1 Reservoir water in Singapore contains ESBL-producing and
2 carbapenem-resistant bacteria with conjugatable conserved
3 gene cluster transfer between different species.

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18 **Abstract:**

19 As the role of the aquatic environment in the “One Health” approach has called
20 increasing attention, the studies of Antimicrobial resistance (AMR) spreading in
21 the water bodies have been reported worldwide. However, there are still limited
22 studies on the AMR carrier in the reservoir water in Singapore. Since 2018, our
23 group has collect water samples from six reservoirs in Singapore and isolated
24 the beta-lactam resistant bacteria from them. We then characterized the isolates
25 with Whole-genome sequencing (WGS) and successfully identified ESBL-
26 producing bacteria from three sampling reservoirs, and confirmed their
27 resistance with both phenotypic and sequencing methods. To better understand
28 the AMR spreading locally, we compared our isolates with isolates from other
29 WGS studies in Singapore covered humans, food, and the enviroment. From
30 there, we noticed the same sequence type (ST) as ST10, ST23, and ST38 has
31 been shared among the environment, food, and humans, as well as the same
32 beta-lactamase genes, are widely distributed among multiple sources. Further
33 genetic environment comparison of beta-lactamase has suggested their
34 spreading as conserved gene clusters among different species and sources. And
35 this hypothesis has been supported by the successful conjugation of *bla*_{CTX-M-15}
36 from *Klebsiella pneumonia* to *Escherichia coli* (*E .coli*). We also applied the
37 shotgun metagenomic sequencing to understand the community of bacteria in
38 reservoir water and detect the AMR genes. The composition of bacteria has
39 shown different diversity among different samples. Besides, different beta-
40 lactamase genes have been identified compared to culture depended methods.
41 Here, we suggest that sequencing analysis has great potential in understanding

- 42 AMR spreading in the “One-Health” approach. A genetic-based AMR risk
- 43 assessment is in urgent need in Singapore.
- 44

45 **Introduction:**

46 As stated by the World Health Organisation (WHO), by 2050, antimicrobial
 47 resistance (AMR) will result in an extra burden of 1.2 trillion USD on health
 48 expenditure every year(Adeyi, Baris et al. 2017). Significantly, the AMR
 49 problem has become a serious challenge to public health, and the concert on
 50 AMR from food and the environment to humans has called increasing
 51 attention(FAO - OIE - WHO 2010). Based on the WHO report “Antimicrobial
 52 Resistance: An Emerging Water, Sanitation and Hygiene Issue,” the aquatic
 53 environment can perform like a reservoir of AMR bacteria from both humans
 54 and animals. On the other hand, the aquatic environment can also provide a
 55 platform for exchanging resistance markers between bacterial
 56 species(Organization 2014). The presence of AMR genes in water bodies has
 57 been reported globally(Herrig, Fleischmann et al. 2020). A quantitative risk
 58 assessment of AMR spreading from aquatic environment to human is in urgent
 59 need with the application of sequencing technology(Ashbolt, Amézquita et al.
 60 2013). The hazard identification for AMR risk assessment from reservoir water
 61 in Singapore is still missing. In the genetic-based risk assessment model,
 62 investigation on both pathogenetic and non-pathogenetic bacteria is needed, as
 63 the non-pathogenetic bacteria can act as “vectors” to pass the AMR genes to
 64 human pathogens(Manaia 2017). In this perspective, the sequencing data,
 65 including the whole genome sequencing (WGS) of the resistant bacteria from
 66 the reservoir, and the shotgun metagenomic sequencing data of the reservoir
 67 microorganism community, is in urgent need to support the risk assessment
 68 system, as well as a phylogenetic-based source attribution surveillance.

69 As the antibiotic with the most extended history of application, beta-lactam is
 70 still one of the most common options for treating bacterial infectious diseases.
 71 Take Demark as an example; based on the “WHO report on Surveillance of
 72 Antibiotic Consumption,” penicillin contributes 61% of total antimicrobials
 73 consumption in Denmark(Organization 2018). Simultaneously, the resistance to
 74 beta-lactams is increasingly reported every year, and harboring beta-lactamases
 75 is one of the most prevalent mechanisms causing the resistance(Noval, Banoub
 76 et al. 2020). As resistant to the “Highest Priority Critically Important
 77 Antimicrobials,” 3rd generation cephalosporin(Organization 2017), the
 78 Extended-Spectrum Beta-Lactamase (ESBL) and carbapenemase producers
 79 have been reported with great clinical importance.

80 Besides the antimicrobial susceptibility test (AST), next-generation sequencing
 81 (NGS) application can play an important role in AMR investigation. The
 82 whole-genome sequencing (WGS) on resistant strains could help track
 83 evolution history, compare the isolates from different sources, and classify them
 84 into different clades. Moreover, the DNA alignment between contigs harboring
 85 AMRs from different isolates can help to investigate the correlated AMR gene
 86 clusters. This application can benefit the surveillance of transferable resistance
 87 genes, including beta-lactamase. Compare to the mutations, the beta-lactamase
 88 is easier to spread through transferable vectors like plasmids and transposons.
 89 The horizontal gene transfer (HGT) of beta-lactamase is widely reported
 90 between different species, which brings the challenge to prevent their epidemic
 91 as the genes can be exchanged between pathogen, no-pathogen, and even some
 92 unculturable bacteria. The NGS analysis can break the boundary of species and
 93 geography and find further links between AMR gene clusters from different

94 sources. As the sequencing technology developed, besides the WGS,
95 metagenomic sequencing has also been widely applied in AMR studies. Besides
96 providing the abundance level of different microorganisms, the functional gene
97 analysis could specifically target AMR genes in complex environmental
98 samples.

99 Even though NGS has been applied for AMR investigation in human and food
100 studies in Singapore, few studies target the beta-lactam resistant bacteria from
101 water bodies, like reservoirs, with sequencing technology. This research
102 collects beta-lactam resistant isolates from six reservoirs and identified beta-
103 lactamase harboring isolates with WGS. We compared their phenotype and
104 genotype through traditional AST and sequencing technology. Furtherly,
105 identified the specific gene cluster of beta-lactamase share between different
106 species. Also, we have proved their transferability with the conjugation test.
107 The multiple source comparison of sequence type and beta-lactamase genes has
108 presented widespread beta-lactam resistance at both isolate and genetic levels.
109 Besides, the metagenomic analysis has provided more information on the
110 bacteria composition and AMR distribution. With our research, we aim to call
111 national attention to control the AMR spreading through the aquatic
112 environment in Singapore and provide more information to build up the risk
113 assessment of AMR in the future.

114 **Materials and Methods**

115 **Water sample collection.**

116 Six water samples were collected from six reservoirs respectively in Singapore
117 during November 2018. The locations of the reservoirs were shown in
118 supplementary. Water from 0.5 m to one meter depth to the surface, less than
119 five meter from the bank were collected with a 1 L sterile bottle. The samples
120 were kept at 4°C and transported to the lab within four h before counting,
121 isolation, and DNA extraction.

122 **Microorganism community collection with filtration.**

123 800 ml of each water sample was filtered with 0.45 µm filter membranes to
124 collect the microorganisms. The bacteria left on the filter membrane were
125 dissolved in two ml saline (0.85% NaCl). The concentrated microorganism
126 solution was further processed for colony counting and enrichment.

127 **Microdilution colony counting**

128 After bacteria collection with membrane, 100 µl of concentrated microorganism
129 solution were series diluted and drop-plated on Brilliance™ ESBL Agar
130 (Thermo Fisher, USA) as ten µl/drop according to a 3×4 drop-plating design.
131 For each drop with colonies between two to 20 was taken into calculation. The
132 colonies formed on the selective were subcultured for disc diffusion.

