A preliminary investigation of major bacterial antibiotic resistance genes present in Australian fecal samples using shotgun metagenomics and targeted sequencing.

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Abstract

The gut microbiota is an immense reservoir of antimicrobial resistance genes (ARGs); however, in Australia the profile of the gut 'resistome', or ensemble of ARGs, has not been investigated. This study provides a first preliminary mapping of the major bacterial ARGs present in human, domestic dog and wild duck fecal samples collected from south-eastern Victoria, Australia; and evaluates the use of shotgun metagenomics sequencing (SMS) and targeted amplification of ARGs. We analysed SMS data using an in-house method and web-based bioinformatics tools: ResFinder and KmerResistance. We examined targeted sequences using One Codex or the PanBacterialAnalysis Torrent Suite plugin. All methods detected ARGs in all samples, with resistance to up to 13 classes of antibiotics detected overall. ARGs were more abundant in the human and dog samples than the duck samples. They mostly conferred resistance to three classes of antibiotics that are the most frequently prescribed in Australia: tetracycline, β-lactams and MLS_B (macrolide, lincosamide, streptogramin B). Targeted sequencing significantly improved sensitivity for detection of ARGs included in the panel; however, SMS provided quantitative information and allowed tentative identification of the host bacteria. For SMS, web-based and in-house methods gave comparable results, with discrepancies mostly due to different reference databases. The in-house method allowed manually checking results and potential errors, while web-based methods were user-friendlier and less time-consuming. More samples need to be investigated to fully describe the resistome in humans and animals in Australia.

Keywords:
AMR; antimicrobial resistance; metagenomics; NGS; resistome; resistance genes

Introduction

Antimicrobial resistance (AMR) occurs naturally in bacteria and other microorganisms, but increases when antimicrobials (antibiotics, antivirals, antifungals) are used[1]. According to WHO, AMR accounts for an estimated 700,000 deaths per year and is one of the ten threats to global health in 2019[2]. Reservoirs of multi drug-resistant (MDR) bacteria are ubiquitous, and the concept of an "antibiotic resistome" was first coined to describe the large collection of bacterial antimicrobial resistance genes (ARGs) found in a specific environment[3]. Although AMR is most clinically relevant in pathogenic bacteria, ARGs exist in both pathogenic and non-pathogenic bacteria, and large reservoirs of these genes exist in all ecosystems, including in commensals at all sites of the human or animal body[4].

The adult human gastrointestinal tract harbours up to 1,000 molecular species or phylotypes often referred to as operational taxonomic units (OTUs) in 16S rRNA metagenomics[5,6]. This vast array of resident bacteria responds to environmental conditions inside the host, including altered dietary intake and antibiotic-induced disturbances. That can result in modifications in the microbial community structure and genetics, including the enrichment of ARGs within the gut microbiota. Recent studies have suggested potential links between the gut resistome and the spread of AMR: horizontal transfer of ARGs from commensal bacteria mediated by mobile genetic elements may affect potentially pathogenic organisms[7-9]. Although the development of culture-independent sequencing techniques has expanded our knowledge of the different reservoirs of ARGs, it is currently unclear how widespread commensal bacteria carrying ARGs are in the gut microbiota, and how they develop over time[10].

There is however considerable evidence that antibiotic exposure promotes the development of a resistome in the gut microbiota[11-13]. Different features such as class, potency, spectrum and regimen of the antibiotics can influence the observed pattern of resistance of the gut microbiota[13]. Geographical differences also exist between countries in relation to the ARGs detected in human fecal samples[14]. For example, the number and abundance of ARGs was higher in the fecal samples of Chinese individuals than in the Spanish and Danish groups studied[15]. Variation in the total usage of antibiotics between countries may be one explaining factor for those differences. Indeed, both antibiotic exposure and the number of ARGs were higher in individuals from Spain, France and Italy than in individuals from Denmark, Japan and the United States[16]. In other countries such as Australia, very little is known about the resistome of humans and animals, with available studies focusing primarily on ARGs in pathogenic bacteria (e.g. *Campylobacter* or *Escherichia coli*) or in environmental samples, e.g. studying the soil resistome after swine, cattle or poultry manure usage[17-19]. One recent study however assessed the diversity and abundance of ARGs transcribed in the fecal microbiota of water birds of Australia[20], but currently no study focused on the human fecal microbiota and its associated resistome in Australia. This clearly represents a significant knowledge gap. Regular surveillance of the resistome in Australia could be used to monitor trends in AMR over time and the emergence of resistance alleles. Resistome

surveillance could also inform future prescribing guidelines for doctors in hospitals and community clinics, and veterinarians in companion and production animal settings, to help preserve the effectiveness of valuable antibiotics.

The declining costs of metagenomic sequencing technologies has led to increased molecular investigation of ARGs and has enabled a shift from phenotype to genotype-based approaches to investigate AMR[21,22]. The ultimate goal of ARG sequencing and related bioinformatic analyses is the accurate detection of the resistome and prediction of the antibiogram from genomic and metagenomic data[23]. Several major databases provide collections of ARGs covering broad categories of AMR mechanisms including the Comprehensive Antibiotic Resistance Database (CARD)[24], Antibiotic Resistance Gene-ANNOTation (ARG-ANNOT)[25], the Bacterial Antimicrobial Resistance Reference Gene Database[26] hosted by the National Center for Biotechnology Information (NCBI) as part of the National Database of Antibiotic Resistant Organisms (NDARO), and ResFinder[27] (see [23] for a more exhaustive list of available AMR bioinformatics software, databases, and data-sharing resources). These resources and their associated prediction tools enable detection and annotation of specific AMR elements in sequenced samples by comparing user-submitted sequences against representative sets of ARGs sequences. While identifying ARGs from a bacterial isolate from a sick patient is reasonably straightforward using whole genome sequencing, the exhaustive description of a resistome in complex environments with high bacteria diversity and relatively low abundances of ARGs is challenging. Two different strategies have been developed to study AMR in complex environments like the gut. Deep shotgun metagenomic sequencing is used to produce millions of reads that are filtered to map the ARGs. However, because reads corresponding to ARGs can be low (e.g. less than 2% of the total sequencing data in [28]), targeted approaches (including an initial amplification of a panel of ARGs) with increased sensitivity have been developed. As new methods and new commercial panels become available, it is currently unclear what method is best to study resistomes when no pre-existing information is available, e.g. in Australia.

We investigated for the first time the diversity and abundance of ARGs in a broad range of fecal microbiota samples (a number of infants, healthy pregnant women, domestic dog, wild ducks) collected in south-eastern Victoria, Australia. We used both non-targeted NGS (analyzed in-house and with web-based tools) and targeted NGS (using two commercially available panels) and assessed and compared the performance of these tools for the analysis of bacterial resistomes in an Australian context. This preliminary study will pave the way for a broader analysis of bacterial resistomes in humans and animals in Australia.

Methods

Sample collection and processing

127 *Sample collection*

128 Human and animal samplings were performed in accordance with relevant guidelines and
129 regulations. We collected stool samples from two infants and seven adults from the southeast of
130 Australia (Table 1). The children were: one infant sampled at 4 weeks of age (ST5-1mo) and again
131 at 18 months (ST5-18mo), and another infant sampled at 3 months of age (ST4-3mo). The study
132 involving these samples was deemed negligible or low risk by the Barwon Health Human
133 Research Ethics Committee and therefore exempt from full committee review (HREC approval
134 17/119). The adult samples originated from pregnant women at 36-weeks gestation between 2010
135 and 2013 with informed consent for study participation as part of the Barwon Infant Study (BIS), a
136 birth cohort study–eligibility criteria and cohort profile are described elsewhere[29]. Ethics approval
137 for this study was granted by the Barwon Health Human Research and Ethics Committee (HREC
138 10/24). All human stool samples were stored at -80ºC prior to DNA extraction.

139

140 **Table 1. List of tested human and animal samples** (n = 13). Fecal samples were DNA extracted
141 using two different methods, as described.

Sample ID	Host	Extraction method
ST5-1mo	Human - child 1 month of age	PowerSoil® DNA Isolation Kit - Mo Bio
ST4-3mo	Human - child 3 months of age	QIAamp Fast DNA Stool Mini Kit
ST5-18mo	Human - child 18 months of age	QIAamp Fast DNA Stool Mini Kit
HS21	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
HS22	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
HS23	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
HS24	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
HS25	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
HS26	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
HS28	Human - adult	PowerSoil® DNA Isolation Kit - Mo Bio
ST1-MAD*	Pool from 6 Pacific black ducks (*Anas superciliosa*)	QIAamp Fast DNA Stool Mini Kit
ST3-MUD*	Muscovy duck (*Cairina moschata*)	QIAamp Fast DNA Stool Mini Kit
DFS**	Pool from 2 dogs	QIAamp Fast DNA Stool Mini Kit

142 HS: human sample; DFS: dog fecal sample. * See [30]. ** See [31].

143

144 Samples from ducks and dogs were collected in the south-eastern part of Victoria, Australia, as
145 part of other projects investigating fecal microorganisms. Fresh fecal samples from one Muscovy
146 duck (MUD) and a pooled sample from six juvenile Pacific black ducks (MAD) were collected in
147 November and December 2016 respectively[30]. Bird sample collection was approved under Deakin
148 University's Animal Ethics Committee project number B43–2016 and Department of Environment,
149 Land, Water and Planning permit number 1008206.

150 A pooled dog fecal sample (DFS) originating from two pups (7 weeks of age) was collected from
151 the ground immediately after being deposited, with the consent of the owner[31]. These puppies
152 were healthy at the time of collection, but they had been sick just 1-2 weeks before and an
153 astrovirus was detected in the 7-week sample[31]. However, they were originally thought to have a
154 *Campylobacter* infection and were likely treated with antibiotics at 5 weeks of age. Swabs from
155 dog samples were placed in Universal Transport Medium (UTM) and stored on ice overnight. The
156 following morning, swabs were aliquoted and frozen at -80ºC until DNA extraction.

