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Environmental antimicrobial resistance gene detection from wild bird habitats using two methods: A commercially available culture-independent qPCR assay and culture of indicator bacteria followed by whole-genome sequencing

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ABSTRACT

Objectives: A variety of methods have been developed to detect antimicrobial resistance (AMR) in different environments to better understand the evolution and dissemination of this public health threat. Comparisons of results generated using different AMR detection methods, such as quantitative PCR (qPCR) and whole-genome sequencing (WGS), are often imperfect, and few studies have analysed samples in parallel to evaluate differences. In this study, we compared bacterial culture and WGS to a culture-independent commercially available qPCR assay to evaluate the concordance between methods and the utility of each in answering research questions regarding the presence and epidemiology of AMR in wild bird habitats.
Methods: We first assessed AMR gene detection using qPCR in 45 bacterial isolates from which we had existing WGS data. We then analysed 52 wild bird faecal samples and 9 spatiotemporally collected water samples using culture-independent qPCR and WGS of phenotypically resistant indicator bacterial isolates.
Results: Overall concordance was strong between qPCR and WGS of bacterial isolates, although concordance differed among antibiotic classes. Analysis of wild bird faecal and water samples revealed that more samples were determined to be positive for AMR via qPCR than via culture and WGS of bacterial isolates, although qPCR did not detect AMR genes in two samples from which phenotypically resistant isolates were found.
Conclusions: Both qPCR and culture followed by sequencing may be effective approaches for characterising AMR genes harboured by wild birds, although data streams produced using these different tools may have advantages and disadvantages that should be considered given the application and sample matrix.

1. Introduction

Antimicrobial-resistant (AMR; abbreviation also used for antimicrobial resistance) bacteria are a threat to human, animal, and environmental health [1]. Selection, either through direct antibiotic use in clinical and agricultural settings or through environmental exposure to antibiotic residues and biocides, has accelerated the proliferation of AMR bacteria globally [2]. Surveillance of AMR in humans and domestic animals in some countries has been ongoing for decades, whereas investigations of AMR in the environment and wildlife has generally received far less attention. Only recently has understanding the selection for, and proliferation of, environmental AMR become a collective priority among agencies tasked with understanding human, animal, and environmental health [3–5].

The evolution of AMR is an ancient process that has occurred over millennia in natural ecosystems [6,7]. However, through horizontal gene exchange and selection due to modern antibiotic use,

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AMR genes have more recently become prolific in clinical settings and widely disseminated in new bacterial clones [8–10]. New mosaics of AMR genes and bacterial clones may be circulated back to the environment and wildlife, although it is unclear if these sectors play an epidemiologically meaningful role in the transmission of AMR to humans [11,12]. AMR contamination in nature may also have yet unknown consequences [13].

Tools for detecting AMR are numerous, and each has its own advantages and disadvantages [14]. Screening programs among food-producing animals, for example, most often include detection of indicator bacteria (e.g., *Escherichia coli*) that are analysed for resistance towards a panel of clinically relevant antibiotics. Such programs most often include selective, and thus more sensitive, screening for emerging and especially clinically important AMR bacteria (e.g., extended-spectrum beta-lactamase–producing *E. coli* or *Klebsiella pneumoniae*). This culture-based approach, when combined with subsequent whole-genome sequencing (WGS), enables high-resolution analysis of all AMR genes and their immediate genetic environment within the indicator bacteria, including information about the bacterial host, although the combination of culture and sequencing may be time and cost prohibitive. Quantitative PCR (qPCR), on the other hand, relies on a limited number of targeted genes but facilitates higher throughput and detects AMR genes present in any organism in the sample. Additionally, the ability to measure the abundance of AMR genes in environmental samples is a major advantage of qPCR, as has been demonstrated in a variety of matrices, including water, faeces, and soil [15–17]. Shotgun metagenomics offers the benefits of detecting all AMR genes in any organism in the sample, although this method often has low sensitivity. While most methodologies may be readily applied to a variety of hosts and sample matrices, potential biological differences in the sample type may affect the performance and the interpretation of results [3]. Implementation of a single methodology [18] or a combination of step-wise approaches [19] have been employed; however, there is a dearth of studies comparing methodologies in parallel.