133 **Culture enriched and bacteria isolation.**

134 After collected with the filter membrane, one ml of concentrated
135 microorganism solution was added into 20 ml Mueller Hinton (MH) plus Lysed
136 horse blood (LHB) broth (Thermo Fisher, USA) and cultured at 37°C overnight.
137 The enriched culture was then streaked on Brilliance™ ESBL Agar (Thermo

138 Fisher, USA) and incubated at 37 °C overnight. The isolates were purified by re-
139 streaked on MH agar and pick the single colonies for further steps. The purified
140 isolates were subcultured in MH broth at 37 °C overnight, following by mixed
141 with 75% glycerol in a 3:1 volumetric ratio to make glycerol stock.

142 **Antimicrobial susceptibility test.**

143 The ESBL screening was performed with disc diffusion according to CDS
144 methods. The amoxillin+clavulanic acid (AMC 30 µg) disc and 4 type
145 cephalosporin discs including ceftriaxone (CRO, 30 µg), ceftazidime (CAZ, 30
146 µg), cefotaxime (CTX, 30 µg), and cefepime (FEP, 30 µg) were used for testing.
147 All the discs are standard discs from Thermo Fisher, USA. Briefly, 2-3 single
148 colonies on the overnight culture plates were picked with sterile cotton swaps
149 and suspended in saline (0.85% NaCl) solution to reach turbidity equal to
150 McFarland 0.5. The bacteria solution was then spread on standard MH agar
151 homogeneously, and the antimicrobial discs mentioned before were placed on
152 the agar surface. The agar plates were then turned over and cultured at 37 °C
153 overnight. The diameter of the inhibit zone around discs below 9 mm was
154 considered as the cut-off value. Isolates showed non-susceptibility to at least
155 two antimicrobial tested were selected for sequencing and further confirmation.

156 The minimum inhibitory concentration tests were performed with microdilution
157 methods and Sensititre™ ESBL Plate (Thermo Scientific, USA). Briefly, 100
158 µl of pure bacteria solution with turbidity equal to McFarland 0.5 was diluted
159 with 11 ml of MH broth and mixed well. 50 µl of the diluted bacteria solution
160 was added to each well of the MIC panel. The 96-well plates were sealed well
161 and culture at 37 °C overnight. The lowest concentration to inhibit the visible
162 growth was recorded as the MIC results.

163 **DNA extraction and sequencing**

164 The DNA extraction was applied for both the microorganism solution and pure
165 culture selected after the disc diffusion test. The DNA extraction followed the
166 modified protocol published previously(Zhong 2019) with the Qiagen DNA
167 Mini Kit (Thermo Fisher, US). The library preparation and sequencing
168 progress were described before(Guo, Tay et al. 2019).

169 **Sequencing analysis of WGS.**

170 The sequencing analysis was processed with the Center for Genomic
171 Epidemiology Server (<https://cge.cbs.dtu.dk/services/>). The Illumina pair-end
172 raw reads were firstly assembled with Assembler1.2
173 (<https://cge.cbs.dtu.dk/services/Assembler/>) in Trim mode. The contigs were
174 further processed for typing with KmerFinder 3.1
175 (<https://cge.cbs.dtu.dk/services/KmerFinder/>)(Hasman, Saputra et al. 2014),
176 SpeciesFinder 2.0 (<https://cge.cbs.dtu.dk/services/SpeciesFinder/>) and MLST
177 2.0 server (<https://cge.cbs.dtu.dk/services/MLST/>) (Larsen, Cosentino et al.
178 2012). The genome annotation was processed by the RAST server
179 (<http://rast.theseed.org/FIG/rast.cgi>) (Aziz, Bartels et al. 2008). The
180 antimicrobial genes were detected by ResFinder
181 (<https://cge.cbs.dtu.dk/services/ResFinder/>) (Zankari, Hasman et al. 2012) with
182 filter setting as identification over 80% and mini length over 60%. The origin of
183 replication (Ori) of plasmids was detected with PlasmidFinder
184 (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>) (Carattoli, Zankari et al. 2014),
185 setting as identification over 95% and mini length over 60%. The phylogenetic
186 tree among different species was built based on Average Nucleotide Identity
187 (ANI) with the Genome-based distance matrix calculator (<http://enve->

188 omics.ce.gatech.edu/g-matrix/), the phylogenetic analysis within the species
189 was processed with CSI Phylogeny 1.4
190 (<https://cge.cbs.dtu.dk/services/CSIPhylogeny/>)(Kaas, Leekitcharoenphon et al.
191 2014). The annotation of the phylogenetic tree was processed with iTol editor.
192 The contigs contain AMR genes were Blast with Blastn
193 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Kmer to determine the location of
194 AMR genes. The sequencing data are available in the National Center for
195 Biotechnology Information (NCBI) genome database under BioProject
196 PRJNA719938.

197 **Conjugation test.**

198 The conjugation test was performed with the co-culture method, as described
199 previously(Zhong, Guo et al. 2021). *E. coli* strain J53 was chosen as the
200 receptor strain. Briefly, both the donor and receptor strains were picked from
201 overnight culture plates for liquid culture in MH broth until the early log phase.
202 Around one ml of J53 culture was added into three ml of the donor cultures,
203 cultured at 37 °C overnight. After that, 100 µl of overnight co-culture were
204 spread on MacConkey agar contain sodium azide (AZ, 200 mg/ml) and
205 ceftazidime (TAZ 16 mg/ml). The successful transconjugants were confirmed
206 with specific-primer PCR. The primers and progress of PCR were shown in
207 Table S1.

208 **Metagenomic analysis.**

209 The shotgun metagenomic sequencing was performed with Illumina Miseq as
210 described previously. The raw reads were quality checked with FastQC and
211 processed with MG-RAST pipeline ([https://www.mg-](https://www.mg-rast.org/mgmain.html?mgpage=analysis#)
212 [rast.org/mgmain.html?mgpage=analysis#](https://www.mg-rast.org/mgmain.html?mgpage=analysis#)) as the default setting. The taxa ID

identified to genus level was extracted and plotted in the phylogenetic tree with
(<https://phylot.biobyte.de/index.cgi>). The relative abundance was calculated as
normalized to total bacteria reads. The alpha-diversity was presented with
Shannon index, Simpson index, species richness, and 80% abundance coverage,
calculated based on the species reading list from MG-RAST. The AMR genes
annotation was performed with ResFinder from the CGE server.

Bacteria community analysis

The overlap analysis of the MLST type of *E. coli* from different sources was
plotted with InteractiVenn (<http://www.interactivenn.net/index.html>) (Heberle,
Meirelles et al. 2015). Besides our study, WGS data from another five studies
done in Singapore were included (Bioprject number: PRJNA476828(Mo, Seah
et al. 2019), PRJNA398288(Harris, Ben Zakour et al. 2018), PRJEB26639(Guo,
Tay et al. 2019), PRJEB34067, PRJNA625931(Ong, Khor et al. 2020)). Studies
were grouped into three main sources as human (healthy donor and clinic
patients), food (the ready-to-eat food and raw meat), and the environment (the
reservoir water and wild animals). The MLST type was extracted from their
publications.

To better address the beta-lactamase shared between different sources, seven
studies covering six isolation sources were selected for analysis. Besides the
five studies mentioned previously, two more studies as PRJNA351910 (Wyres,
Wick et al. 2016)and PRJNA431029(Heng, Chen et al. 2018), were included.
These two studies mainly collected the *Klebsiella* spp. isolates from the clinic
in Singapore. The beta-lactamase genes information was extracted from their
publication.

237 **Genetic environment characterization of *bla*_{CTX-M}**

238 The multi-alignment of contigs carrying *bla*_{CTX-M} was processed with Blast+
239 and plotted with EasyFig. The gene annotation was processed with the RAST
240 server and Blast+ to the NCBI nucleotide database.