157
158 *DNA extraction*
159 DNA was extracted from fecal samples and swabs using the Qiagen Powersoil® DNA Isolation Kit
160 (Cat#12888-100) (QIAGEN Pty Ltd., Victoria, Australia) or the QIAamp Fast DNA Stool Mini Kit
161 with MoBio bead shearing (Mo Bio, Carlsbad, CA USA). The manufacturer's protocol was slightly
162 modified following optimization: for the inhibitors' removal steps, 375 μL of Solution C2 and 335 μL
163 of Solution C3 were used instead of the prescribed volumes. DNA concentrations were measured
164 using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Scoresby, VIC, Australia), and
165 the quantity and size distribution of the fragments after DNA extraction was visualized on an
166 Agilent Bioanalyzer DNA 7500 chip (Agilent Technologies) using the High sensitivity DNA kit
167 (Agilent Technologies).
168
169 **Library preparation and sequencing**
170 Extracted DNA was sequenced using three different methods relying on Next Generation
171 Sequencing (NGS). We used a non-targeted sequencing of sheared DNA, hereafter referred to as
172 "Shotgun Metagenomic Sequencing" (SMS) method, as well as a targeted metagenomic
173 sequencing method using two commercially available Ion Ampliseq™ (PCR) panels (Life
174 Technologies).
175
176 *Non-targeted sequencing: Shotgun Metagenomic Sequencing*
177 Genomic libraries were prepared separately for each genomic sample from 100 ng of DNA. DNA
178 was fragmented using the Ion Shear™ Plus Reagents (Life Technologies, Grand Island, NY).
179 Aiming for a 200 base-read library, the chosen reaction time for the enzymatic incubation at 37°C
180 was 15 minutes, as advised in the manufacturer's protocol. Sheared DNA was purified with
181 Agencourt AMPure® XP Reagent (1.8x sample volume) (Beckman Coulter, Lane Cove, NSW,
182 Australia). The quantity and size of sheared material was visualized on an Agilent Bioanalyzer
183 DNA 7500 chip (Agilent Technologies) using the High sensitivity DNA kit (Agilent Technologies)
184 assuming a targeted 200 base-read library.
185 The adapter ligation and nick repair were performed using the Ion Plus Fragment Library kit and
186 Ion Xpress barcode adapters following the manufacturer's recommendations (Life Technologies).
187 Ligated and nick repaired DNA was purified with Agencourt AMPure® XP Reagent (1.4x sample
188 volume) (Beckman Coulter) assuming a targeted 200 base-read library. The ligated and nick
189 repaired DNA was size-selected individually with the E-Gel® SizeSelect™ Agarose Gel (Life
190 Technologies). The size selected (and unamplified) libraries were quantified as per the
191 manufacturer's instructions using the Ion Library qPCR Quantitation Kit (Life Technologies) to
192 determine if library amplification was required.
193 If required (i.e. final library quantity was < 50 pM), the size selected libraries were further amplified
194 using Platinum® PCR SuperMix High Fidelity and Library Amplification Primer Mix as per
195 manufacturer's instructions (Life Technologies). This was necessary for four samples (ST4-3mo,

196 MUD, MAD and DFS), which were amplified for 10 PCR cycles prior to purification and
197 quantification.
198 All libraries were standardized to a concentration of 100 pM. Libraries were then pooled and
199 diluted to 50 pM prior to loading onto Ion 530™ or 540™ Chips using the Ion Chef Instrument and
200 Ion 530™ or Ion 540™ Kit (Thermo Fisher Scientific). Following template preparation, the chips
201 were run on the Ion Torrent S5xl System (Thermo Fisher Scientific) as per company protocols.
202 NGS and associated reactions were performed at the Geelong Centre for Emerging Infectious
203 Diseases (GCEID), Geelong, Victoria, Australia.
204
205 *Targeted sequencing: IonAmpliSeq™ panels*
206 We used two commercially available community panels designed to target AMR determinants,
207 both kindly donated by Life Technologies. The first panel used was the Ion AmpliSeq™ Pan-
208 Bacterial Research panel, consisting of two primer pools: the one pool contained 269 amplicons
209 targeting 21 specific bacterial species and 716 amplicons targeting 364 known ARGs; the other
210 pool was comprised of 24 amplicons for 16S rRNA gene profiling of up to approximately 400,000
211 16S rRNA sequences. From this panel, we only used the first primer pool targeting the ARGs.
212 The second panel used was the Ion AmpliSeq™ Antimicrobial Resistance (AMR) Research panel
213 that also consisted of two primer pools comprising a total of 815 amplicons targeting 478 known
214 ARGs[32]. Both primer pools were used from this panel. The list of primers is available upon request
215 from Life Technologies (https://www.thermofisher.com/).
216 Ten nanograms of extracted DNA were PCR-amplified with the Ion AmpliSeq™ Library Kit (Life
217 Technologies) and Ion AmpliSeq™ Panels, both panels including a 5X Ion AmpliSeq™ HiFi Master
218 Mix. With the Pan-Bacterial Research panel, PCR conditions were as follows: enzyme activation at
219 99ºC for 2 minutes followed by 18 cycles of 99ºC for 15 seconds and 60ºC for 8 minutes before
220 holding at 10ºC. With the AMR Research panel, PCR conditions were as follows: enzyme
221 activation at 99ºC for 2 minutes followed by 19 cycles of 99ºC for 15 seconds and 60ºC for
222 4 minutes before holding at 10ºC. The following steps (adaptors and barcodes ligation, purification,
223 and qPCR quantification) were done as per the manufacturer's instructions. None of the
224 AmpliSeq™ libraries required further amplification at this stage. Libraries were then pooled prior to
225 loading onto an Ion 530™ Chip and loading into the Ion Chef Instrument. Following template
226 preparation, the chip was run on the Ion Torrent S5xl System (Thermo Fisher Scientific) following
227 company protocols. We submitted reads obtained from the SMS and targeted sequencing to the
228 European Nucleotide Archive (ENA) under project accession number PRJEB36405.
229
230 **Next generation sequence analyses**
231 We obtained three sets of sequence data that we analysed using different tools and methods. A
232 schematic flowchart summarizing the different sequencing and analysis methods is presented in
233 Figure 1.
234

235 **Figure 1. Flowchart describing three methods to identify the presence of the most common**
236 **ARGs in clinically healthy human/animal samples using NGS without culturing.** (A) Non-
237 targeted sequencing of sheared DNA or "Shotgun Metagenomic Sequencing" (SMS) method,
238 versus (B) and (C) targeted sequencing using two commercially available AmpliSeq™ panels
239 targeting ARGs. Sequences obtained with the SMS method were analysed using different web-
240 based tools (ResFinder and KmerResistance, https://cge.cbs.dtu.dk/services/), and an in-house
241 method based on the comparison of reads and contigs with a NCBI-curated ARGs database
242 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047). *Targeted sequencing was tested on
243 human samples only: 4 with the AMR Research panel, 8 with the Pan-Bacterial panel. ** refers to
244 Ion Torrent Suite plugins available from the Thermo Fisher Scientific Plugin store. *** refers to the
245 "One Codex" online platform (available at: https://app.onecodex.com/) used with the AMR
246 Research panel. "ID": percentage identity threshold; "minL": minimum length: minimum percentage
247 of coverage compared to reference length or minimum number of nucleotides (nt); "ref": reference
248 sequences used for comparison with reads and/or contigs.
249
250 *Analysis of the sequences obtained by Shotgun Metagenomic Sequencing*
251 Raw sequences were analysed using the Ion Torrent Server linked to the Ion Torrent S5xl
252 instrument, based on each sample having a unique barcode. We developed an "in-house method"
253 based on the comparison of our data with references in the NCBI-curated AMR gene database
254 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA313047) containing a total of 4,528 sequences
255 (downloaded on the 2nd of August 2018).
256 The reads were queried against the NCBI's ARGs database using Magic-BLAST[33] with
257 parameters as follows: 98% minimum identity between the read and the reference, and 70
258 nucleotides minimum length. Concomitantly, the Torrent Suite AssemblerSPAdes 5.6.0 plugin was
259 used to generate contigs from the reads for each sample[34]. These contigs were also queried
260 against the NCBI's ARGs database but using nucleotide BLAST (BLASTN)[35,36] with parameters as
261 follows: 80% minimum identity between the contig and the reference and 1e-10 minimum E-value.
262 The contigs identified to carry ARGs were queried against the "Microbes" reference database
263 using BLAST Genomes in order to tentatively identify the bacterial species carrying ARGs.
264 After identifying the ARGs present, FASTA files were created by adding chromosomes X, 21 and
265 mitochondrion from the human genome reference sequence (hg19) to each pre-selected ARG
266 sequence (listed in Supplementary Table S1). This was done in order to artificially increase the
267 length of the reference used for the mapping, as it is our experience that mapping issues may
268 arise from mapping reads against small references such as e.g. ARGs of only around 1,000 to
269 2,000 bp due to the scaling factor algorithm used by the Torrent Suite TMAP plugin. The extended
270 FASTA references were added to the Ion Torrent Server and used for the mapping of sequence
271 reads using the Torrent Suite TMAP plugin. Mapped reads and consensus sequences were
272 visualised and analysed using the Integrative Genomics Viewer (IGV)[37].
273
274 *ARGs coverage and depth*

275 Individual ARGs coverage and depth metrics were calculated using the Torrent Suite
276 CoverageAnalysis v5.6.0.1 plugin using the filters: library type = whole genome; minimum aligned
277 length = 100; minimum mapping quality = 20. The “average base read depth” obtained as an
278 output was used to standardize reads counts. A “mean base depth per 5 million reads” (or BD5M)
279 was calculated for each ARG in each sample following the formula: (average base read depth *
280 5,000,000) / total reads. Contrary to the common RPKM (number of reads per kb of gene per
281 million sequenced reads), BD5M is ‘per base’ and not ‘per read’ and its interpretation is
282 independent of the size of the reads or the reference. As an example, if 5 million reads were
283 obtained for a sample, BD5M = 2 for a specific gene is equivalent to reads mapping the whole
284 gene twice or reads mapping half of the gene four times. To avoid confusion between the raw
285 coverage from the sequencer and our calculated BD5M, we hereafter refer to the latest as an
286 abundance rather than a coverage.
287
288 *Additional AMR analyses*
289 We analysed the sequences obtained with the SMS method using two web-based tools,
290 KmerResistance (available at https://cge.cbs.dtu.dk/services/KmerResistance/)[38,39] and ResFinder
291 3.1 (available at https://cge.cbs.dtu.dk/services/ResFinder/)[27], in order to compare the results with
292 those obtained with our “in-house method” (Figure 1). Both tools rely on the same reference
293 database (available at https://cge.cbs.dtu.dk/services/data.php)[27]. Raw sequences (FASTQ reads)
294 from each sample were screened for ARGs present in the ResFinder database with the default
295 settings of 70% identity threshold and 10% depth correction using “bacteria” as the host database
296 and “resistance genes” as the gene database. Scoring method was “species determination on
297 maximum query coverage”. Contigs (FASTA files) from each sample were also screened for ARGs
298 present in the ResFinder database using the settings of 90% minimum identity and 20% minimum
299 length.
300
301 *Microbiota analysis and host identification*
302 The Torrent Suite Ion Reporter™ software was used to process reads for metagenomics analysis
303 of the gut microbiota. Data files (Binary Alignment Map, BAM) generated by the Ion Torrent S5xl
304 with the SMS method were uploaded to Ion Reporter (https://ionreporter.thermofisher.com/ir/). All
305 analyses were run using a “150-90-3” setting, i.e. minimum read length = 150; minimum alignment
306 coverage between hit and query = 90%; minimum number of unique reads (or ‘read abundance
307 filter’) = 3. This means that only reads found as triplicates or more were included in the analysis. A
308 minimum percentage identity of 97% was required for genus identification of a ribosomal
309 sequence, and a minimum percentage identity of 99% for species identification. When there was a
310 difference of less than 0.2% match between the top hit and the next best hit, the sequence was
311 identified as 'species slash ID' (unidentified species). The relative abundance of the species/genus
312 identified in the gut microbiota was determined from Ion Reporter™.