Wildlife, particularly wild birds, have been identified as indicators of AMR in the environment and as bridge hosts that may facilitate the persistence of AMR in the habitats they occupy [20–22]. An improved understanding of AMR in wild birds and their habitats relies on accurate detection methods and the ability to compare results from studies employing different methodologies. Thus, in this study, we aimed to compare two commonly used methods for studying AMR in natural wild bird habitats. Our culture-based screening method focused on clinically relevant Gram-negative indicator bacteria, including both a non-selective screen and a selective screen for extended-spectrum cephalosporin-resistant bacteria as well as carbapenemase-producing bacteria. Cultured bacteria were subjected to WGS to characterise AMR genes per a common workflow for clinically relevant isolates. We also employed a culture-independent commercially available qPCR assay for the detection of AMR genes. Our methods employed two sample types collected from wild bird habitats: environmentally deposited gull faeces and surface waters adjacent to where birds were observed. We first compared the concordance of WGS and qPCR for AMR detection among isolates to better understand potential differences in sensitivity and specificity between approaches. We next applied culture-based sequencing and qPCR workflows to environmental samples collected from wild bird habitats to compare AMR data streams. In this second study, we did not directly compare the detection of AMR genes between the two methods, given the different starting material, as the culture-based method only reflects resistance in specific Gram-negative indicator bacteria, whereas the qPCR assay detects resistance within the whole sample microbiome. Rather, we compared the utility of each approach for answering research questions regarding the presence and epidemiology of AMR in wild bird habitats.

2. Materials and methods

2.1. Isolate selection

We queried existing WGS data from 385 AMR *E. coli* and *K. pneumoniae* isolates cultured from wild bird faeces as part of prior research [21,23–25] and selected at least two isolates to represent as many gene targets in the Microbial DNA qPCR Array for Antibiotic Resistance Genes (Qiagen, USA) as possible. A total of 45 isolates were selected, in which one or more of 25 gene targets represented in the qPCR array were previously detected using WGS. Fifty-nine qPCR gene targets were not previously identified using WGS in any isolate characterised by our research group. DNA was extracted from the 45 isolates using the MagnaPure nucleic acid kit (Roche, Stockholm, Sweden), the same method that was used to extract DNA prior to WGS.

2.2. qPCR and concordance analysis of bacterial isolates

We analysed 45 *E. coli* or *K. pneumoniae* isolates, using a commercially available qPCR assay, for the presence of 84 AMR genes, representing eight antimicrobial classes or resistance mechanisms. Six SHV gene variants were excluded, as a direct comparison between SHV nucleotide variants (as determined by WGS) and SHV codon variants (as determined by qPCR) was deemed unnecessary to answer our research questions. DNA from bacterial isolates was analysed for AMR genes according to the manufacturer's instructions for the Microbial DNA qPCR Array for Antibiotic Resistance Genes assay (Qiagen). Briefly, 500 ng of sample DNA was added to Microbial qPCR Mastermix (Qiagen) and sterile water to make the PCR reaction mix. To all 96 wells of the PCR plate, 25 µL of reaction mix was added. Plates were sealed and run on a Bio-Rad CFX96 RT-PCR thermocycler with the following thermal cycling conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and then 60 °C for two min. A cycle threshold value of 34 was selected based on the 16S cut-off recommended by the manufacturer. Cycle threshold values of ≤34 were considered positive; 35–40, equivocal. No amplification was considered negative.

WGS data were reinterrogated using three independent bioinformatic programs: ResFinder and the ResFinder database [26], AMRfinderPlus and the NCBI database [27], and SRST2 and the CARD database [28]. Assembled contigs were used as input into Resfinder and AMRfinderPlus, whereas raw reads were used as inputs into SRST2, using default settings for all programs. An AMR gene was defined as present if detected in at least two of the three AMR gene detection programs. Results from the qPCR assay were compared to WGS results using sensitivity, specificity, positive predictive value, negative predictive value, and Cohen's Kappa statistic. Sensitivity was defined as the proportion of isolates with a targeted AMR gene that had a positive detection with qPCR. Specificity was defined as the proportion of isolates without the targeted AMR gene that had a negative detection with qPCR. Positive predictive value was defined as the probability that samples with the targeted AMR gene had a positive detection with qPCR, and negative predictive value was defined as the probability that samples without the targeted AMR gene had a negative detection with qPCR. We used the Kappa statistic as a measure of inter-method reliability to assess how WGS and qPCR agreed compared to chance alone. The Kappa statistic was categorised according to McHugh [29]. All metrics were calculated using SAS 9.4 with the 'proc freq' command.