241 **Results:**

242 **The concentration of ESBL-producing bacteria.**

243 On the six counting plates, 95 isolates were picked in total. Only 11 were
 244 confirmed as beta-lactam resistant by disc diffusion and then sent for whole-
 245 genome sequencing. Seven out of 11 were detected to harbor beta-lactamase
 246 genes in the genome. However, only two isolates were found to carry ESBL
 247 genes, and the number of isolates is too small to have statistical significance.
 248 The two ESBL positive isolates were picked from the drop-plating plate of
 249 Marina Bay and Punggol, respectively. These two colonies were formed in one
 250 of the triplicate ten µl drops from the filter collection without dilution. With a
 251 brief calculation, the estimation concentration of ESBL-producing bacteria in
 252 the Marina Bay and Punggol samples was Log₁₀ 1.93 CFU/ L. While the other
 253 four water samples were below the detection limit of this method. Based on the
 254 proposed standard for water quality in the Singapore reservoir provided by the
 255 PUB, the fecal coli count should below 1000 MPN/L(Alliance). Briefly, the
 256 positive rate of ESBL among bacteria possibly below 1%, but the result is not
 257 acute due to the different measurement methods. Also, the concentration we
 258 obtained of ESBL carrier from the reservoir is at least 10,000 times smaller than
 259 the reported concentration of hospital wastewater effluent (10³ to 10⁶ CFU/ml).

260 **Basic information about the isolates.**

261 Around 350 isolates were picked from ESBL selective agar. After disc diffusion,
 262 44 isolates were sent for whole-genome sequencing (Table S1). 13/44 were
 263 Enterobacteriaceae, including *E. coli* (9), *Klebsiella* spp(3). and *Citrobacter*
 264 *freundii* (1), and 31/44 (70.45 %) were non-Enterobacteriaceae. The top three
 265 prevalent species were *Pseudomonas* spp. (34%) , *E. coli* (20%) and *Aeromonas*

spp. (14%). One of the isolates was typed to be *Bacillus pumilus* as gram-positive. All the other 43 isolates were gram-negative based on the KmerFinder result.

Among the sequenced isolates, 17 isolates were not defined as any sequence type (ST), including *Pseudomonas* spp. , *Stenotrophomonas* spp., *Citrobacter freundii*, *Acinetobacter* spp. *Bacillus pumilus*, *Burkholderia cepacia*, *Chromobacterium violaceum*, and *Ochrobacterum* spp. , as they haven't been included in the CGE server database. Six of the isolates cannot be clustered as any known ST. The STs of all the *E. coli* and *Klebsiella pneumonia* isolates have been confirmed. Two isolates of *E. coli* from two different locations (Marina and Chinese Garden) were defined as the same ST (ST1193). Meanwhile, ST1193 has been reported as an emerging human pathogen(Johnson, Elnekave et al. 2019).

The numbers of coding genes belonging to different subsystems are presented in Fig 1. Around 3000 to 7000 coding sequences were detected in the genome of isolates. The phylogenetic analysis is agreed with KmerFinder and SpeciesFinder. The isolates belonging to the same species are located on the same branch of the phylogenetic tree. Significantly, more coding genes were detected related to metabolism functions as four subsystems, including 1) Cofactors, Vitamins, Prosthetic Groups, Pigments; 2) Protein Metabolism; 3) Amino Acids and Derivatives; 4) Carbohydrates. Enterobacteriaceae were detected to have higher numbers of coding genes related to potassium metabolism, while lower numbers of genes related to stress responses compare to Non-Enterobacteriaceae.

290 Interestingly, there is no significant difference among different species for
291 numbers of genes related to transportable elements and resistance to toxicity
292 compounds. But in further analysis on the Ori with PlasmidFinder (Table 4),
293 Ori was only detected in *E. coli* and *Klebsiella pneumonia* isolates. The
294 numbers of AMR genes subjected to different classes were also plotted in the
295 heatmap.

296 **The AMR genes profile of isolates**

297 The acquired AMR genes were detected with ResFinder and are shown in Table
298 1. Eleven out of 44 isolates failed to show any acquired AMR genes, the 11
299 isolates including *Pseudomonas* spp. (8), *Chromobacterium violaceum* (2),
300 and *Burkholderia cepacia* (1). Nine isolates were detected only to carry one
301 resistance gene; 20 isolates were carrying AMR genes subject to more than one
302 class. Ten isolates were harboring more than ten resistance genes. The
303 *Klebsiella pneumoniae* strain SrichB-1 was carrying the highest number of
304 AMR genes as 23. Colistin, fosfomycin, and rifampicin are less prevalent as
305 detected in less than four isolates. Other classes of AMR genes were detected in
306 more than ten isolates, and significantly, beta-lactamase genes are the most
307 dominant AMR genes. From a genetic angle, 16 isolates are multi-drug resistant.

308 Thirty out of 44 isolates were detected with known beta-lactamase genes or
309 novel variants. The top 3 prevalent species to harbor beta-lactamase genes were
310 *E. coli* (9/9), *Aeromonas* spp. (6/6), and *Pseudomonas* spp. (5/15).
311 *Pseudomonas* spp. strains have shown a very high rate of disagreement between
312 disc diffusion test and harboring of beta-lactamase genes. 13/30 isolates were
313 detected to carry only one beta-lactamase gene, the other 17 isolates were
314 harboring at least two beta-lactamase genes. The different classes of beta-

lactamase genes were shown in Table 3. Class A is the most abundant, as 29 class A beta-lactamase genes detected in 17 isolates. Enterobacteriaceae have shown a preference for class A beta-lactamase, as all the Enterobacteriaceae isolates carry at least one class A beta-lactamase. The top 2 prevalent beta-lactamase genes are *bla*_{CTX-type} (14) and *bla*_{OXA-type}(13), which have been detected in multiple species. Except for one isolate carrying class D beta-lactamase gene, all the other *Pseudomonas* spp. were harboring class B beta-lactamase, including *bla*_{PAM-type} and *bla*_{POM-type}. Interestingly, *bla*_{PAM-type} and *bla*_{POM-type} were highly reported to be specific for *Pseudomonas* spp. Besides, some other beta-lactamase genes also seem specific for certain species, including *bla*_{L1-type} for *Stenotrophomonas* spp., *bla*_{ADC-type} for *Acinetobacter* spp., and *bla*_{OCH-type} for *Ochrobactrum* spp. Some beta-lactamase genes have only been detected in one species like *bla*_{MOX-type}, *bla*_{PER-type}, and *bla*_{VEB-type} genes were only detected in *Aeromonas* spp., also, *bla*_{SHV-type} genes were only detected in *Klebsiella* spp. *bla*_{CTX-type}, *bla*_{OXA-type}, and *bla*_{TEM-type} genes were shared among multiple species. Some variants have shown correlation to specific species like *bla*_{OXA-427-like} genes have been detected in all *Aeromonas* spp. Isolates. Twenty-eight of the detected beta-lactamase are novel variants without 100% identification.

Two of the isolates have been detected with *mcr-3-like* genes. Both of them are *Aeromonas* spp. isolates and confirmed as ESBL-producing with both genotypic and phenotypic tests. Later MIC test of colistin has shown the isolates are still sensitive to colistin.

Phenotypic and genotypic comparison

339 Thirty isolates determined to harbor beta-lactamase genes were subjected to the
340 microdilution MIC test. The MIC results were shown in Table S1. The
341 interpretation of resistant, intermedium, and susceptible were based on the
342 CLSI M100 and M45 standards. Except for the Enterobacteriaceae isolates, the
343 interpretation of susceptibility for *Stenotrophomonas* spp. and *Acinetobacter*
344 spp. isolates were following the specific catalog for their species, while
345 *Aeromonas* spp. is specified in CLSI M45. The susceptibility of all other
346 isolates was interpreted based on the Non-Enterobacteriaceae catalog in CLSI
347 M100. The comparison of phenotype and genotype is shown in Table 2. The
348 phenotype prediction by ResFinder was also shown together with the real
349 phenotype obtained by measures MIC for comparison.