313 The contigs generated from SMS allowed tentative identification of host bacteria carrying the
314 ARGs. This was achieved by running the contigs against the "Microbes" database using BLAST
315 Genomes or the ResFinder tool.
316 *Analysis of the sequences obtained by Ion AmpliSeq™ panels*
317 Following targeted amplification, sequences obtained from the Ion Torrent S5xl were analysed
318 based on each sample having a unique barcode and using dedicated platforms or plugins, i.e. (i)
319 the "One Codex" web-based platform (available at: https://app.onecodex.com/) for the sequences
320 obtained from using the AMR Research panel, or (ii) the "PanBacterialAnalysis" Torrent Suite
321 plugin from the Ion Torrent Server for the sequences obtained from using the Pan-Bacterial
322 Research panel (see Figure 1). One Codex reported the percentage of the ARG sequence
323 covered by reads in the sample, the percentage identity to the reference ARG sequence, and the
324 depth of read coverage. The "PanBacterialAnalysis" plugin reported the "read counts" matching
325 each ARG in the database.
326

327 ## Results

328
329 All 13 samples were processed using the SMS method; with eight samples processed using the
330 Pan-Bacterial Research panel, and four of these eight also processed using the AMR panel (Table
331 2). SMS generated a total of 3.8 to 11 million reads per sample, with average read lengths of 162-
332 201 nucleotides (Table 2). Whatever the method, all samples had reads that mapped to ARGs,
333 with abundance and composition varying between samples. SMS overall identified ARGs to 2-11
334 classes of antibiotics in individual samples (Figure 2 and Table 3), with ARGs to two additional
335 classes identified using targeted sequencing methods.
336
337 **Table 2. Summary of NGS sequencing results using three methods:** (1) shotgun
338 metagenomics sequencing (SMS) or (2) targeted sequencing using community panels from Life
339 Technologies: (2a) the Ion AmpliSeq™ Antimicrobial Resistance (AMR) Research panel and (2b)
340 the Ion AmpliSeq™ Pan-Bacterial Research (PBR) panel. BC: barcode adapter identifier; Reads:
341 number of short read sequences per sample; MRL: mean read length in nucleotides. The four
342 human samples tested with all three methods are in dark grey, the four human samples tested with
343 two methods are in light grey and the five samples tested only with the SMS method are in white.
344

Sample ID	Host	SMS method			AMR panel		PBR panel	
		BC	Reads	MRL	BC	Reads	BC	Reads
ST4-3mo	Human - child	BC062	5,057,157	184	-	-	-	-
ST5-1mo	Human - child	BC002	10,998,563	201	BC049	1,486,710	BC053	505,192
ST5-18mo	Human - child	BC063	5,656,858	195	-	-	-	-
HS21	Human - adult	BC021	3,829,581	182	BC050	523,118	BC054	524,591
HS22	Human - adult	BC022	5,721,672	184	-	-	BC055	377,819
HS23	Human - adult	BC023	9,313,335	197	-	-	BC056	262,060
HS24	Human - adult	BC024	7,303,016	195	BC051	709,047	BC057	491,234

HS25	Human - adult	BC025	6,986,354	193	-	-	BC058	174,895
HS26	Human - adult	BC026	9,411,554	198	BC052	730,272	BC059	431,487
HS28	Human - adult	BC028	7,952,415	180	-	-	BC060	110,975
ST1-MAD	Pacific black ducks	BC065	5,671,145	162	-	-	-	-
ST3-MUD	Muscovy duck	BC066	8,438,563	169	-	-	-	-
DFS	Pool from 2 dogs	BC070	8,570,346	192	-	-	-	-

Figure 2. Overview of antimicrobial resistance genes (ARGs) abundance and composition in human and animal samples (n = 13) using the shotgun metagenomics sequencing (SMS) method. (A) Major *versus* (B) minor ARGs. ARGs abundance is calculated as a “mean base depth per 5 million reads” (BD5M) for each reference gene in each sample, and then summed for ARGs belonging to the same class of antibiotics.

Table 3. Antimicrobial resistance genes (ARGs) identified from the shotgun metagenomics sequencing (SMS) using the "in-house" analysis (based on queries against the NCBI's "Bacterial Antimicrobial Resistance Reference Gene Database" BioProject PRJNA313047)**.** Mean depth coverage per 5 million reads (BD5M) between 1 and 10 is highlighted in yellow, BD5M > 10 is highlighted in red. Additional ARGs identified with the ResFinder and/or KmerResistance tools but not the in-house method are not listed here (see text).

Class of antibiotics	Gene	ST4-3mo	ST5-1mo	ST5-18mo	HS21	HS22	HS23	HS24	HS25	HS26	HS28	MAD	MUD	DFS
Aminoglycosides	aac(6')	0	0	0	0	0	0.9[R]	0	0	0	0	0	0	0
	aadA1	0	0	0	0	0	0	0	0	0	0.9	9*	1.2	0
	aadE (or ant6-Ia)	0	0	0	6.7[KR]	1.3[K]	3.4[R]	10.2[KR]	0.4	1.7[KR]	2.6[K]	0	0	1.1[R]
	aadS [§]	0	0	0	0	0	1.0[§]	41.7[§]	0	0	0	0	0	18.2[§]
	spw for ant9 [§]	0	0	0	0	0	0.1[§]	8.9[§]	0	2.2[§]	0	0	1.3[§]	0
	aph(3')-Ia	0	0	0	0	0	0	0	0	0	0	0	0	2.7[R]
	aph(3')-IIIa	0	0	0	1.0	1.4	1.2	0.2	1.0[R]	0.1	0.2	0	0	0
	aph(6)-Id	0	0	0	0	0	0	0	0	0	0	0	0	0.6
	aac(6')-Ie / aph(2")-Ia [§]	0	0	0.5[§]	0	0.1[§]	0	0	0	0.9[§]	0.2[§]	0	0	0.1[§]
Beta-lactams	blaEC [§]	46.8[§]	95.9[§]	0	2.9[§]	0	0.4[§]	0	0.6[§]	0.3[§]	0	0.5[§]	0.1[§]	0
	blaOXA	0	0	0	0	0	0	0	0	0	0	0	1.2[K]	0
	blaTEM	53.7[KR]	0	0	0	0	0	0	0	0.1	0	0.2	0	2.7
	cblA [§]	0	0	32.4[§]	1.6[§]	4.4[§]	0.7[§]	0	1.2[§]	0.5[§]	2.8[§]	0	0	0
	cepA	0	69.5[KR]	11.8[KR]	0	0.3	0.1	0.9	0.1	0.2	0	0	0	0
	cfxA	0	0	35.9[KR]	16.2[K]	4.0[K]	1.7[R]	0	1.5	1.5[R]	0	0	0.5	66.5
Chloramphenicol	catD [§]	0	0	0.2[§]	0	0.7[§]	4.6[§]	0	0	0.1[§]	0.8[§]	0	0	0
	catP	0	0	0.2	0	0.7	6.4	0	0	0	0.8	0	0	0
Fosfomycin	fosA	1.0[R]	0	0	0	0	0	0	0	0	0	0	0	0
Glycopeptides	vanG [§]	0	0	0	0	0.5[§]	0	0	0	0	0	0	0	0
	vanS-D	0	0	0.4	0	0.1	0.2	1.1	0.2	0	0	0	0	0
	vanY-D	0	0	0.7	0	0	0	0	0	0	0	0	0	0
MLS_B	eat(A) [§]	0	0	0	0	0	1.0[§]	0	0	0	0	0	0	0.1[§]
	ere(D)	0	0	0	0	0	1.0[R]	0	0	0	0	0	0	0
	erm(B)	0	0	1.4	0.3	2.8[KR]	1.2[R]	20.8[KR]	5.2[KR]	21.4[KR]	13.4[KR]	0	0.2	0
	erm(F)	0	0	0	0	0	0	29.4[KR]	0.3	0	0	0.2	4.2[KR]	0
	erm(G)	0	0	0	0	0	0.1	0	0	0	0.1	0	0.5	26.4[KR]
	erm(Q)	0	0	0	0	0	0	0	0	0	0	0	2.2[KR]	0.6
	lnu(A)	0	0	0	0	0	0	0	0	0	0	0	0	2.3[R]
	lnu(AN2) [§]	0	63.2[§]	29.5[§]	0	0.7[§]	1.2[§]	14.6[§]	0.2[§]	25.0[§]	1.0[§]	0	1.1[§]	56.6[§]
	lnu(C)	0	0	0	0	1.1	0.4	0	4.9[KR]	0.7	0.2	0	5.8[KR]	64.2[KR]