Table 1
qPCR performance statistics in detecting antimicrobial resistance genes in bacterial DNA using whole-genome sequencing detection of antimicrobial resistance genes as a gold standard

Antibiotic Class	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value	Kappa	Kappa Rating
Aminoglycosides	0.889	0.944	0.686	0.985	0.739	Moderate
Class A Beta-lactams	0.833	0.999	0.987	0.984	0.895	Strong
Class B Beta-lactams	1	0.998	0.917	1	0.955	Almost perfect
Class C Beta-lactams	1	0.980	0.286	1	0.437	Weak
Class D Beta-lactams	0.857	0.995	0.667	0.998	0.747	Moderate
Fluoroquinolones	0.892	0.961	0.647	0.991	0.726	Moderate
Macrolides	1	0.996	0.750	1	0.855	Strong
Tetracycline	0.968	0.983	0.968	0.983	0.951	Almost perfect
All genes combined	0.886	0.988	0.802	0.994	0.832	Strong

NOTE: Erythromycin, MDR Efflux Pump, and Van and Staph were not detected in this study and therefore have been excluded from this table.
Gene-specific statistics are provided in Supplemental Table 1.

2.3. Sample collection

We collected 52 gull (*Larus* Spp.) faecal samples from locations on the upper (n = 2) and lower Kenai River (n = 50) during July 2021 by placing two sterile swabs into recently deposited gull faeces found on rocks or the beach adjacent to the river. One swab was placed into Longmire buffer ('A' samples), and the other, into chilled Luria broth ('B' samples). Vials of Luria broth were kept cool on ice for up to 6 h and then frozen at -80°C. Vials of Longmire buffer were stored at room temperature for approximately six months.

Water samples (500 mL) were collected in triplicate from two locations on the upper Kenai River and one location on the lower Kenai River, kept cool on ice for up to 6 h, and then stored at 4°C overnight. The following day, 200 mL of water from each sample was i) filtered through a 0.2 µm filter (MilliporeSigma, USA) that was subsequently placed in a vial of Luria broth and ii) filtered through a 0.2 µm filter that was subsequently placed in a vial of Longmire buffer. Vials of Luria broth were frozen at -80°C, and vials of Longmire buffer were stored at room temperature for approximately six months.

2.4. Bacterial culture and phenotypic antimicrobial susceptibility testing

Gull faecal swab samples were inoculated in 2 mL of brain heart infusion (BHI) broth (Becton Dickinson, USA) supplemented with vancomycin (16 mg/L; Sigma-Aldrich, Merck, Sweden) for the selection of Gram-negative bacteria and incubated for 18–24 h at 36°C. Following incubation, 10 µL of BHI broth was streaked onto CHROMagar C3GR plates (CHROMagar, France), a selective growth medium that supports the growth of bacteria with reduced susceptibility to extended-spectrum cephalosporins, and Uriselect plates (Bio-Rad Laboratories), a non-selective growth medium. More than one isolate was retained from C3GR plates if colonies with different phenotypes were observed. Presumptive *E. coli* and *Klebsiella* isolates were subjected to matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF-MS) for species identification [30].

Antimicrobial susceptibility testing of confirmed *E. coli* and *K. pneumoniae* isolates was performed using the following antibiotic discs: nalidixic acid (30 µg), nitrofurantoin (100 µg), piperazillin-tazobactam (36 µg), tetracycline (30 µg), trimethoprim (5 µg), trimethoprim-sulfamethoxazole (25 µg), meropenem (10 µg), ciprofloxacin (5 µg), ampicillin (10 µg), cefadroxil (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), and mecillinam (10 µg) (Thermo Fisher Scientific Oxoid Ltd, Hants, UK), according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [31]. Inhibition zone diameters were interpreted according to EUCAST breakpoints [32], or to breakpoints defined by the Normalized Resistance Interpretation method [33] for antibiotics with no defined clinical breakpoints. Phenotypic characterisation of extended-spectrum beta-lactamase–producing *E. coli* or *K. pneumoniae* isolates from the CHROMagar C3GR plates was performed using the EUCAST disc diffusion method with the following four antibiotic discs: ceftazidime (10 µg), cefotaxime (5 µg), cefepime (30 µg), and amoxicillin/clavulanic acid (30/1 µg).