350 As suggested by CLSI M100, when the MIC_{cefotaxime} or MIC_{ceftazidime} is at least
351 eight times of the MIC_{cefotaxime/clavulanic acid} or MIC_{ceftazidime/clavulanic acid}, the isolate
352 is confirmed as ESBL positive. According to this definition, 17 isolates were
353 detected to harboring ESBL genes, but three out of 17 isolates didn't show
354 ESBL-producing phenotype. This may be due to the novel variant they carried,
355 in which the mutations change the substrate and capability of beta-lactamase.
356 Two of the isolates show the ESBL-producing phenotype without known ESBL
357 genes. The briefly disagree rate for ESBL is around 12%. Among the ten
358 isolates harboring potential carbapenemase, only four were finally found to be
359 resistant to meropenem. In total, we got five carbapenem-resistant isolates; two
360 of them were defined as *Stenotrophomonas* spp. , and were harboring *bla_{L1}*-like
361 carbapenemase, which is B3 type Metallo-beta-lactamase (MBL). Another two
362 are *Pseudomonas* spp. isolates with *bla_{POM-1}*-like and *bla_{PAM-1}*-like, respectively,
363 which both belong to B3 type MBL. The other isolate is *E. coli* with *bla_{NDM-5}*

364 carbapenemase, and this is the only carbapenem-resistance-Enterobacteriaceae
365 (CRE) isolate we got. The *Ochrobactrum* spp. isolate with AmpC type *bla*_{OCH}-
366 *like* genes were also shown resistant to meropenem but still sensitive to
367 imipenem. In all, among the six sampled reservoirs, 5/6 were contaminated
368 with ESBL-producing isolate, which has been confirmed with both phenotypic
369 and genotypic methods.

370 All the isolates were harboring beta-lactamase, except for *Acinetobacter* spp.
371 strains were resistant to at least one of the beta-lactams tested. Cephalothin,
372 cefpodoxime, cefazolin, cefoxitin, and ampicillin were also included in tests,
373 but there was no MIC breakpoint for non-Enterobacteriaceae in CLSI; thus,
374 they were not shown in the comparison table of phenotype and genotype.
375 Briefly, the MIC of all the isolates to cephalothin and ampicillin were all over
376 the detection range (16 µg/ml). The MIC of all the isolates except
377 *Acinetobacter* spp. were all over the detection range as 32 µg/ml. The MIC of
378 all the isolates except MarinaQ1(*E. coli*) were all over the detection range as
379 16 µg/ml. For cefoxitin, all the Enterobacteriaceae isolates except strain
380 LsrichA-4Y were sensitive to it. All the *Aeromonas* spp. isolates except one
381 were also susceptible to cefoxitin. For the other non-Enterobacteriaceae, the
382 MICs were over 64 µg/mL.

383 All the isolates containing *bla*_{CTX-M-type} genes were resistant to both ceftriaxone
384 and cefotaxime, regardless of species. All the *Klebsiella* spp. isolates were
385 resistant to ceftazidime as harboring *bla*_{CTX-M-15} and *bla*_{SHV-type} genes. At the
386 same time, three *Aeromonas* spp. isolates (LsrichE-8G, MrichA-1Y, and
387 MaB1001-1) contain both *bla*_{OXA-427-like} and *bla*_{MOX-5-like} genes were also
388 resistant to ceftazidime, two *Aeromonas* spp. isolates (Prich7-2 and SrichE-2G)

389 contained *bla_{MOX-6}-like* instead and were less resistant to ceftazidime,
 390 suggesting that the *bla_{MOX-5}-like* gene is important for the resistance to
 391 ceftazidime. Interestingly, the MrichA-1Y strain, which contains the
 392 combination of *bla_{OXA-427}-like* and *bla_{MOX-5}-like* genes together with *bla_{PER-3}*,
 393 has shown resistance to cefepime. However, when changed the *bla_{MOX-5}-like* to
 394 *bla_{MOX-6}-like* in the combination, as strain Prich7-2, the isolate becomes
 395 sensitive to cefepime. All the other *E. coli* isolates with the combination of
 396 *bla_{CTX-M}* genes, and *bla_{TEM}* genes have shown resistance to cefepime. However,
 397 this combination seems to be less effective on cefepime in *Klebsiella* spp.
 398 isolates as their MIC only reach the intermedium level. Except for the
 399 *Ochrobactrum* spp. isolates and SrichA-1 strain with *bla_{NDM-5}*, all other isolates
 400 are sensitive to the combination of piperacillin and tazobactam constant(4
 401 µg/ml). The SrichA-1 strain has shown resistance to all the antimicrobials tested,
 402 besides, due to the carbapenemase, the ESBL-producing phenotype has been
 403 covered up. The *Ochrobactrum* spp. isolates were resistant to all the
 404 antimicrobials tested except imipenem, this may attribute to the novel variants
 405 of the *bla_{OCH-type}* gene.

406 The strains have shown different responses to beta-lactamase inhibitor,
 407 clavulanic acid. All ESBL-producing isolates have been strongly inhibited of
 408 the resistance to cefotaxime by clavulanic acid, as the MIC decreased 512 or
 409 1024 times when cefotaxime combined with clavulanic acid, except SrichE-2G
 410 strain, still decreased 128 times. The MICs of ceftazidime were less influenced
 411 by inhibitor as decreased no more than 256 times, except for the MaB1001-1B
 412 strain, which has a MIC decreased by 2048 times, even over the decrease of
 413 cefotaxime. Interestingly, the MaB1001-1B were failed to detect with known

414 ESBL-genes. Besides, the strain LsrichE-8G, who carrying the same type of
415 beta-lactamase as MaB1001-1B has shown a slight response to the inhibitor.
416 For isolate LsrichA-4Y, which harboring a variant of *bla*_{CTX-M-151} showed a very
417 slight response to the clavulanic acid.

418 **Phylogenetic analysis of *E. coli* isolates with AST result from multiple** 419 **sources**

420 To better describe the relatedness of *E.coli* from different sources, WGS data of
421 isolates with AST result from PRJNA39288, PRJEB26639 was extracted and
422 compared with isolates from reservoir water based on core-genome SNP. The
423 reservoir isolates included current research and the isolates reported by our
424 group previously(Zhong, Guo et al. 2021). As in Fig 2A, in total, 95 isolates
425 were included in the phylogenetic analysis, as 18 isolates from the reservoir, 23
426 isolates from the raw meat, and 44 isolates from the clinical patients. Based on
427 the phylogenetic tree, there is no clear border between different sources. Based
428 on the susceptibility interpretation, all except three clinical isolates were
429 resistant to CTX. However, two of the three sensitive isolates except Mer-99
430 were harboring the beta-lactamase gene, as OXA-type combine CTX-M-15 and
431 CTX-M-27, respectively. Meanwhile, all the other isolates carrying the same
432 type of genes were resistant to CTX. This study has no explanation as to why
433 these genes are not functional in the three isolates. Around 96% of the 95
434 isolates were susceptible to TZP, except four isolates from the reservoir shown
435 resistance to TZP. However, there is no clear correlation between the beta-
436 lactamase gene they are carrying and their resistance to TZP, as their beta-
437 lactamase genes are diverse. To better understand the relationship between
438 reservoir isolates and human source isolates, a phylogenetic analysis on

439 isolates from both reservoirs and healthy donors was performed (Fig S1). The
440 same STs were found to be shared between different sources. The detailed
441 comparison of ST type from multiple sources has been shown in Fig 2B.