	lnu(P)	0	0	0	0	0	0	0	0	0.3	0	0	3.0[KR]	0
	lsa(A)	0	0	0	0	0	0	0	0	0	0	6.1[KR]	0	0
	mef(A)	0.5	1.1[KR]	0.1	0	0.1	0.7	14.4[KR]	0.6	3.7[KR]	0.1	0	10.0[KR]	20.5[KR]
	mef(En2) [§]	0	61.8[§]	32.8[§]	0.4[§]	0.3[§]	1.3[§]	15.7[§]	0.2[§]	17.3[§]	2.2[§]	0	2.7[§]	65.9[§]
	msr(C)	0	0	0	0	0	1.3[R]	0	0	0	0	0	0	0.3
	msr(D) (or mel)	0.3	0.6[KR]	0.2	0	0.1	0	0	0.1	0.1	0.1	0.1	1.3	23.9[KR]
Quinolone	oqxA	0.6	0	0	0	0	0	0	0	0	0	0	0	0
Sulfonamide	sul1	0.2	0	0	0	0	0	0	0	0	0.4	0	0	0
	sul2	0	0	0	0	0	0	0	0.1	0	0	0	0	0.8[R]
Tetracyclines	tet(32)	0	0	24.8[KR]	3.5[K]	12.1[KR]	43.9[KR]	14.7[R]	12.5[K]	25.4[KR]	38.0	0	0.3	7.2
	tet(40)	0	0	3.3[KR]	1.6	9.1[KR]	2.6[R]	7.5[KR]	1.4	13.0[KR]	4.1[KR]	0	0.1	1.7[R]
	tet(44)	0	0	0	0.2	0.2	0.1	0.3	0.4	1.1	1.4[R]	0	0.1	0.7
	tet(A)	279.8[KR]	0	0	0	0.1	0	0	0	0	0.2	0	0	0
	tetA(P)	0	3.4[KR]	0	0.7	0.2	0	0	0	0.3	0	0	40.8[KR]	1.1[R]
	tetB(P)	0	0	0	0.6	0.4	0.6	0	0	0.3	0	0	42.3[KR]	1.2[R]
	tetB(46)	0.2	0	0	0.2	0.5	0	0	1.3[R]	0.1	0	0	0	0
	tet(C)	0	0	0	0	0	0	0	0	0	0.6[R]	0	0	0
	tet(M)	0.2	0	1.4[R]	0.2	0.7	1.5	0.9	0.2	1.5	2.0	0	0.1	0.9[R]
	tet(O)	0.1	0.1	34.2[K]	5.2	16.1[KR]	73.9[KR]	19.4[KR]	5.9[KR]	29.8[K]	67.5[KR]	0	0.5	15.6[R]
	tet(Q)	0.2	54.5[KR]	52.9[KR]	29.8[KR]	33.7[KR]	6.2[KR]	68.4[KR]	9.7[K]	11.7[KR]	2.7[KR]	0	5.1[KR]	14.9[KR]
	tet(W)	0	0	13.3[KR]	7.4[KR]	43.3[KR]	76.6[KR]	55.6[KR]	43.7[KR]	13.1[KR]	67.5[KR]	0	3.8[KR]	12.2[KR]
	tet(X)	0	0	0	0	0	0	96.2[KR]	0	0	0	0	0	20.5[KR]
Trimethoprim	dfrF [§]	0	0	17.6[§]	0	2.2[§]	2.0[§]	1.9[§]	2.9[§]	5.3[§]	0.9[§]	0	0	0.2[§]
Trimethoprim/ streptothricin	estX/sat1 [§]	0	0	0	0	0	0	0	0	0	0	0	1.5[§]	0

HS: human sample; MAD: Pacific black duck; MUD: Muscovy duck; DFS: dog fecal sample. [K] ARGs detected from the SMS that were also detected with the KmerResistance tool. [R] ARGs detected from the SMS that were also detected with the ResFinder tool. [§] ARGs that were absent from the ResFinder database. * Gene aadA1 was calculated to be present in bird MAD at BD5M = 9 but the mapping shows that only 23% (226 / 989 bp) of the gene (reference NG_047324) is present.

359 **SMS-based analysis of ARGs**
360 All 13 samples were processed using the SMS method, allowing comparison of ARGs diversity
361 between the human samples; three young infants and seven adults; as well as the pooled
362 domestic dog fecal sample (DFS), and two bird samples (MAD being a pool of six birds). We
363 identified ARGs from both the reads and the contigs (see Table S1).
364
365 *Resistance - antibiotic classes*
366 Using the "in-house method", we detected genes conferring resistance to 11 classes of antibiotics
367 (out of 16 classes included in the NCBI's ARGs database). ARGs conferring resistance to at least
368 one class of antibiotics at BD5M >50 were found in 12 samples (Figure 2 and Table 3). ARGs
369 conferring resistance to tetracycline (BD5M 49 to 280), macrolide, lincosamide, streptogramin B
370 (MLS_B) (BD5M 0.7 to 260), β-lactams (BD5M 1.9 to 165) and aminoglycoside (BD5M up to 61)
371 were found at higher abundances in all 13 samples (Figure 2A, Table 3). ARGs to six other
372 classes of antibiotics were found in ten samples at lower abundance (Figure 2B): trimethoprim
373 (BD5M up to 17.6), chloramphenicol (BD5M up to 11), glycopeptides, quinolones, fosfomycin and
374 sulfonamide (all last four classes with BD5M ≤ 1) (Figure 2B and Table 3). An eleventh class of
375 antibiotics was only detected as a bifunctional gene estX/sat1 conferring resistance to
376 trimethoprim/streptothricin.
377 The wild ducks (MAD) and the 1-month old human (ST5-1mo) carried genes conferring resistance
378 to the fewest classes of antibiotics –three classes (or two classes at BD5M ≥ 1 for sample MAD)–
379 while HS23, HS24, duck MUD and dog DFS carried ARGs attributable to the most classes of
380 antibiotics, i.e. seven classes (or six classes at BD5M ≥ 1) (Table 3).
381
382 *Resistance - gene level (ARGs)*
383 At the gene level, 62 ARGs were detected from the SMS-generated reads: 54 using the "in-house
384 method" (or 45 at BD5M ≥ 1), 43 using the ResFinder tool and 28 using the KmerResistance tool
385 (Figure 3B). Within a same class of antibiotics, the lowest diversity of ARGs was observed for
386 fosfomycin, quinolones and trimethoprim/streptothricin (n = 1), while the highest diversity of ARGs
387 was observed for MLS_B (n = 15) and tetracycline (n = 13) (Table 3).
388 Out of 54 ARGs identified with the in-house method, 18 could not be detected using the web-
389 based tools. Of these, twelve were absent from the ResFinder database and thus could not have
390 been detected using the web-based tools (Figure 3B). Conversely, eight ARGs identified from the
391 SMS reads using the ResFinder and KmerResistance tools could not be detected using the in-
392 house method (Figure 3B).
393
394 *Incongruent results between methods at the individual level*
395 ARGs identified by all three methods at the gene level could sometimes be identified by only two
396 or one method at the individual level (see supplementary Table S2 for detailed results).
397 Gene cfxA (β-lactam resistance) was detected with all three methods in sample ST5-18mo but
398 was detected only with the in-house method in dog DFS (BD5M = 66.5). The best match in the

399 NCBI's AMR reference database (NG_047636.1) was only 89% similar to the consensus
400 sequence, while the best match by BLASTN against the full NCBI nucleotide database (98.9%
401 similarity) was gene cfxA6 (GQ342996), which was absent from the NCBI's AMR database.
402 Gene tet(32) (tetracycline resistance) was detected only with the in-house method in HS28 (BD5M
403 = 38.0) with the best match in the NCBI's AMR reference database being NG_048124.1. However,
404 reads coverage against NG_048124.1 was very high for the first 350 bp and last 700 bp only,
405 probably related to recombination events with other 'tet' genes (see Discussion). The best match
406 with BLASTN (97.8% similarity) was a recombinant tet(O/32/O) gene (JQ740052). Even though
407 the ResFinder database includes five recombinant tet(O/32/O) gene references, it was not
408 detected in HS28 with the web-based tools.
409
410 **Figure 3. Resistance patterns identified from shotgun metagenomics sequencing (SMS)**
411 **using three analysis methods (A) per gene and per sample or (B) per gene.** SMS reads were
412 screened for the presence of antimicrobial resistance genes using (i) an "in-house method" (based
413 on queries against the NCBI's AMR reference gene database BioProject PRJNA313047), (ii)
414 ResFinder and/or (iii) KmerResistance tools.
415
416 At the individual level, ARGs identified using the web-based tools but not the in-house method
417 were: aadA24 and aph(3')-III (aminoglycosides); cfxA6 (β-lactams); catS (chloramphenicol);
418 mdf(A) (MLS_B); tet(O/W), tet(O/32/O) and tet(W/32/O) (tetracycline). Out of these, seven genes
419 were absent from the NCBI's AMR database, and thus could not have been detected using the in-
420 house method (Table S2). The remaining gene (aadA24) was detected only with the web-based
421 tools despite being present in both reference databases, but further analysis showed that this gene
422 was detected as an aadA1 gene with the in-house method.
423
424 *Resistance profiles between and within species*
425 Different species (human / birds / dogs) and different samples showed different resistance profiles
426 (Table 3). With the in-house method, HS23 showed the highest AMR diversity within the human
427 samples, with 30 ARGs (or 19 at BD5M ≥ 1) belonging to six classes of antibiotics detected,
428 followed by HS26 (28 ARGs or 15 at BD5M ≥ 1), and HS22 (28 ARGs or 12 at BD5M ≥ 1). HS24
429 showed the highest abundances of individual ARGs, with 12 out of 20 ARGs detected at BD5M ≥
430 10. When accounting for the additional genes detected using the ResFinder and KmerResistance
431 tools, the number of ARGs increased for most samples, HS22 becoming the sample with the
432 highest AMR diversity (34 ARGs) (Table S2).
433 Most of the adult samples were similar to each other in their resistance profile, except for HS24
434 (Figure 2). The three infants showed higher abundances for fewer ARGs compared to the adults,
435 e.g. three ARGs at BD5M ≥ 1 in infant ST4-3mo: two ARGs conferring resistance to β-lactams
436 (BD5M = 47 and 54) and one to tetracycline (BD5M = 280) (Table 3).
437

438 The sample MAD (pool from 6 Pacific black ducks) had the lowest AMR diversity, with two ARGs
439 at BD5M ≥ 1 (BD5M = 9 for aminoglycosides; 6.4 for MLS_B), while the sample MUD (from a
440 Muscovy duck) showed the presence of 16 ARGs at BD5M ≥ 1 (resistance to aminoglycoside,
441 MLS_B, β-lactams and tetracycline). ARGs in highest abundance in the sample MUD were tetA(P)
442 and tetB(P) (tetracycline resistance; BD5M > 40), two genes also found in humans and dog DFS
443 but at very low levels (Table 3).
444
445 The pooled DFS showed one of the highest AMR diversity, with 29 ARGs detected (or 20 at BD5M
446 ≥ 1) belonging to six classes of antibiotics; this number increased to 32 ARGs when accounting for
447 additional genes detected using the ResFinder and KmerResistance tools. The dog sample also
448 contained 12 ARGs at high abundance (BD5M ≥ 10), including 6 MLS_B genes. This is comparable
449 to 5 MLS_B genes at BD5M ≥ 10 found in HS24, but with only three ARGs in common, and those in
450 the dog DFS typically at higher abundances than in the human HS24.
451
452 **Identification of bacteria carrying ARGs**
453 The metagenomics analysis of the gut microbiome for selected samples (n = 7) using the Torrent
454 Suite Ion Reporter™ software is summarized in Table 4. Because adult human microbiotas are
455 diverse and complex, we are detailing one example for which the ARGs carrying bacteria were
456 likely identified (HS24).
457
458 **Table 4. Percentages of major bacterial Orders in the fecal microbiome of selected samples**
459 **(n = 7).** Bacterial orders likely carrying antimicrobial resistance genes (ARGs) identified in each
460 sample are highlighted as a grey box.
461