WGS was performed on isolates resistant to one or more beta-lactam antibiotic. Libraries were prepared using the NexteraXT kit (Illumina, USA), and sequencing was performed on MiSeq, using a 600 cycle v3 kit (Illumina). All sequences are publicly available in the sequence read archive under BioProject ID PRJNA870156. Raw reads were trimmed and filtered using fastp [34] using default settings and then assembled de novo with Unicycler [35]. AMR genes were detected from assembled contigs using Resfinder [26], and multilocus sequence types (MLSTs) were determined using SRST2 [28].

2.5. qPCR analysis of gull faecal samples and water

DNA was extracted directly from gull faeces and filters stored in Longmire buffer using the PowerFecal DNA extraction kit (Qiagen, USA) and PowerWater DNA extraction kit (Qiagen, USA), respectively, according to the manufacturers' instructions, to a final elution of 100 µL. DNA was quantified using a Qubit 3.0, and qPCR was performed as described above.

3. Results

3.1. Baseline concordance

Forty-five *E. coli* or *K. pneumoniae* isolates were analysed by both WGS and a commercially available qPCR assay for the presence of 84 AMR genes. With WGS results considered biological reality, estimates for sensitivity and specificity of qPCR (excluding equivocal results) were 88.6% and 98.8%, respectively, and agreement between qPCR and WGS was 0.832 ('strong') as determined by Cohen's Kappa statistic. The positive predictive value and negative predictive value were 80.2% and 99.4%, respectively (Table 1).

Positive predictive values fluctuated among individual genes (Supplementary Table 1) and 11 antibiotic classes, ranging from 28.6% (Class C beta-lactams) to 98.7% (Class A beta-lactams). Kappa values similarly reflected differences among antibiotic classes. Including equivocal results as detections (i.e., higher qPCR cycle threshold cut-off) increased the sensitivity and decreased the specificity and Kappa values (Supplementary Table 1). qPCR failed to identify an AMR gene detected through WGS on 10 occasions (eight genes) (Table 2; Supplementary Fig. 1). Several discordant

Table 2
Number of concordant, equivocal, and discordant antimicrobial resistance (AMR) gene detections among bacterial isolates from wild bird faeces using a qPCR assay compared to whole-genome sequencing

Gene	Concordant: qPCR positive/WGS positive (proportion)	Equivocal: qPCR weak positive/WGS negative or positive (proportion)	Discordant: qPCR positive/WGS negative (proportion)	Discordant: qPCR negative/WGS positive (proportion)
AAC(6)-lb-cr	13 (0.29)	1 (0.02)	2 (0.04)	0
aacC1	0	0	0	0
aacC2	16 (0.36)	3 (0.07)	1 (0.02)	0
aacC4	0	0	0	0
aadA1	3 (0.07)	0	10 (0.22)	2 (0.04)
ACC-1 group	0	0	0	0
ACC-3 group	0	0	0	0
ACT 5/7 group	0	1 (0.02)	0	0
ACT-1 group	0	0	0	0
aphA6	5 (0.11)	0	0	0
BES-1	0	0	0	0
BIC-1	0	0	1 (0.02)	0
ccrA	0	0	0	0
CFE-1	0	0	0	0
CMY-10 group	0	0	0	0
CTX-M-1 group	12 (0.27)	6 (0.13)	0	1 (0.02)
CTX-M-8 group	1 (0.02)	1 (0.02)	0	0
CTX-M-9 group	4 (0.09)	9 (0.20)	0	1 (0.02)
DHA	4 (0.09)	7 (0.16)	0	0
ereB	0	0	0	0
ermA	0	0	0	0
ermB	3 (0.07)	4 (0.09)	1 (0.02)	0
ermC	0	1 (0.02)	0	0
FOX	0	1 (0.02)	0	0
GES	0	0	0	0
IMI NMC-A	0	0	0	0
IMP-1 group	0	0	0	0
IMP-12 group	0	0	0	0
IMP-2 group	0	0	0	0
IMP-5 group	0	0	0	0
KPC	4 (0.09)	0	0	0
LAT	0	3 (0.07)	9 (0.20)	0
lukF	0	0	0	0
mecA	0	1 (0.02)	0	0
mefA	0	0	0	0
MIR	0	0	0	0
MOX	0	0	1 (0.02)	0
msrA	0	2 (0.04)	0	0
NDM	10 (0.22)	4 (0.09)	0	0
oprj	0	0	0	0
oprm	0	0	0	0
OXA-10 group	0	1 (0.02)	0	1 (0.02)
OXA-18	0	0	0	0
OXA-2 group	3 (0.07)	0	0	0
OXA-23 group	0	0	0	0
OXA-24 group	0	0	0	0
OXA-45	0	0	1 (0.02)	0
OXA-48 group	3 (0.07)	0	1 (0.02)	0
OXA-50 group	0	0	0	0
OXA-51 group	0	0	0	0
OXA-54	0	0	0	0
OXA-55	0	0	0	0
OXA-58 group	0	0	0	0
OXA-60	0	0	1 (0.02)	0
Per-1 group	0	0	0	0
Per-2 group	0	0	0	0
QepA	0	0	0	1 (0.02)
QnrA	1 (0.02)	1 (0.02)	0	0
QnrB-1 group	7 (0.16)	3 (0.07)	4 (0.09)	1 (0.02)
QnrB-31 group	0	0	1 (0.02)	0
QnrB-4 group	4 (0.09)	6 (0.13)	0	0
QnrB-5 group	3 (0.07)	6 (0.13)	9 (0.20)	2 (0.04)
QnrB-8 group	0	1 (0.02)	1 (0.02)	0
qnrC	0	0	0	0
qnrD	0	0	0	0
qnrS	5 (0.11)	6 (0.13)	1 (0.02)	0
SFC-1	0	0	0	0
SFO-1	0	1 (0.02)	0	0
SHV	10 (0.22)	3 (0.07)	0	0
SME	0	0	0	0
spa	0	0	0	0
StaphA	0	0	0	0