442 To further investigate the potential transferring of *E. coli* among different
443 sources, we briefly compared the ST type of *E. coli* collected in Singapore from
444 various sources, including food, humans, and the environment. Each source
445 contains two subgroups: food: ready to eat (RTE) food and raw meat, human:
446 healthy donors and clinic patients, and environment: reservoir water and wild
447 animals. The involved Bioproject details have been described in the materials
448 and methods. Three ST types have been found to distribute among humans,
449 food, and animals, including ST10, ST38, and ST23, especially ST10 has been
450 found in 5/6 subgroups that exclude the wild animals. Besides these three ST,
451 human and food sources shared other nine ST as ST93, ST48, ST101, ST117,
452 ST349, ST394, ST354, ST2179, ST2732, and ST3580. While except the three
453 ST share by all sources, the numbers of shared ST between human and the
454 environment, food, and the environment was smaller, as four and five
455 respectively. As detailed, ST1011, ST127, ST155, ST1730, ST206 only shared
456 between food and the environment, while ST131, ST410, ST648, ST1193 only
457 shared between the humans and the environment. This may due to the larger
458 sample size of human and food isolates, but it may also raise the question of
459 whether the bacteria from environmental sources are more difficult to colonize
460 on humans. However, if the percentage of ST for overlap is compared, 75% of
461 the ST found in reservoir water can be found in other sources as both humans
462 and food. Comparatively, only 41% of the ST found in ready-to-eat food is
463 shared with other sources. 37.5% of the ST from reservoir water can be found

in either clinic patients or healthy donors, while 19.7% of the ST from raw meat and 17.6% of the ST from RTE food, respectively. This is different from our hypothesis; traditionally, RTE food should have more exportation on humans. The sample size of wild animals is the smallest as only five ST has been reported, but contain an ST23 shared among four subgroups exclude reservoir water and clinic patients. Except the three ST only belong to the wild animal group, the other ST, like ST602 from the wild animals, can be found in the raw meat group. On the other hand, the raw meat and the wild animal are the only two groups containing ST602. Due to the different sample sizes, it is difficult to evaluate which source contributes more to the *E. coli* spread to humans based on current data. Interestingly, within the subgroup, the RTE food and raw meat only shared six ST. Relatively, the raw meat group shares seven ST with the reservoir water; considering the large sample size of raw meat, the isolates type between the cooked and raw materials could be quite different. Even though many limitations for the overlap analysis, we can still find the wide distribution of specific types of *E.coli* like ST10 regardless of sources and has caused human infections in the clinic.

Phylogenetic analysis of *Klebsiella pneumoniae* from humans and reservoir water

To better indicate the relationship between *Klebsiella* spp. from different sources, a phylogenetic analysis based on core genome SNP was performed on our isolates from the reservoir and other isolates from the clinic (Fig S2). As one of the top ESBL-producing pathogens in the clinic, the sequencing record of *Klebsiella pneumoniae*. we extracted can be up to the year 2011 to 2012. Unlike the *E .coli* strains, no same ST has been found between humans and

489 reservoir water. Interestingly, except for isolate SRR7371314, neither the same
 490 ST was found between healthy donors and patients. It seems like the isolation
 491 spreading bound existing not just between the environment and humans, but
 492 also between individuals with different health conditions. But the same
 493 combination of beta-lactamase genes has appeared in various sources. For
 494 example, the combination of *bla*_{OXA-1}, *bla*_{TEM-1B}, and *bla*_{CTX-M-15} has presented
 495 in 14/95 isolates in the analysis, regardless of sources. This raises the
 496 hypothesis that *Klebsiella pneumoniae* is acting more like a “vector” of passing
 497 AMR genes instead of directly colonizing humans. This hypothesis has been
 498 further supported by beta-lactamase distribution analysis and the conjugation
 499 tests. The *bla*_{SHV-like} genes were not plotted in the phylogenetic tree as all of the
 500 isolates are carrying *bla*_{SHV}, and most of them are novel variants. Besides, the
 501 *bla*_{SHV} of our *Klebsiella pneumoniae* strain are all determined to be located on
 502 the chromosome, and the variant type has shown ST specification in both
 503 human and reservoir isolates, which make it more like an intrinsic resistance
 504 gene, the rich mutations of the *bla*_{SHV} may due to the evolution of the genome.

505 Quite a pity that we didn’t find many sequencing data reports of *Klebsiella* spp.
 506 from other environmental sources and food in Singapore, which stop us from
 507 tracking their transportation among different sources.

508 **Ori detection and location determination of beta-lactamase**

509 The beta-lactamase genes' locations were determined by the best hits (Table S4)
 510 found by NCBI Blastn, also with the Ori detected by PlasmidFinder (Table S3)
 511 as reference. Briefly, based on the best hits found by Blastn, the
 512 Enterobacteriaceae are more likely to carry transferable beta-lactamase located
 513 on the plasmid. The Non-Enterobacteriaceae are mainly detected with beta-

514 lactamase located on the chromosome. This is also agreed with the numbers of
 515 Ori detected in different groups. Known from the Blastn result, the
 516 *Pseudomonas* spp., *Stenotrophomonas* spp. and *Ochrobactrum* spp. isolates
 517 each has a chromosome-located beta-lactamase. As mentioned previously, these
 518 beta-lactamases are mainly specific to the species. Each of the two
 519 *Acinetobacter* spp. isolates were harboring new variants of *bla*_{OXA-type} and
 520 *bla*_{ADC-type} genes, co-located on the chromosome.

521 Except for *bla*_{TEM-1B} detected in Prich7-2 strain were similar to a plasmid
 522 reported, all other beta-lactamases were probably located on the chromosomes.
 523 This is not exactly in agreement with the PlasmidFinder results as no Ori has
 524 been detected in *Aeromonas* spp. isolates. Interestingly, the *bla*_{PER-3} genes
 525 detected in Prich7-2 and MrichA-1Y strain were likely from an integron
 526 sequence, a highly similar integron has been reported in *Aeromonas hydrophila*
 527 strain RJ604. The integron sequence is a very specific transferable cluster,
 528 which could acquire AMR genes and insert them into the genome.

529 Three out of five *bla*_{CTX-M-15} genes in *E. coli* isolates were determined to be
 530 located on the chromosome. The phenomena have been found and addressed by
 531 our previous research(Zhong, Guo et al. 2021). The *bla*_{CTX-M-15} genes were
 532 inserted into the chromosome driven by a specific insert sequence. The
 533 structure and genetic environment were further compared then (Fig 6). All the
 534 other beta-lactamase except *bla*_{CMY-1} were found highly similar to the sequences
 535 of plasmids from multiple species and sources. Also, except for Sblue-3, all the
 536 *E. coli* isolates have been detected to have at least one Ori. This may suggest
 537 their rich capability for genetic communication. Even though the contigs
 538 carrying *bla*_{CTX-M-55} of Sblue3 were found to be similar to plasmid pCFSA1096

539 from *Salmonella enterica* strain CFSA1096, as no Ori was detected in the
540 Sblue-3 strain, this may support our conclusion from the previous research
541 (Chapter II), that this gene cluster may initially located on a plasmid and then
542 jump to the chromosome during HGT.

543 Different from *E. coli*, the *bla*_{CTX-M-15} genes in *Klebsiella* spp. isolates were all
544 co-located with *bla*_{TEM-1B} on the contigs similar to the sequence of the plasmid,
545 also the contigs from different strains are highly similar to each other, which
546 may suggest they are transferred from the same ancestor. This widely
547 distributed plasmid has also been shown to be conjugatable then.

548 **Conjugation capability test.**

549 The *Klebsiella pneumoniae* isolates have shown the capability of passing ESBL
550 genes to a different species. The genes have been conjugated to *E. coli* strain
551 J53 successfully in a frequency around 10^{-6} . The successful transconjugants *E.*
552 *coli* can survive on agar plates containing 16 µg/ml of ceftazidime, a further
553 PCR and MIC test has also found the ESBL phenotype and resistance to
554 cephalosporin, which may reflect the donor has obtained the resistance and the
555 beta-lactamase can be successfully activated in a different species.

556 **Transferable beta-lactamase is distributed with different prevalence in** 557 **different sources.**

558 From the conjugation tests, we proved conversed ESBL gene clusters to be
559 conjugated among different species. Based on this conclusion, we compare the
560 beta-lactamase shared between isolates from different species, including *E. coli*
561 and *Klebsiella* spp. , we also include the beta-lactamase found in the
562 *Aeromonas* spp. in this study (Fig 3). However, there are pretty limit WGS

report of *Aeromonas* spp. from Singapore, so we failed to find sequencing information for comparison. Besides, we also noticed that the beta-lactamase carried by our *Aeromonas* spp. isolates can be found in *Klebsiella* spp. isolates from the clinic, this is also one reason we included *Aeromonas* spp. in this analysis. Due to each study's sample size being different, we use the positive rate of the gene carrier to plotted the gene communication and distribution among various sources.