Phylum	Order	ST5-1mo	ST5-18mo	ST4-3mo	HS24	DFS	ST1-MAD	ST3-MUD
Firmicutes	Clostridiales	< 1%	35%		41%	4%	9%	54%
	Erysipelotrichales						4%	18%
	Lactobacillales	< 1%				42%	74%	
	Selenomonadales					12%		
	Veillonellales			2%				2%
Bacteroidetes	Bacteroidales	34%	51%		56%	30%		
Proteobacteria	Burkholderiales		5%					
	Campylobacterales						2%	19%
	Enterobacteriales	66%		46%				
	Gammaproteobacteria					5%		
	Kopriimonadales						1%	
	Legionellales							2%
Verrucomicrobia	Verrucomicrobiales		6%		2%			
Actinobacteria	Bifidobacteriales		1%	51%				
Cyanobacteria	Synechococcales						5%	
	Nostocales						3%	
Deinococcus-Thermus	Deinococcales						1%	

462 ST5-1mo: infant sample one month old; ST5-18mo: infant sample 18 months old; ST4-3mo: infant sample three months
463 old; HS: human sample; DFS: dog fecal sample; MAD: pool from 6 Pacific black ducks; MUD: Muscovy duck.
464
465 *Human infants samples*

466 The composition of the humans gut microbiota was age-related (Table 4). At the taxonomic level
467 'Order', there were two overwhelmingly dominant bacterial populations in ST5-1mo:
468 Enterobacteriales (66%) and Bacteroidales (34%). ARGs were identified in both populations:
469 Order Enterobacteriales (possibly Species *Escherichia coli*) carrying the blaEC gene for
470 cephalosporin-hydrolyzing class C β-lactamase; and Order Bacteroidales (possibly Species
471 *Bacteroides fragilis*) carrying three ARGs, one to β-lactams, and two to MLS_B antibiotics.
472 In ST5-18mo (same child as ST5-1mo but older) Order Enterobacteriales was absent, but new
473 Orders appeared: Bacteroidales (51%), Clostridiales (35%), Verrucomicrobiales (6%) and
474 Burkholderiales (5%). ARGs to three β-lactams and two MLS_B antibiotics were most likely carried
475 by bacteria in the Order Bacteroidales, while ARGs to tetracyclines were most likely carried by
476 bacteria in various taxa, predominantly Order Clostridiales and Order Bacteroidales.
477 In ST4-3mo, we detected no or little bacteria in Order Bacteroidales, but many bacteria in
478 Bifidobacteriales (51%) and Enterobacteriales (46%). ARGs were identified in bacteria belonging
479 to Order Enterobacteriales only: two ARGs to β-lactams, one to fosfomycin, one to MLS_B
480 antibiotics and one to tetracycline. Of note, genes blaTEM and tet(A) were likely carried by a
481 plasmid, as identified by a BLASTn query of the contigs carrying these genes.
482
483 *Human adult samples*
484 In sample HS24, the two main Orders were Bacteroidales (56%) and Clostridiales (41%). The
485 most abundant ARGs appeared to be carried by bacteria in the Order Bacteroidales (resistance to
486 aminoglycosides, MLS_B and tetracyclines). Of note, gene erm(B) (conferring resistance to
487 macrolides) was likely carried by a plasmid.
488
489 *Animal samples*
490 The gut microbiota and resistant bacterial species in the dog sample DFS were somewhat similar
491 to the human samples, with bacteria in the Order Bacteroidales likely carrying one β-lactam-
492 resistance gene, while 6 MLS_B-resistance genes were likely carried by bacteria in the Phylum
493 Firmicutes (Order unidentified). Bacteria carrying ARGs to tetracycline and aminoglycosides could
494 not be definitively identified. The aph(3')-Ia gene (conferring resistance to aminoglycosides) was
495 likely sitting on a plasmid also carrying a transposase, and the aadS gene (aminoglycosides) was
496 likely sitting on a transposon.
497 The birds' microbiotas were different from the mammals (Table 3). Bacteria from the Order
498 Lactobacillales (possibly Species *Enterococcus faecalis*) were likely carrying gene lsa(A)
499 (conferring multidrug resistance) detected in the wild pacific black ducks (MAD). Bacteria from the
500 Order Clostridia (possibly Species *Clostridium perfringens*) was likely carrying genes erm(Q)
501 (resistance to MLS_B) and tetA(P) and tetB(P) (resistance to tetracycline) detected in the Muscovy
502 duck (MUD), with other less abundant MLS_B-resistance genes likely carried by unidentified
503 bacteria (Phylum Firmicutes).
504
505 **Comparison of the SMS method and the targeted sequencing methods to detect ARGs**

506 Four human samples were analysed using all three methods. For the SMS method, we focused on
507 the in-house analysis and ARGs at BD5M ≥ 1. The results are summarized in Table 5 (see Table
508 S3 for detailed information per sample and per gene).
509 Combining all methods, we detected ARGs conferring resistance to 13 different classes of
510 antibiotics, with two additional classes not detected with the SMS-based in-house analysis:
511 Mupirocin and Quaternary Ammonium Compounds (QAC). The number of ARGs identified from
512 the four individuals was 29 to 54 using the Ion AmpliSeq™ AMR panel, 14 to 30 using the Ion
513 AmpliSeq™ Pan-Bacterial panel, and 9 to 28 using the SMS-based in-house analysis (Table 5).
514 Two to seven ARGs were found with all three methods, while four to 16 ARGs were found with
515 both the SMS-based in-house analysis and the AMR panel (Table S3). The AMR panel was the
516 most sensitive, identifying 21 to 38 ARGs not found with any other method, representing 50-66.7%
517 of the overall identified genes. Only two to three ARGs were identified with the Pan-Bacterial panel
518 alone (4.4-7.1% of all genes) while 5 to 11 genes were identified with the SMS-based in-house
519 analysis alone (11.9-16.2% of all the genes).
520
521 **Table 5. Number of antimicrobial resistance genes (ARGs) identified with three sequencing**
522 **methods from human samples (n = 4).** Reads obtained from the shotgun metagenomics
523 sequencing (SMS) were queried against the NCBI's AMR reference gene database BioProject
524 PRJNA313047. Other methods were targeted sequencing using the Ion AmpliSeq™ AMR
525 Research panel or the Ion AmpliSeq™ Pan-Bacterial panel.
526

	Method	ST5-1mo	HS21	HS24	HS26
Number of ARGs identified with each method	AMR Research panel *	29 (14)	39 (33)	34 (28)	54 (44)
	Pan-Bacterial panel	14	26	23	30
	SMS + in-house **	9 (7)	17 (10)	20 (16)	28 (15)
Number of ARGs identified with any method		34	45	39	64
Number of ARGs identified exclusively with one method	AMR Research panel only	24 (66.7%)	27 (55.1%)	21 (50.0%)	38 (55.9%)
	Pan-Bacterial panel only	2 (5.6%)	3 (6.1%)	3 (7.1%)	3 (4.4%)
	SMS + in-house only	5 (13.9%)	6 (12.2%)	5 (11.9%)	11 (16.2%)

527 * The number of genes with a depth >1 is given in brackets. ** The number of genes with a mean base depth per 5
528 million reads (BD5M) >1 is given in brackets.
529
530 General performances of two methods (the SMS-based in-house analysis and the AMR panel)
531 were compared in Figure 4.
532
533 **Figure 4. Performance summary** of the in-house analysis from the shotgun metagenomics
534 sequencing (SMS), compared to one targeted sequencing method using the Ion AmpliSeq™ AMR
535 panel (Life Technologies). Results obtained with the AMR panel were analyzed with the One
536 Codex online platform (available at: https://app.onecodex.com/); depth is defined as the average
537 number of reads piled up across the entire gene.
538
539 **Discussion**

540
541 Although only including a relatively low number of samples (n = 13), we identified a high number of
542 ARGs (34 to 64) predicted to confer resistance to 13 classes of antibiotics. All sequencing
543 methods were able to detect the most abundant genes, showing that ARGs were common in the
544 gut microbiota of humans and dogs in Australia. Australian birds also carried ARGs in their gut, at
545 a very low level in six wild Pacific black ducks from a non-urban environment, and at higher
546 abundances in a Muscovy duck from an environment close to humans (semi-urban).
547
548 **ARGs conferring resistance to tetracycline, β-lactams and MLS_B are widespread**
549 The most abundant ARGs present in the samples conferred resistance to tetracycline, β-lactams
550 and MLS_B antibiotics. Interestingly, antibiotics within these classes are used frequently in human
551 and veterinary medicine in Australia. Between 2015 and 2017, in human medicine, the most
552 commonly dispensed antibiotics in community-based outpatient practice were β-lactams (57.3%),
553 tetracycline (8.0%) and macrolides (5.1%), similar to those prescribed in hospitals (63.3% β-
554 lactams; 8.2% tetracycline; 4.0% macrolides)[40]. Antimicrobial use in veterinary medicine in
555 Australia is not as well documented, however the same three classes of antibiotics made up the
556 majority (50.5 to 65.4%) of antimicrobials sold for animal use between 2005 and 2010 but ranked
557 differently: macrolides and streptogramins (18.8 to 27.1%), followed by tetracyclines (16.3 to
558 28.6%) and β-lactams (9.2 to 14.6%)[41]. Studies have reported that subjects from countries with
559 tighter policies on antibiotic usage in humans and animals have considerably less ARG levels[14]. In
560 line with this, we would argue that the common usage of specific classes of antibiotics as noted
561 above is likely driving the high abundance of matching ARGs in the gut microbiota of people and
562 animals in Australia.
563
564 A large proportion of ARGs in our human samples were likely carried by bacteria within the Order
565 Bacteroidales that is common in the human lower gut[42]. ARGs conferring resistance to tetracycline
566 are one of the most commonly found in human gut microbiota[15] and their prevalence has steadily
567 increased in Bacteroidales since the 1970's[43]. A study suggested that once acquired, ARGs to
568 tetracycline can persist in Bacteroidales in the absence of antibiotic selection[43]. This could explain
569 why ARGs to tetracycline were the most abundant in our data (detected in 12 samples out of 13,
570 with up to 11 tetracycline ARGs in a single sample), despite tetracycline only being the third most
571 common class of antibiotics prescribed in the Australian community behind several β-lactams[40].
572
573 Mosaic tetracycline resistance genes, presumably arising by recombination between wild-type
574 genes, have been discovered recently, and studies have shown that mosaic genes comprising
575 tet(O), tet(W), and tet(32) sequences were abundant in DNA extracted from pig and human fecal
576 samples[44]. In our data, mosaic genes tet (O/W), tet(O/32/O) and tet(W/32/O) were identified using
577 the ResFinder tool. There is probably more undetected, as reads mapping against tet genes
578 sometimes showed incomplete coverage with clear truncations. Targeted sequencing techniques