(*continued on next page*)

Table 2 (*continued*)

Gene	Concordant: qPCR positive/WGS positive (proportion)	Equivocal: qPCR weak positive/WGS negative or positive (proportion)	Discordant: qPCR positive/WGS negative (proportion)	Discordant: qPCR negative/WGS positive (proportion)
tetA	26 (0.58)	13 (0.29)	0	0
tetB	4 (0.09)	4 (0.09)	1 (0.02)	1 (0.02)
TLA-1	0	0	0	0
vanB	0	0	0	0
vanC	0	0	0	0
VEB	0	0	0	0
VIM-1 group	1 (0.02)	1 (0.02)	0	0
VIM-13	0	0	1 (0.02)	0
VIM-7	0	0	0	0

detections could be attributed to gene variants, such as the detection of *aadA1* in a sample when using qPCR and the detection of gene variants *aadA2* or *aadA5* from the same sample when using WGS.

3.2. AMR gene detection using qPCR of environmental DNA extracts and WGS of cultured bacterial isolates

AMR gene detection in gull faecal samples (n = 52) and river water samples (n = 9) was performed using i) qPCR of direct DNA extracts and ii) bacterial culture followed by phenotypic antimicrobial susceptibility testing and WGS of resistant isolates. The qPCR assay detected one or more AMR gene from 59% (30/51) of faecal samples and 11% (1/9) of surface water samples (Fig. 1). One faecal sample failed amplification of positive controls and was therefore omitted from comparisons. A total of 27 different genes were detected among direct DNA extracts from faecal and water samples, ranging from detection in a single sample (e.g., *qnrB-1* group) to detection in as many as 16 samples (e.g., *ermB*). Twenty-eight genes were not detected in any sample.

Five *E. coli* isolates (from four gull faecal samples) were cultured from selective enrichment samples for extended-spectrum cephalosporin resistance and exhibited additional phenotypic resistance to up to five different antibiotic classes other than the beta-lactams for which it was selected in the screening process. An additional 30 *E. coli* and 12 *K. pneumoniae* isolates were cultured from non-selective enrichment samples, of which four *E. coli* isolates and one *K. pneumoniae* isolate (from four gull faecal samples) exhibited phenotypic resistance to one or more antibiotics tested (Supplementary Table 2). WGS of these 10 isolates resulted in detection of one or more AMR genes from 15% (8/52) of faecal samples and 0% (0/9) of surface water samples, with AMR genes conferring resistance to aminoglycosides, beta-lactams, quinolones, macrolides, tetracyclines, and other antibiotics that do not have AMR genes represented in the qPCR assay (Fig. 2). Identical clones, defined as isolates with identical AMR gene profiles and MLSTs, were identified on two occasions. In one case, *E. coli* ST963 was found in gull faeces collected on the upper and lower Kenai River, approximately 16 km apart (Fig. 2).