Exclude the wild animals and RTE food, the other four subgroups have been found to contain a rich type of beta-lactamase. As the most common beta-lactamase gene, *bla*_{TEM-1B} has been found in all six subgroups, including all three species. Besides, most of the beta-lactamase was shared between all three sources, exclude *bla*_{CTX-M-27} and *bla*_{CTX-M-3} are only shared between the environment and humans, *bla*_{CTX-M-1}, *bla*_{TEM-1A}, and *bla*_{DHA}, only shared between food and human. This result reflects there is no bound for the beta-lactamase spreading among different sources and species.

Genetic environment analysis of the *bla*_{CTX-M} gene.

As the capability of passing ESBL genes between different species was shown, a DNA alignment of contigs harboring *bla*_{CTX-M} genes was also investigated. A conserved gene cluster containing the same types of genes has been detected in *Aeromonas* spp. *E. coli* and *Klebsiella* spp. Including the ISEcp1 insert sequence, a *bla*_{CTX-M} gene, and a *Wubc-like* gene. Interestingly, the sequence of this cluster found on the plasmid of *Klebsiella* spp. is the same as the one located on the chromosome of *E. coli* isolates, which possibly suggests this gene cluster could be moved from the plasmid to the chromosome. The same gene cluster has been detected in eight (36%) isolates among all the isolates

588 harboring *bla*_{CTX-M} genes collected in this and previous research of reservoir
589 bacteria, suggesting the wide local spreading of this gene cluster.

590 A further blast has been done between isolates from different sources and
591 species (Fig 4). The genetic environment of CTX-M-15 and CTX-M-55 was
592 found to be highly similar and conserved. They were both highly evolved with
593 the *ISEcpI* and followed by a *WbuC* gene. And this gene cluster has been
594 widely reported, including in our previous research(Zhong, Guo et al. 2021).
595 The proximity of CTX-M-15 or CTX-M-55 is widely presented among
596 different species and sources. The genetic environment of CTX-M-65 has also
597 been shown conserved among different species but highly different from CTX-
598 M-15 and CTX-M-55 (Fig S2).

599 **Taxonomy analysis of shotgun metagenomic data of reservoir samples**

600 Based on the MG-RAST taxonomy analysis result, around 1500 species taxa
601 unit belong to 590 genera has been identified (Fig 5). The total reading number
602 is between 2588981 to 5000134 was called. Proteobacteria are the dominant
603 group. Besides, Firmicutes, Actinobacteria, and Bacteroides are also detected
604 with rich abundance. The Shannon index, Simpson index, species richness, and
605 80% abundance coverage was calculated based on the taxa unit identified to
606 species level. The community of the Serangoon reservoir has shown unexpected
607 low diversity due to dominated by *Microcystis aeruginosa*, which has covered
608 20% of the relative abundance; this reminds us of the light green color of the
609 sample from Serangoon. Also, the species richness of Serangoon is the highest,
610 which presents as a significantly lower score of Shannon, Simpson and, 80%
611 abundance coverage.

612 **Functional gene annotation of shotgun metagenomic data of reservoir** 613 **water**

614 Between 1106317 to 1930613 functional unit hits were detected based on the
615 SEED database (Fig S4). Like the WGS data, the dominant group is related to
616 the essential metabolism of nutrients, like carbohydrate metabolism. We
617 specifically look into the function group of antimicrobial resistance and found
618 the beta-lactamase can be detected in all six samples with different relative
619 abundance. The multi-drug resistance efflux is still the most common group
620 among the functional hits belonging to the “Drug-resistant” group (Fig S5).

621 A further annotation targeting the AMR genes with ResFinder is shown in
622 Table 3. Interestingly, the AMR genes detected are entirely different from the
623 ones seen in isolates. Bedok and Lower Seletar samples failed to detect AMR
624 genes with assembled contigs. Even though the sample was not applied with
625 enrichment and selection, the beta-lactamase genes were still prevalent.
626 However, the types of the gene are different from the genes found in the
627 isolates. The *oqxA*-like gene was detected in some isolates from Punggol and
628 Serangoon. Significantly, the raw reads with KMA methods can help to detect
629 more AMR genes but lack genetic information. Besides, the assessable contigs
630 carrying AMR genes are too short to determine their source organism.

631 **Discussion:**

632 Based on the information from the PUB water supply
633 (<https://www.pub.gov.sg/watersupply>) there are four main “national taps” of
634 water in Singapore, including water from the local catchment, water imported,
635 highly-purified reclaimed water known as NEWater, the desalinated seawater.
636 The local water catchment refers to the underground rainwater collection
637 system and the reservoirs. The water imported will also be stocked at the
638 reservoir before treated for human consumption. From these two points,
639 reservoir water plays an essential role in the freshwater supply in Singapore.
640 This is also the initial motivation to build a completely separate system for
641 pollution, to protect the reservoir water quality better. Besides, the Singapore
642 government generously encourages people to get close to the natural
643 environment, many reservoirs like Chinese Garden, Pongee were built with the
644 population entertainment functions. This increases the anthropogenic influence
645 on the aquatic environment and, on the other hand, also increases the exposure
646 of humans to microorganisms from the aquatic environment. As reported by
647 Laurence Haller, et al previously, the wastewater from hospitals contains many
648 types of CRE and ESBL carrier and genes(Haller, Chen et al. 2018), even
649 though the rainwater collection is a separate system, there is still raise the
650 concern if the reservoir waters have been contaminated with dangerous AMR
651 pathogens. Unfortunately, the same ST isolates collected from different
652 locations regardless of sources and time, in some way supported the hypothesis.

653 Refer to the current situation, in which the same ST resistant bacteria can be
654 detected in all three sources in “One Health” approach, a genetic-based risk
655 assessment system from the environment to humans is in urgent need. The

656 present research is an example to process the first step of risk assessment as
 657 “hazard identification”, which refer to understand the potential risk bacteria to
 658 human health and also their concentrations. Even though the estimation is not
 659 working as expected, it still provided a brief idea for the upline of concentration.
 660 For the genetic-based risk assessment model, the non-pathogenetic bacteria
 661 which can transfer the AMR genes as a “vector” is also included. From our
 662 analysis, different species may play a different role in this model. The
 663 difference between *E. coli* and *Klebsiella pneumoniae* can give us some new
 664 sight of their different fate in AMR spreading. Based on the phylogenetic
 665 analysis with the same species from different sources, and the transferable beta-
 666 lactamase gene analysis, the *E. coli* from the reservoir has shown some
 667 genome-or isolate-level relatedness to the isolates from humans and food, as the
 668 same sequence type found among different sources. However, refer to
 669 *Klebsiella pneumoniae*, the isolates from different groups rarely shared the
 670 same ST. Instead, the same combination of resistance genes has been found
 671 among different isolates, and also, we have proved their capability to pass beta-
 672 lactamase to isolates to other species. From the limit data, the *E. coli* is
 673 performed more like a direct risk and spread at the genome/isolate-level,
 674 relatively, *Klebsiella spp.* perform more like a “vector” to contribute the
 675 resistance gene instead of colonized at different sources directly. However, all
 676 these hypotheses were based on the limited data we got, a national level of
 677 isolates collection and surveillance is needed to build the risk assessment
 678 system and generate a more accurate conclusion. The exposure model for
 679 contacting reservoir water is also missing, and more studies are expected. Also,

680 at that stage, we can complete the source distribution with more data support to
681 find AMR's main sources for humans.