579 might be required in order to properly assess tetracycline resistance that might otherwise be
580 underestimated.
581
582 **Detected ARGs in humans and animals are diverse**
583 The diversity of ARGs detected in humans was generally higher in adults compared to infants.
584 This might be correlated with higher diversity of the gut microbiota in adults (the range of
585 microorganisms found in the intestine changes dramatically between birth and the age of 3
586 years[45]) and/or a growing antibiotic selection pressure with age. Although the ARGs in infants
587 were less diverse, they were at very high relative abundances, having the 2nd, 3rd and 4th highest
588 mean coverage of ARGs (BD5M) of all the human samples. In Australia, antibiotic usage in
589 children aged 0 to 9 years is almost equivalent to one antibiotic prescription per child and per
590 year[46], which is relatively high when compared to similar countries[47]. Although, the youngest infant
591 still had ARGs evident when it had never been exposed to antibiotics.
592
593 The ARGs detected in the dog sample were somewhat similar to the ones detected in adult
594 humans. This might reflect similarities in the diversity and composition of the dog and human fecal
595 microbiota, with microbial exchange facilitated by direct contact[45] as well as a similarity in the
596 antibiotics used (as previously mentioned). Very few ARGs were detected in the Pacific black duck
597 MAD (a pool of faeces from wild ducks collected in rural areas) compared to the Muscovy duck
598 MUD (common duck collected in a semi-urban area). This is consistent with previous studies
599 demonstrating that proximity to human activity increases the number of the antibiotic-resistant
600 bacteria that are associated with wild birds. For example, gulls and geese nesting near waste or
601 agricultural water harbor more antibiotic-resistant *E. coli* than do birds associated with unpolluted
602 water[48,49]. Interestingly, ARGs to macrolide and tetracycline were the most abundant in the
603 Muscovy duck MUD sample, overlapping with the antimicrobials used for therapeutic purposes in
604 farm animals in Australia[41]. Most of the macrolides and tetracyclines are administered in food or
605 water to pigs and poultry in Australian farms [41], and environmental contamination near farms with
606 both antibiotics and ARGs has been demonstrated, as well as the higher abundance of ARGs in
607 wild animals residing in the vicinity of farms[50].
608
609 **The different sequencing methods have advantages and limitations**
610 We tested three sequencing techniques and analysed the SMS-based data with three methods.
611 The SMS-based in-house method and the AMR panel detected the greatest range of ARGs, with
612 the highest diversity of ARGs obtained with a combination of the two methods. However, each
613 method had advantages and disadvantages. The SMS-based in-house analysis and the AMR
614 panel both detected abundant ARGs, while discordant results occurred for ARGs at low
615 abundances, because of different detection sensitivities of the methods.
616 Discordant results between methods also occurred from the use of different AMR reference
617 databases. Of the ARGs identified with the SMS-based in-house analysis only, many were absent
618 from the AMR panel, and therefore could not have been detected by this method. Similarly, many

619 of the ARGs identified with the AMR panel only were absent from the NCBI's AMR database,
620 therefore could not have been detected using the in-house analysis (Figure 4).
621
622 ***The two targeted sequencing methods relying on AmpliSeq™ panels*** were very sensitive
623 because of the pre-amplification step by PCR. The AMR panel identified the highest number of
624 ARGs and captured >50% more genes not identified by the other methods. This included genes
625 present in the NCBI's AMR database but nonetheless undetected with the in-house method, e.g.
626 erm(G), sat4, tet(M), lnu(C). Despite amplification, those genes were found with the AMR panel at
627 a depth below 6,000 i.e. ≤ 1% of the total amplified reads per sample (see Table S3 for details). As
628 a comparison, in sample HS24, all ARGs represented 0.2% of total SMS reads. With a non-
629 targeted method, sensitivity can only be increased by increasing the number of reads.
630
631 On the contrary and less intuitively, four genes were detected at relatively high abundances with
632 the SMS-based in-house method but at a low depth with the AMR panel: mef(A), aadE, aadS, tetQ
633 (Figure 4). This might indicate poor efficiency of some primers. In sample HS24, the "One Codex"
634 online platform reported a mean depth of 328 for tet(M) gene when in reality two sets of primers
635 were amplified and detected at different depths (38 and 608). Other sets of primers may be
636 working poorly, leading to false negative results or underestimated depths. Also, genes used to
637 design the primers might also have been being quite different from the one present in our samples.
638 This seemed to be the case for gene aadE detected in sample HS24, with the best match using
639 the SMS-based in-house method (NG_047378.1 *Pediococcus acidilactici* aadE gene for
640 aminoglycoside 6-adenylyltransferase) being only 69% similar to the aadE gene reference
641 AF516335 used to design the AMR panel.
642
643 Targeted methods are limited by their inability to identify novel AMR determinants, whereas a
644 functional SMS approach might provide more detail[52]. Using an in-solution probe-and-capture
645 strategy[53], researchers suggested that designed probes should target sequences with up to 15%
646 nucleotide sequence divergence from a reference sequence, which would widen their applicability
647 and target capacity toward newly characterized members of AMR gene families, which often differ
648 from other members by only a few nucleotides[28].
649
650 Because the depth analysis follows an amplification step, targeted methods are not fully
651 quantitative compared to SMS. Nevertheless, it can be considered semi-quantitative as the depth
652 generally gives a good idea of the initial quantity of the ARGs present.
653
654 ***The SMS-based in-house analysis*** was not as sensitive as the targeted methods, being that the
655 sequencing is random with no amplification step. However, (i) it is quantitative and (ii) it potentially
656 allows identification of the bacteria carrying ARGs. Limitations of the SMS-based in-house analysis
657 include reliance on manual checking of the ARGs detected. In the NCBI database used for the
658 current study, references were constructed to include the ARG but also flanking regions of

659 approximately 100 bp each side (if such regions were available in the original sequence report).
660 This could trigger identification of false-positives for the presence of ARGs. That was the case for
661 nimJ gene in HS24 that was originally identified at a BD5M = 0.3, but after manual checking, all
662 reads mapped against the first 100 bp only of reference NG_048017 (*Bacteroides fragilis* nimJ
663 gene for nitroimidazole resistance), i.e. the bacterial host genome.
664
665 Manual checking was also essential to avoid overestimation of a gene's abundance. Gene tet(C)
666 was identified in ST4-3mo at a BD5M = 13, but all reads mapped against a limited region of the
667 gene and the consensus sequence was only 80.8% similar to the best reference in the NCBI's
668 AMR database NG_048174 (*Francisella tularensis* tet(C) gene for tetracycline efflux MFS
669 transporter). The best match overall using BLASTN was gene tet(A) (98.9% similarity; reference
670 MG904997). A tet(A) gene (reference NG_048158) was actually identified in ST4-3mo at a BD5M
671 = 279.8, confirming that the reads mapped to NG_048174 / tet(C) were in fact not the best match.
672
673 Library post-amplification (done when the initial library quantity was too low) triggered unexpected
674 problems and should be avoided when possible. Reference NG_047324 (*Escherichia coli* aadA1
675 gene for ant(3")-Ia family aminoglycoside nucleotidyltransferase) was identified in MAD at a BD5M
676 = 9 but the mapping result showed that all reads matched the same 200 bp region in the middle of
677 the gene, so that the aadA1 gene coverage was only 22.7%. This specific sample (as well as ST4-
678 3mo, MUD and DFS) was post-amplified, meaning that there might have only been a small amount
679 of aadA1 gene in the initial DNA extract, and one fragment only was post-amplified and
680 sequenced, leading to overestimating the depth of this gene in MAD.
681
682 In contrast, the depth may have been underestimated for some genes. In HS24, reference
683 NG_047625 (*Bacteroides fragilis* cepA gene for beta-lactamase) was identified at a BD5M = 0.9
684 using a Q-value = 20, with NG_047625 being only 80.9% similar to the consensus sequence. This
685 could be verified at a Q-value = 0, with good mapped reads, only quite different from the reference
686 used for mapping; BD5M increased to 4.5, which is probably more accurate. One way to overcome
687 this issue and obtain the real depth is to use the consensus sequence as the reference for re-
688 mapping the reads. Identification from reads or contigs relies heavily on the reference databases,
689 and this example shows that the closest match with a non-perfect reference can still allow the
690 detection of a hypothetical ARG. But because only distantly related to the original curated ARG,
691 further explorations are required to assess if the gene is actually a functional resistance gene.
692
693 Genes for which there is no good match in the reference database or in the NCBI general
694 nucleotide database might be totally missed. When conducting read mapping coverage analyses
695 at a Q-value = 0 instead of 20, we detected in some samples mixed populations of reads, i.e. one
696 population matching the reference quite well, and the second population being more dissimilar. A
697 coverage analysis at a Q-value = 20, showed the real depth of population 1, but discarded
698 population 2 for which no better reference was available.

699
700 ***The ResFinder tool,*** especially when combined with the ***KmerResistance tool***, had a sensitivity
701 similar to the SMS-based in-house analysis, albeit with the ARGs detected being slightly different.
702 Even though the ResFinder and KmerResistance tools relied on the same database, results did
703 not fully overlap, probably because they used different input data, i.e. contigs versus reads
704 respectively. Compared to the in-house analysis, the online tools are more user-friendly and
705 provide quick and relatively accurate results. Provided that the reference database is regularly
706 updated to include missing genes (in our case the obvious ones being aminoglycoside/aadS,
707 lincosamide/lnu(AN2), macrolide/mef(En2) and β-lactam/blaEC) these are valuable tools for
708 scientists and diagnosticians requiring fast results. However, they don't allow manually rechecking
709 the mapping quality, and do not provide information on whether the genes are chromosomal or on
710 plasmids, nor the taxa of the host bacteria. Even though the ResFinder tool provides an accession
711 number corresponding to a good match for the host bacteria (from the contig sequence), it does
712 provide a single match even though several other (potentially very different) species might show
713 the same percentage of identity with the contig. Also, because we used the ResFinder tool with the
714 contigs only, abundance is not provided, while the KmerResistance tool provides a depth result for
715 the ARGs identified. The SMS-based in-house analysis provided the best accuracy regarding the
716 abundance of ARGs, our calculated "BD5M" coverage taking into account the size of the gene as
717 well as the depth of coverage.
718
719 ***All the methods tested*** shared some common limitations. First, they rely on reference databases
720 that are non-exhaustive, precluding the detection of ARGs very different to those included in the
721 database, and impacting the abundance estimations of distantly related genes or when multiple
722 similar genes are present. ARGs that show many different alleles were challenging to detect (e.g.
723 cfxA / cfxA6 in sample DFS or aadA1 / aadA24 in sample MUD). For targeted methods, new
724 (larger) panels are constantly being released, e.g. 37,826 probes targeting over 2,000 nucleotide
725 sequences associated with AMR using a probe-and-capture strategy[28], or 78,600 non-redundant
726 genes (including 47,806 putative ARGs) using targeted metagenomics[51].
727 Second, genotypic resistance does not necessarily reflect a phenotypic resistance. More studies
728 comparing molecular data with phenotypic resistance are needed to assess how much of the
729 detected resistome is linked to a functional resistant gene. It will also be important in the future to
730 assess if those genes persist over time in an individual, and how much ARGs in the gut microbiota
731 can be horizontally transferred to other bacteria species, potentially pathogenic ones.
732
733 This study is the first to describe the bacterial resistome in humans and animals from Australia,
734 comparing and assessing various methods. A high diversity of ARGs was identified, as well as a
735 high variability between samples, even with a limited number of samples analysed. We show that
736 resistomes from (human) adults and infants are dissimilar, and that resistomes from adults, dogs
737 and urban birds are more similar than that from rural birds. Ongoing surveillance of ARGs
738 abundance in people and animals (including livestock and wild animals) in Australia using the