One AMR gene detected via WGS of a bacterial isolate was also detected in the same sample via qPCR of a direct environmental DNA extract, whereas six genes detected via WGS were not detected in the same samples using qPCR (Fig. 2). In the baseline concordance analysis, these five genes (*aadA1, CTX-M-1* group, *qnrS, tetA*, and *tetB*) had positive predictive values that ranged between 0.231 and 1.000 (Supplementary Table 1). No AMR genes were identified using qPCR in two faecal samples from which a phenotypically resistant bacterial isolate was found (Fig. 1). Results from both qPCR and bacterial culture suggested low levels of resistance in river water samples. Beta-lactam resistance was detected in 39% (20/51) of faecal samples using qPCR and only 12% (6/52) of paired faecal samples using bacterial culture and WGS.

4. Discussion

Using bacterial isolates recovered from wild bird faeces and water samples obtained from wild bird habitats, we assessed the reliability and utility of a commercially available qPCR assay for detecting AMR genes, as compared to a WGS approach. We found strong concordance between the qPCR assay and WGS with regard to detecting AMR genes from individual bacterial isolates. Overall sensitivity and specificity of the qPCR assay was high, although positive predictive values varied among different antibiotic classes. Some genes detected in the qPCR assay appeared to have low specificity, as WGS revealed different gene variants.

While direct comparison between cultured *E. coli* and *K. pneumoniae* isolates and characterisation of AMR genes from DNA extracts of the entire environmental sample is not appropriate given the different starting material (i.e., DNA from a bacterial isolate versus DNA from all organisms in the environmental sample), one would expect to find AMR genes in the metagenomic sample if phenotypic resistance and/or AMR genes were detected in a bacterial isolate cultured from that sample. The latter did not always prove true in our comparisons of analytical approaches, presumably for a variety of reasons. For example, beta-lactamase genes were not detected in two samples using qPCR, although $bla_{\text{CMY-2}}$ or $bla_{\text{TEM-1}}$ (targets not included in the qPCR assay) were found in phenotypically resistant isolates from respective paired samples using bacterial culture and WGS. In numerous other instances, gene targets were not detected in samples using the qPCR assay, despite the finding that phenotypically resistant isolates were cultured from paired samples and found to contain targeted AMR genes through WGS. In these instances, we presume that the qPCR assay did not detect gene targets on account of imperfect sensitivity. As expected, resistance was detected using qPCR in numerous gull faecal samples that were not identified as harbouring resistance genes using bacterial culture. These AMR genes may have been present in bacteria other than the selected indicator bacteria, in unculturable bacteria, in dead bacteria, or as free DNA. *E. coli* was isolated from only 6% (3/50) of gull faecal samples collected from the lower Kenai River in this study using selective enrichment, whereas *E. coli* was isolated from 26% (44/168) of gull faecal samples collected in 2016 from the same general location using the same selective enrichment methodology [24]. The isolation of *E. coli* from non-selective enrichment samples remained relatively unchanged between years, with *E. coli* isolated from 54% (27/50) and 58% (98/168) of samples in 2021 and 2016, respectively. As such, selective enrichment followed by isolation of indicator bacteria may have been limited by overgrowth of non-target species, such as those intrinsically resistant to several antibiotics, such as *Serratia* Spp., *Enterbacter* Spp., *Hafnia* Spp., and *Morganella* Spp., all of which were detected in samples analysed in this study.

The choice of molecular tool for AMR gene detection depends, in part, on the research objective and the sample matrix. Environmental samples may contain bacterial species harbouring