682 The prediction of AMR with genetic data has become a hot topic(Feldgarden,
683 Brover et al. 2019). The new landed version of ResFinder has provided this
684 function(Bortolaia, Kaas et al. 2020). However, the disagreement between
685 phenotypic and genotypic addressed in this study has already been widely
686 discussed in AMR detection(Hazards, Koutsoumanis et al. 2019). As mentioned
687 previously, the novel mutation may change the enzymatic activity and
688 influences the substrate type could be hydrolyzed, further affecting the
689 resistance phenotype(Palzkill 2018). Therefore, further study for protein
690 structure and function would be needed in the future. With more variants
691 documented, the detection accuracy would be increased. Based on all the
692 isolates we collected and their MIC result, we found three factors, which may
693 influence the resistance pattern. First is the variant type of beta-lactamase. The
694 mutation on the known resistance genes may change their resistance pattern,
695 such as the mutation of *bla*_{CTX-M-151} found in our isolates, which may block their
696 sensitivity to beta-lactamase inhibitor, and reduce their resistance to
697 cephalosporin. The second is the genetic environment of the resistance gene. As
698 the different distance of *bla*_{CTX-M} to the insert sequence has been found in our
699 isolates, the distance to the upside gene and promotor is also varied, which may
700 influence the transcription level regulation. The third is the species of the
701 isolates. Significantly, both the *Klebsiella* spp. and *E. coli* isolates contain the
702 same gene cluster of *bla*_{CTX-M-15}, while their susceptibility is different. This
703 phenome has also been observed as the different susceptibility between donors
704 and transconjugants, who contain the same gene cluster. The regulation of beta-

705 lactamase and how it would influence phenotypic resistance would be an
706 interesting topic for further studies.

707 The conversed gene cluster reported here has been reported in ESBL-producing
708 *E. coli* worldwide(Karim, Poirel et al. 2001, Dhanji, Patel et al. 2011).
709 Interestingly, this time, we further proved it could also be detected on the
710 plasmid of *Klebsiella* spp. The conjugation test has approved the transferability
711 of the *bla*_{CTX-M} gene, which may suggest the *bla*_{CTX-M} genes are spreading in
712 this conserved formate in Singapore. This gene cluster could become a potential
713 indicator to evaluate the spreading of ESBL genes between different species.

714 The resistance genes detected from isolates and metagenomic annotation are
715 quite different. There has been an ongoing discussion for the comparison
716 between these two methods. However, as our research's primary focus is the
717 beta-lactamase genes, the shotgun sequencing may not be specific enough for
718 this research. Other studies reported alluding to the need to enrich before
719 sequencing, increasing the possibility of beta-lactamase detection(Noyes,
720 Weinroth et al. 2017). But during the enrichment progress, as the growth rate
721 for different species is different, some types of bacteria will become dominant,
722 which will change the relative abundance of the resistance genes. From this
723 angle, important quantification information is missing. An ideal case is to find a
724 beta-lactamase gene as an indicator so that an amplicon metagenomic
725 sequencing could be used to specifically target it. And this gene could be used
726 to represent or evaluate the complete AMR spreading and would be useful for
727 the AMR risk assessment.

728 There are still some limitations of this study, which can be further improved in
 729 a better setting in the future. Firstly, the missing estimation targeting specific
 730 species. In this study, our estimate focuses on the ESBL-carrier regardless of
 731 species, which lacks the prevalence data of ESBL-producing strain among
 732 certain species. For example, many studies reported the positive rate of ESBL-
 733 carrier among the total *E. coli* population, which didn't present in this study.
 734 Besides, the estimation of the ESBL-carrier is not working as expected; MPN
 735 based methods may be applied to improve the accuracy for estimation.
 736 Secondly, the sample size is not big enough. To have a general idea of the
 737 AMR spreading, we collected the water sample as one reservoir per point
 738 instead of a multi-point collection for each reservoir. This is not just due to the
 739 limited manpower but also borrowed the idea of the outbreak investigation.
 740 Thirdly, we didn't pick a significant mathematic model for tracking the AMR
 741 spreading pathway among different sources. Instead, this paper focuses on
 742 investigating the transferable gene cluster and the similarity of the genetic
 743 environment of beta-lactamase genes from various sources.

744 In conclusion, we have detected ESBL and carbapenem-resistant isolates,
 745 including the ESBL pathogens in reservoir water, which may suggest an
 746 anthropogenic influence on reservoirs. We also present the distributed
 747 spreading of AMR genes among different sources and different species. Besides,
 748 we found the conserved gene cluster and proved its transferability among
 749 different species, which may become an AMR spreading indicator in the future.

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757 All authors declare no conflict of interests.

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895

1 **Reservoir water in Singapore contains ESBL-producing and**
2 **carbapenem-resistant bacteria with conjugatable conserved**
3 **gene cluster transfer between different species.**

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Fig 1. Phylogenetic analysis and coding sequences annotation. The phylogenetic tree was built based on the ANI since including all different species. The coding sequences were annotated by the RAST server grouped based on the subsystem. The AMR genes were detected with ResFinder 4.0. The color presents the numbers of coding sequences and AMR genes that belong to this function group.

22

23

24 **Fig 2. A.** Phylogenetic tree of *E.coli* from different sources annotated with AST results and
 25 beta-lactamase genes. The *E. coli* isolates from human, food, and water sources were aligned
 26 based on core-genome SNP. Only the isolates reported with AST interpretation were extracted
 27 for the phylogenetic analysis. The MG1655 strain is used as the reference. **B.** Overlap analysis
 28 of MLST type of *E. coli* from different sources. The ST types of *E.coli* isolates from multiple
 29 sources, including human (healthy donors, clinic patients), ready-to-eat food, raw meat, wild
 30 animals, and reservoir water, were extracted for the analysis. The total numbers of ST types are
 31 shown in the bracket next to the subgroup label. The numbers of ST shared between more than
 32 one source are labeled in the overlap area.

33

34

35 **Fig 3. The beta-lactamase carrier present in isolates from different sources.** The beta-
36 lactamase genes information of isolates from this study and other studies was collected,
37 including *E .coli*, *Klebsiella* spp. and *Aeromonas* spp (only from this study). Due to the
38 difference in sample size, the positive rate of carrier among total sequenced isolates was used
39 for comparison. The same beta-lactamase gene was linked with line and labeled with the same
40 color. The beta-lactamase detected only in one group was not present here. The total percentage
41 is over 100% is due to the co-existing of genes.

44

45 **Fig 4. DNA alignment of contigs with *bla*_{CTX-M} genes from different sources' isolates.** The
46 contigs contain CTX from different isolates were extracted for DNA alignment with Blast+ and
47 plotted with EasyFig. **A:** Alignment of contigs over 20 kbp contain CTX-M-15 from reservoir
48 isolates of *E. coli* in this study. **B:** Alignment of contigs contain CTX-M-15, CTX-M-55, and
49 CTX-M-65 between 3 kbp to 12 kbp from reservoir isolates of both *E. coli* and *Klebsiella*
50 *pneumoniae*. **C:** The genetic elements of the transferable gene cluster. The conversed gene
51 cluster from multiple species was extracted to present with gene annotation and distance
52 between genetic elements. **D:** Alignment of contigs contain CTX-M-15 and CTX-M-55 from
53 multiple sources and species.

56

57 **Fig 5. Phylogenetic analysis of taxa ID identified to genus level with shotgun metagenomic sequencing**
 58 **analysis on different reservoirs, annotated with relative abundance normalized to total bacteria. A.** The
 59 phylogenetic tree was build based on the taxa unit identified with MG-RAST at the genus level; 584 leaves are
 60 included. The inner-circle presents the phylum of the taxa unit, phylum with over five leaves were annotated
 61 directly on the ring. Phylum with fewer leaves was color labeled as the legend. The six outer-circle presents the
 62 Log₁₀ number of taxa units in six reservoirs, from inside to outside, marked with the color legend. The isolates
 63 from each reservoir were also labeled on the brach with the same color of their reservoir source. B. Alpha-
 64 diversity analysis of the bacteria composition. The composition of bacteria of the water samples was annotated
 65 with the MG-RAST pipeline, and composition at phylum level was plotted. The alpha-diversity indexes were
 66 calculated based on the readings identified to species level. A significantly lower diversity level was found in
 67 the sample of Serangoon.

