome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.02.022.

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