739 techniques described here could help inform policy makers and health care professionals on the
740 most prudent use of these important drugs to ensure we have access to effective treatments of
741 bacterial infections well into the future.
742
743 **Ethics statement**
744 Ethics approval for the use of seven pregnant mothers samples was granted by the Barwon Health
745 Human Research and Ethics Committee (HREC 10/24). The study involving the three infant
746 samples was deemed negligible or low risk by the Barwon Health Human Research Ethics
747 Committee and therefore exempt from full committee review (HREC approval 17/119). Bird sample
748 collection was approved under Deakin University's Animal Ethics Committee project number B43–
749 2016 and Department of Environment, Land, Water and Planning permit number 1008206. Fecal
750 samples from the dogs were environmental samples collected on the ground and did not require
751 ethics approval.
752
753 **Data availability**
754 All data generated or analysed during this study are included in the published article.
755
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Author Contributions

Project design and coordination was performed by SA. Samples were collected by and selected by SA and FC, with adult human samples collection coordinated by the BIS investigator group. Nucleic acid was extracted by VG and FC. VG and FC performed the sample library and NGS DNA sequencing. VG, AC and SA performed the reads analyses. VG wrote the initial manuscript with input from SA, and later versions were based on input and suggestions from all.

Additional information

Competing Interests: The authors declare no competing interests.

Legends to Supplementary Tables

S1 Table: Detailed results from the mapping of Shotgun Metagenomic Sequencing (SMS) against the NCBI's AMR reference gene database using the Torrent Suite TMAP plugin. Reads were queried against the database using Magic-BLAST and contigs were queried against the database using BLASTN. For each reference (line), red squares indicate the samples for which the reference was selected from the reads and/or contigs as the best (or one of the best), while the other samples were mapped against the reference just as a comparison with the other samples. Mean base depth per 5 million reads (BD5M) is highlighted in yellow when between 1 and 10, or red when > 10. When a reference is the best mapping result but the number of reads mapped to the reference is low (< 20), the exact number of reads is provided in brackets.

S2 Table: Detailed comparison of the analyses with the SMS-based method using (i) the KmerResistance tool, (ii) the ResFinder tool and (iii) our in-house method. For ResFinder and KmerResistance the percentages provided are the template coverage. For the in-house method, the calculated "mean base depth per 5 million reads" is provided. * gene not in the NCBI's AMR gene database; § gene not in the ResFinder database.

S3 Table: Results from a SMS-based in-house method and a targeted metagenomic sequencing method using the Ion AmpliSeq™ AMR panel (Life Technologies). Results obtained with the AMR panel were analysed with the One Codex online platform (available at: https://app.onecodex.com/); depth is defined as the average number of reads piled up across the entire gene. SMS: shotgun metagenomics sequencing; BD5M: mean base depth per 5 million reads; MLS: macrolide, lincosamide, streptogramin B; QAC: quaternary ammonium compounds; gni: gene not included in Bioproject database; gnp: gene not included in the AMR panel. Genes detected with both methods are highlighted in green; genes in blue are detected with the AMR panel only; genes in yellow are detected with the SMS method only.

Figure 1.

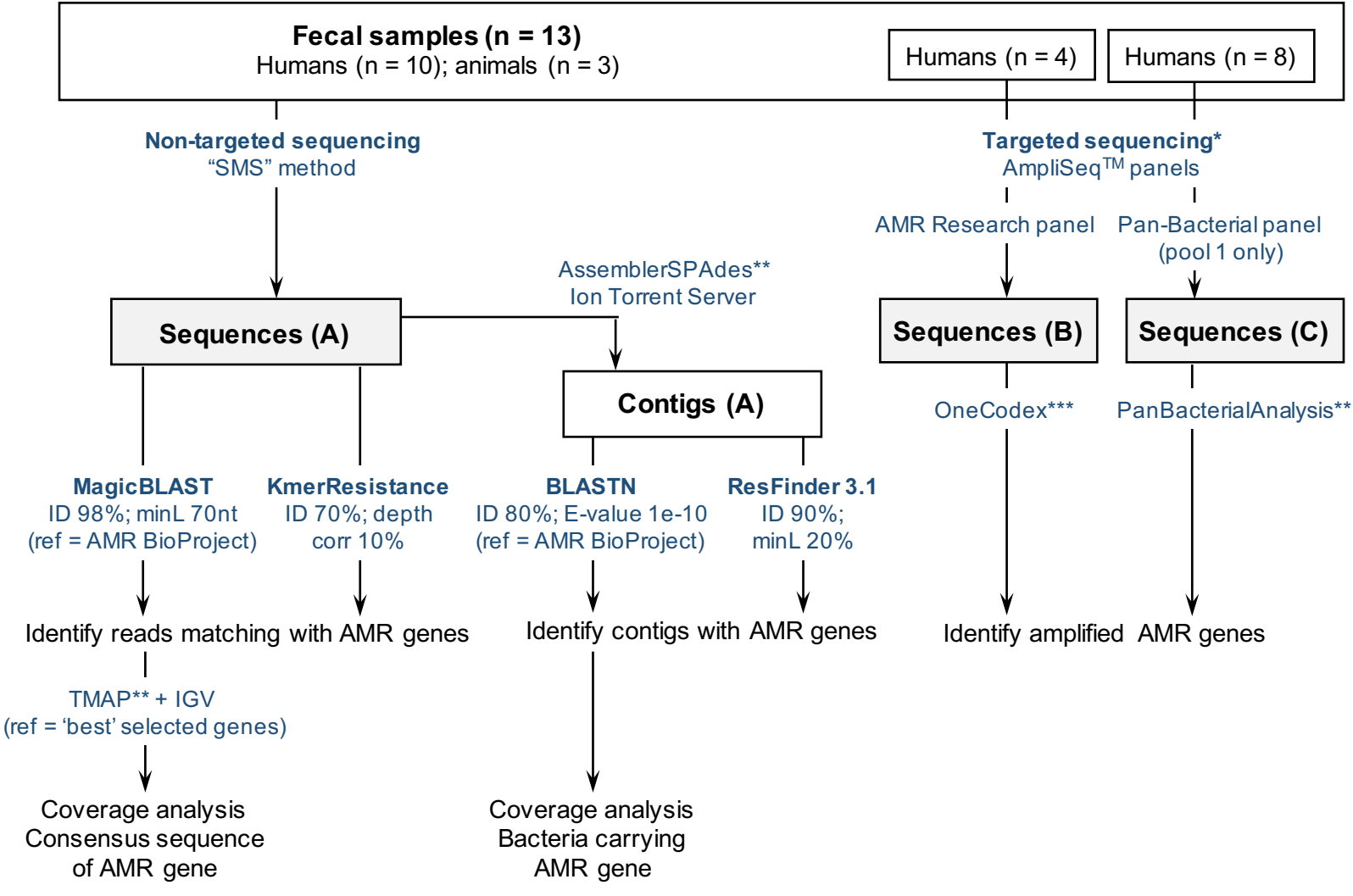

Fecal samples (n = 13)
Humans (n = 10); animals (n = 3)
Humans (n = 4)
Humans (n = 8)
Non-targeted sequencing
"SMS" method
Targeted sequencing*
AmpliSeq™ panels
AMR Research panel
Pan-Bacterial panel
(pool 1 only)
AssemblerSPAdes**
Ion Torrent Server
Sequences (A)
Sequences (B)
Sequences (C)
Contigs (A)
OneCodex***
PanBacterialAnalysis**
MagicBLAST
ID 98%; minL 70nt
(ref = AMR BioProject)
KmerResistance
ID 70%; depth
corr 10%
BLASTN
ID 80%; E-value 1e-10
(ref = AMR BioProject)
ResFinder 3.1
ID 90%;
minL 20%
Identify reads matching with AMR genes
Identify contigs with AMR genes
Identify amplified AMR genes
TMAP** + IGV
(ref = 'best' selected genes)
Coverage analysis
Consensus sequence
of AMR gene
Coverage analysis
Bacteria carrying
AMR gene

Figure 2.