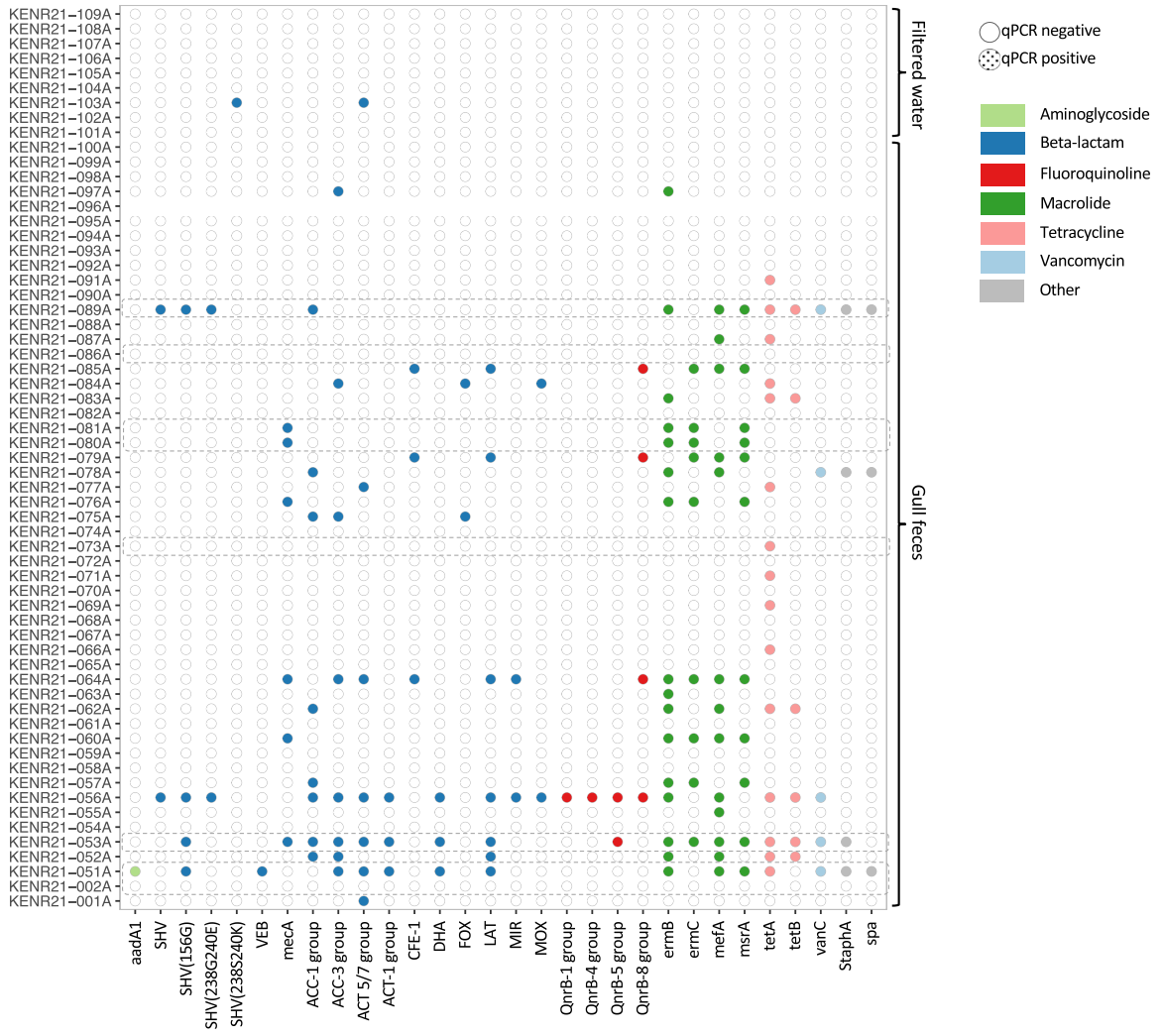

Fig. 1. Quantitative PCR (qPCR) detection of antimicrobial resistance (AMR) genes from 52 gull faecal samples and 9 filtered water samples. Rows represent sample IDs, and columns represent AMR gene targets identified in the qPCR assay. Filled circles represent detections, with colours representing the antibiotic class to which resistance is conferred. Samples that produced phenotypically resistant isolates through bacterial culture are designated by dotted boxes.

naturally occurring resistance genes, as many AMR genes now common in clinical settings initially evolved in environmental bacteria [36]. For some applications, confirmation that AMR genes are present within a pathogen or mobile genetic element is essential, in which case bacterial culture may be required. Similarly, the identification of molecular epidemiological links between samples is facilitated by the identification of bacterial species, sequence type, AMR genes, and the genomic context thereof, which is only provided though WGS. This was exemplified by the identification of identical bacterial clones in bird faeces collected in different locations along the Kenai River. Additionally, research questions that rely on the ability to accurately differentiate between genetic variants that could be epidemiologically informative [37] may require a more detailed analysis of AMR genes, such as that provided by WGS. For other applications that have broader objectives, such as to estimate the overall burden or the relative similarities/differences in AMR genes among locations, qPCR detection of preselected AMR genes present within the entire microbial community may be sufficient and even advantageous [38]. Prior studies have demonstrated that qPCR surveillance of AMR in the environment, particularly in wastewater, can be successfully used to monitor trends in local communities [39]. Our qPCR results indicate that on average, at least 25% of wild birds inhabiting the lower Kenai River harbour beta-lactam, macrolide, and tetracycline resistance genes. Such data could be informative for local management agencies if monitored over time and space to understand potential trends and inform assessments of dissemination risk [21].