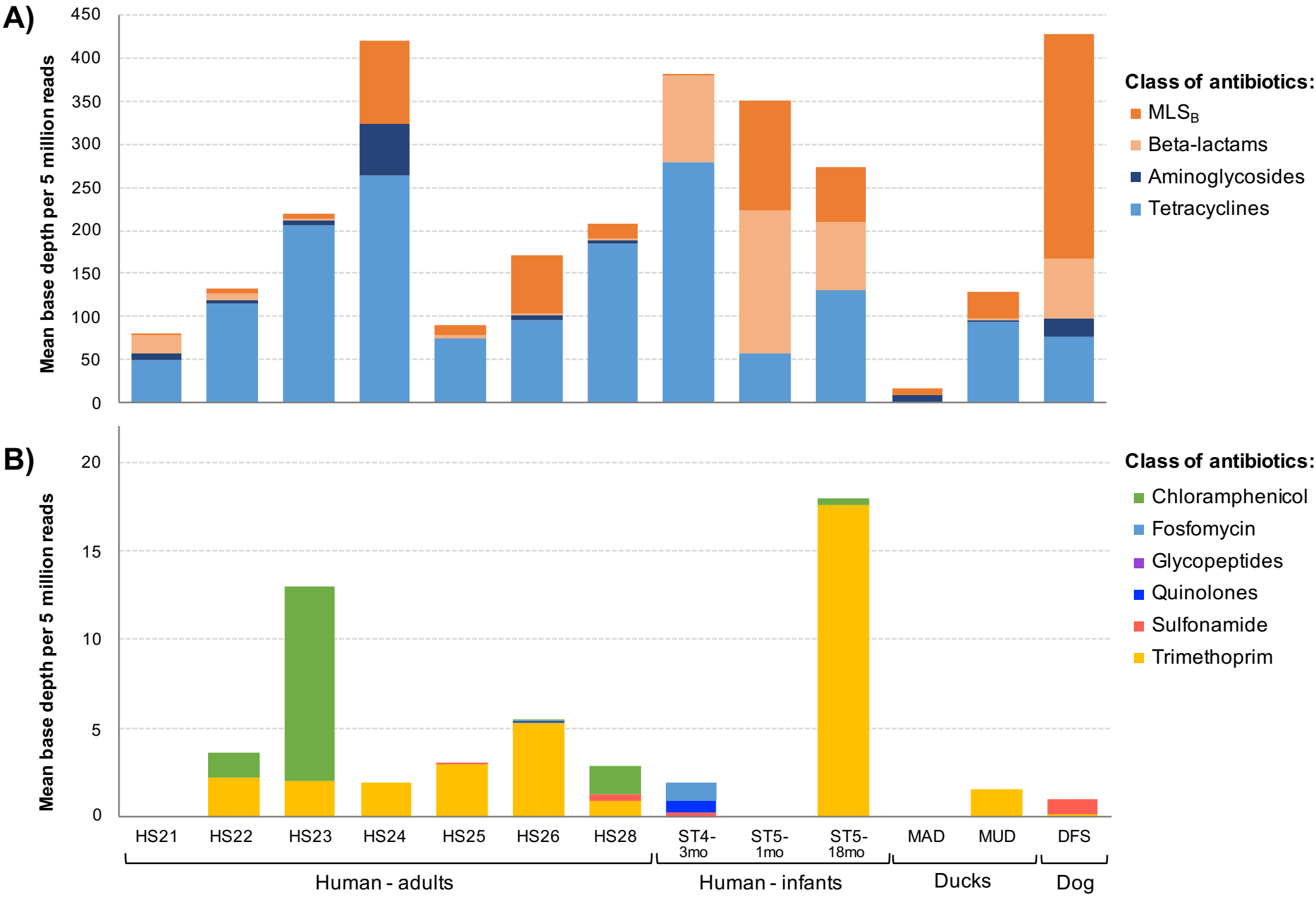

A)
Mean base depth per 5 million reads
450
400
350
300
250
200
150
100
50
0
Class of antibiotics:
MLS$_B$
Beta-lactams
Aminoglycosides
Tetracyclines
B)
Mean base depth per 5 million reads
20
15
10
5
0
Class of antibiotics:
Chloramphenicol
Fosfomycin
Glycopeptides
Quinolones
Sulfonamide
Trimethoprim
HS21
HS22
HS23
HS24
HS25
HS26
HS28
ST4-3mo
ST5-1mo
ST5-18mo
MAD
MUD
DFS
Human - adults
Human - infants
Ducks
Dog

Figure 3.

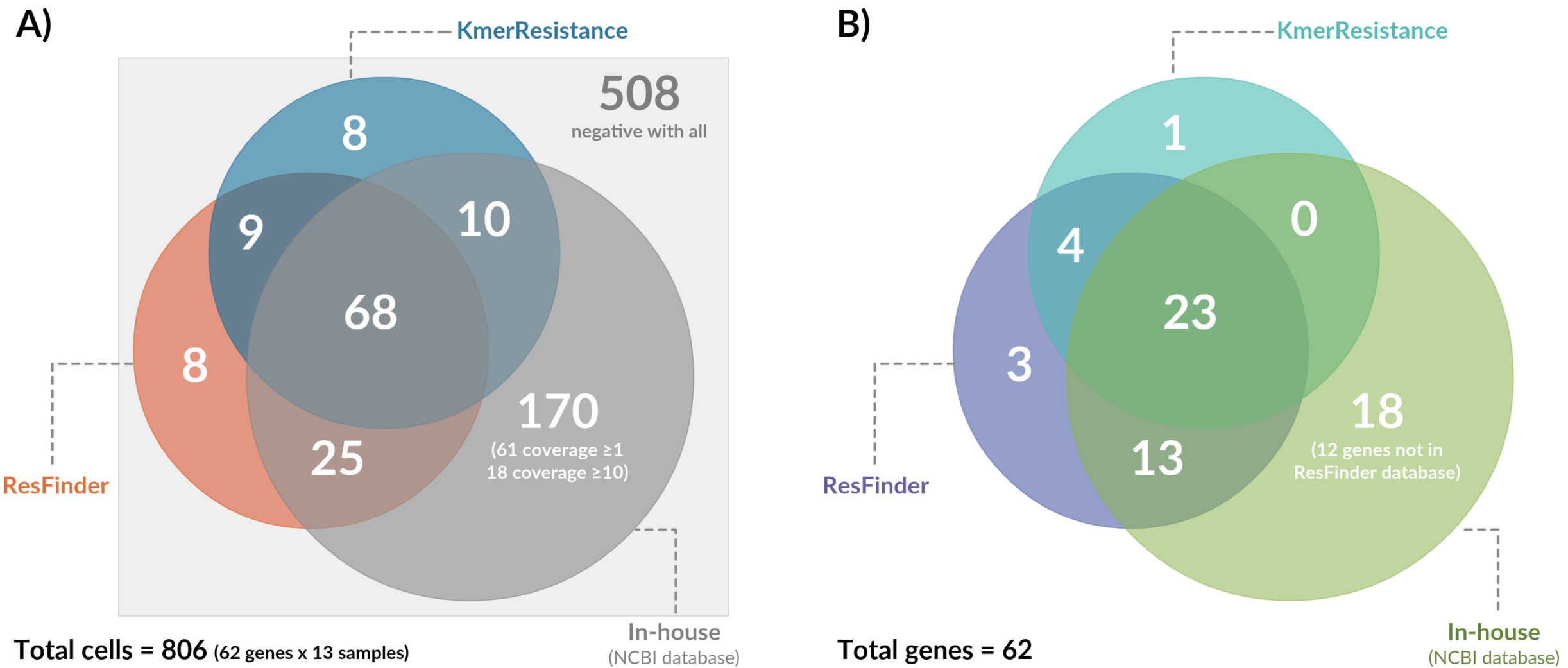
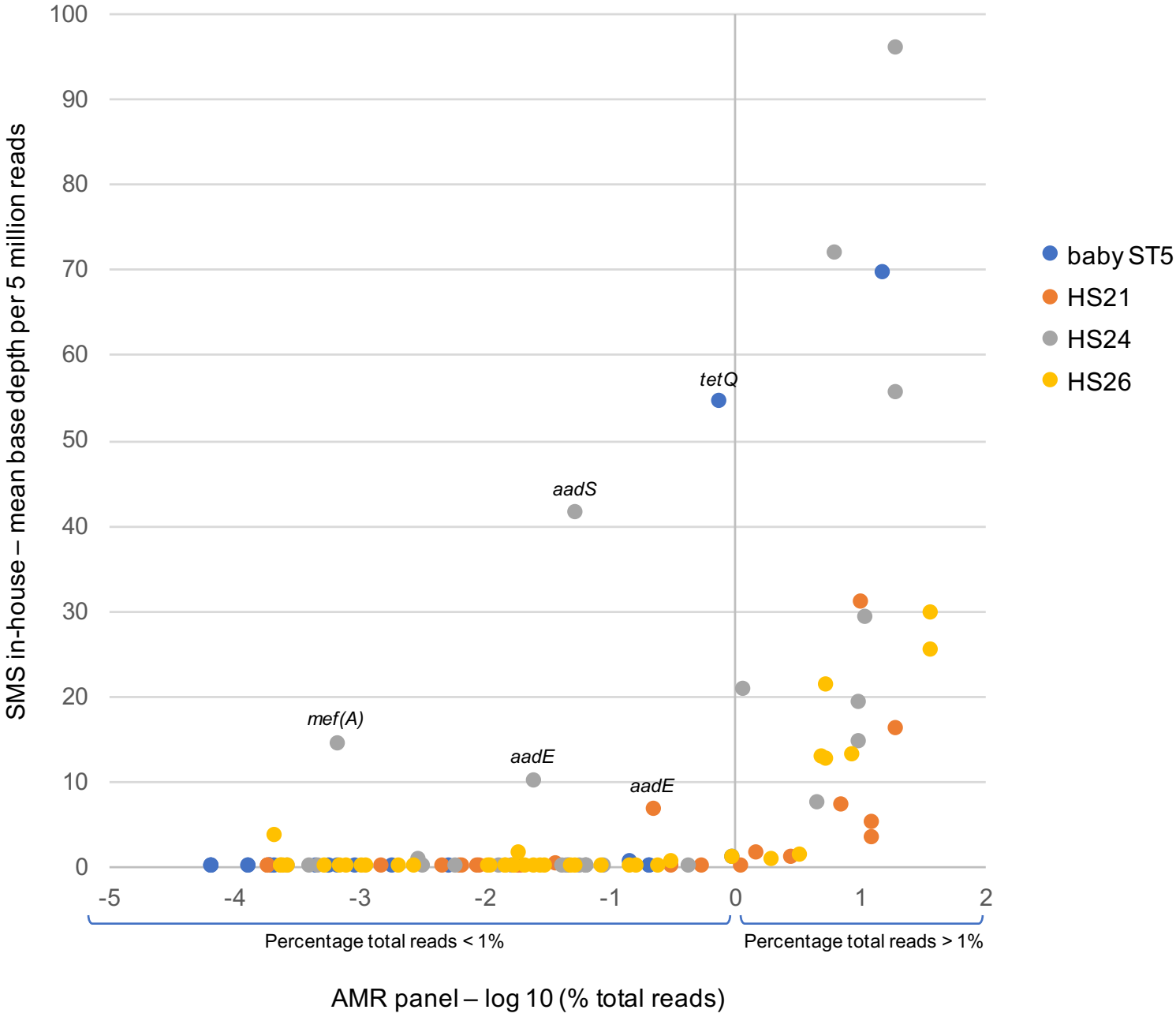

A)
KmerResistance
508
negative with all
8
9
10
68
8
25
170
(61 coverage ≥1
18 coverage ≥10)
ResFinder
In-house
(NCBI database)
Total cells = 806 (62 genes x 13 samples)
B)
KmerResistance
1
4
0
23
3
13
18
(12 genes not in
ResFinder database)
ResFinder
In-house
(NCBI database)
Total genes = 62

Figure 4.


100
90
80
70
60
50
40
30
20
10
0
SMS in-house – mean base depth per 5 million reads
tetQ
aadS
mef(A)
aadE
aadE
-5
-4
-3
-2
-1
0
1
2
Percentage total reads < 1%
Percentage total reads > 1%
AMR panel – log 10 (% total reads)
baby ST5
HS21
HS24
HS26