Our results demonstrate that the commercially available qPCR assay employed in this study is a reliable and rapid tool for the detection of environmental AMR genes. However, this particular commercial qPCR assay did not include some AMR genes (e.g., $bla_{\text{CMY-2}}$ and $bla_{\text{TEM-1}}$) of clinical importance that were present in faecal samples from wild birds inhabiting Alaska, as well as AMR genes conferring resistance to other potentially important classes of antibiotics, such as sulfonamides and colistin [40,41]. WGS and qPCR methods identified both overlapping and different samples harbouring AMR genes and offered different advantages. Depending on research objectives, future studies investigating AMR genes in wild bird habitats may benefit from custom qPCR assays to incorporate measures of target abundance, specific targeted genes of interest, and source tracking markers. However, the selection of AMR genes to target in environmental samples is challenging, given that some genes (e.g., $bla_{\text{CMY-2}}$) may be a serious clinical problem when present among pathogenic clones of *E. coli* or *K. pneumoniae*,

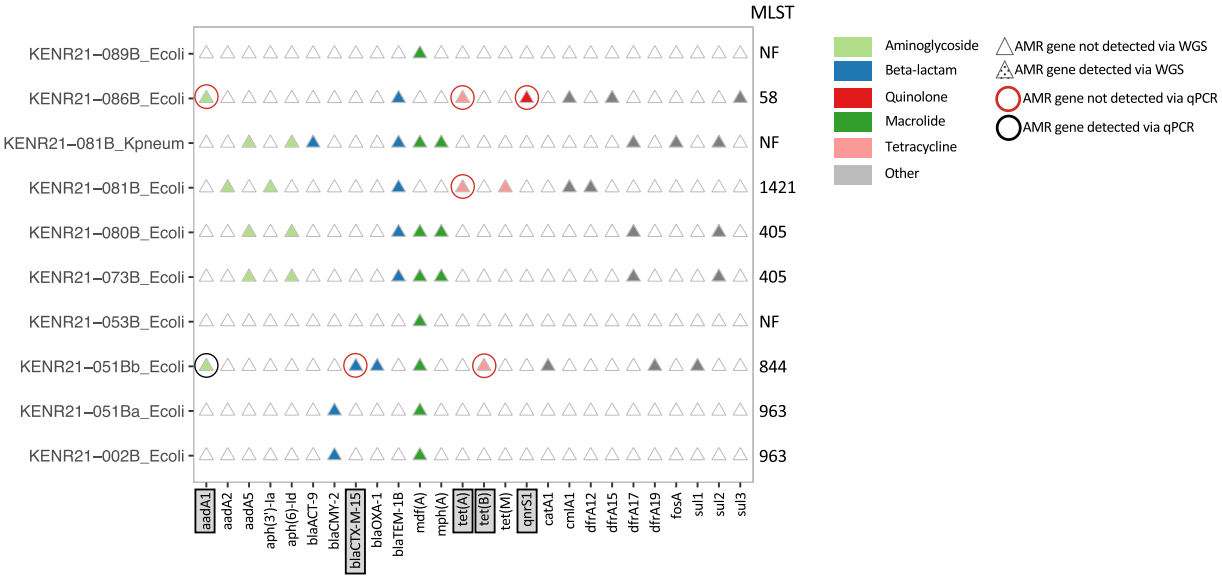

Fig. 2. Whole-genome sequencing detections of antimicrobial resistance (AMR) genes from phenotypically resistant bacterial isolates cultured from 52 gull faecal samples and 9 filtered water samples. Rows represent sample IDs of isolates, and columns represent AMR genes identified using whole-genome sequencing. Filled triangles represent detections, with colours representing the antibiotic class to which resistance is conferred. Shaded AMR gene names indicate that the gene is represented in the qPCR assay, and circles indicate whether the gene was (black) or was not (red) detected in the qPCR assay.

although they may have naturally occurring homologues in common environmental bacteria (e.g., *Citrobacter freundii*). Thus, an alternative approach would be to use step-wise analyses of AMR genes, such as qPCR followed by culture and WGS, on select samples to identify if targeted AMR genes are present among clinically relevant bacteria. Such a step-wise workflow may leverage the advantages of multiple approaches to thoroughly and efficiently evaluate and characterise AMR of clinical importance among environmental samples [19].

Ethical approval

Not Required.

Competing interests

None declared.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jgar.2023.03.009.

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