Isolates ID	Aminoglycoside	Beta-lactam	Colistin	Fluoroquinolone	Fosfomycin	MLS	Phenicol	Rifampicin	Sulphonamide	Tetracycline	Trimethoprim
BB10013-Y		<i>bla</i> _{PAM-1} *									
BedokY-2		<i>bla</i> _{POM-1} *									
BrichB-2Y											
BrichC-1Y				<i>qnrS1</i> *							
BrichC-3Y											
BrichZ-10	<i>aph(3')-IIC</i> *	<i>bla</i> _{L1} *									
BrichZ-7		<i>bla</i> _{L1} *									
LsrichE-8G		<i>bla</i> _{MOX-5} *, <i>bla</i> _{OXA-427} *							<i>sul1</i>		<i>dfrA1</i>
LsrichA-4Y		<i>bla</i> _{CTX-M-151} *									
Lsblue-1	<i>aph(4)-Ia</i> , <i>aadA1</i> , <i>aac(3)-IV</i>	<i>bla</i> _{CTX-M-65} , <i>bla</i> _{TEM-1B} *			<i>fosA3</i>	<i>mdf(A)</i> *	<i>floR</i> *			<i>tet(A)</i> *, <i>tet(X)</i> *	<i>dfrA1</i>
Lsblue-2		<i>bla</i> _{CTX-M-15}		<i>qnrS1</i>		<i>mdf(A)</i> *					
LSB1003-2N											
Lsrich E-2B											
LSB1002-1N		<i>bla</i> _{OXA-457} *									
MrichA-1Y	<i>ant(3'')-II</i> , <i>aac(6')-IId</i> *, <i>aph(6)-Id</i> , <i>aph(3'')-Ib</i> *	<i>bla</i> _{PER-3} , <i>bla</i> _{OXA-427} *, <i>bla</i> _{MOX-5} *	<i>mcr-3.17</i> *	<i>aac(6')-Ib-cr</i> *, <i>qnrS2</i>		<i>mph(A)</i>	<i>floR</i> *, <i>catB3</i>		<i>sul1, sul2</i>	<i>tet(E)</i> *	<i>dfrA1</i>
MarinaQ1	<i>aph(3')-Ib</i> , <i>aadA5</i> , <i>aac(3)-IId</i> *, <i>aph(6)-Id</i>	<i>bla</i> _{TEM-1B} , <i>bla</i> _{CTX-M-15}				<i>mdf(A)</i> *, <i>mph(A)</i>			<i>sul1, sul2</i>	<i>tet(A)</i>	<i>dfrA17</i>

[illegible]

SrichA-1	<i>aac(6')-lb-cr,aadA2,aadA5</i>	<i>bla_{CMY-2}, bla_{NDM-5}, bla_{CTX-M-15}, bla_{TEM-1B}, bla_{OXA-1}</i>	<i>aac(6')-lb-cr</i>	<i>mdf(A)*</i>	<i>catB3*</i>	<i>sul1</i>	<i>tet(B)</i>	<i>dfrA12,dfrA17</i>
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		D	Ampicillin resistance gene	<i>bla</i> _{OXA-1}											
Isolate Information		Genotype			Susceptibility to beta-lactams and beta-lactamase inhibitor									ESBL phenotype ^s	
Isolate ID	Species	Beta-lactamase Class	Gene group	Beta-lactamase gene	CRO	MEM ^e	CTX ^e	CTX+CLA [#]	CAZ ^e	CAZ+CLA [#]	IMI ^e	TZP4	FEP ^e	MIC _{CTX} /MIC _{CTX+CLA}	MIC _{CAZ} /MIC _{CAZ+CLA}
SblueE-7	<i>E. coli</i>	A	ESBL	<i>bla</i> _{CTX-M-55}	R/R	S/S	R/R	S/S	R/R	S	S/S	S/S	R/R	1024	64
SblueE-3	<i>E. coli</i>	A	ESBL	<i>bla</i> _{CTX-M-55}	R/R	S/S	R/R	S/S	R/R	S	S/S	S/S	R/R	1024	128
CGX1	<i>E. coli</i>	A	Ampicillin resistance gene	<i>bla</i> _{TEM-1B}	R/R	S/S	R/R	S/S	R/S	S	S/S	S/S	R/R	1024	32
		A	ESBL	<i>bla</i> _{CTX-M-15}											
MarinaQ1	<i>E. coli</i>	A	Ampicillin resistance gene	<i>bla</i> _{TEM-1B}	R/R	S/S	R/R	S/S	R/S	S	S/S	S/S	R/R	1024	32
		A	ESBL	<i>bla</i> _{CTX-M-15}											
		A	IRS	<i>bla</i> _{SHV-72*}											
PrichB-4	<i>Klebsiella pneumoniae</i>	A	Ampicillin resistance gene	<i>bla</i> _{TEM-1B}	R/R	S/S	R/R	S/S	R/R	S	S/S	R/S	R/I	512	64
		A	ESBL	<i>bla</i> _{CTX-M-15}											
		D	Ampicillin resistance gene	<i>bla</i> _{OXA-1}											
SrichB-1	<i>Klebsiella pneumoniae</i>	A	Ampicillin resistance gene	<i>bla</i> _{TEM-1B}											
		A	ESBL	<i>bla</i> _{CTX-M-15}	R/R	S/S	R/R	S/S	R/R	S	S/S	R/S	R/I	1024	128
		D	Ampicillin resistance gene	<i>bla</i> _{OXA-1}											
CGX2	<i>Klebsiella pneumoniae</i>	A	ESBL	<i>bla</i> _{SHV-40*}											
		A	Ampicillin resistance gene	<i>bla</i> _{TEM-1B}											
		A	ESBL	<i>bla</i> _{CTX-M-15}	R/R	S/S	R/R	S/S	R/R	S	S/S	R/S	R/I	512	64
MarinaZ3	<i>Acinetobacter spp.</i>	D	Ampicillin resistance gene	<i>bla</i> _{OXA-1}											
		A	N.A.	<i>bla</i> _{SHV-61}											
MarinaZ4	<i>Acinetobacter</i>	C	AmpC	<i>bla</i> _{ADC-25*}	S/I	S/S	S/I	S/S	S/S	S	S/S	S/S	S/S	2	1
		D	N.A.	<i>bla</i> _{OXA-506*}											
MarinaZ4	<i>Acinetobacter</i>	C	AmpC	<i>bla</i> _{ADC-25*}	S/I	S/S	S/I	S/I	S/S	S	S/S	S/I	S/S	1	1

CGA-6	<i>baumannii</i> <i>Ochrobactrum</i> spp.	D	N.A.	<i>bla</i> _{OXA-343}	S/R	S/S	S/R	S/R	S/R	R	S/S	S/R	S/R	1	1
CGA-5	<i>Ochrobactrum</i> spp.	C	AmpC	<i>bla</i> _{OCH-2*}	S/R	S/R	S/R	S/R	S/R	R	S/S	S/R	S/R	1	1

15 The class of beta-lactamase is based on the gene type. The gene group definition is based on the literature review of functional studies on the gene listed. MBL: metallo-beta-
16 lactamase. IRS: inhibitor resistance. N.A. : no publication about its function has been found. €: phenotype prediction by Resfinder 4.0/susceptibility is determined based on
17 the breakpoint of CLSI with MIC. The disagreement between Resfinder 4.1 prediction and MIC test results is highlighted with a grey color background. #: no standard range
18 refers to the combination of beta-lactam and the beta-lactamase inhibitor; the determination is based on the beta-lactam itself. *: without 100% identification. \$: How many
19 fold difference between these two MIC values. Based on the CLSI standard, when the fold different over 8, the isolate consider as ESBL positive. CRO: ceftriaxone, MEM:
20 meropenem, CTX: cefotaxime, CTX+CLA: cefotaxime / clavulanic acid, CAZ: ceftazidime, CAZ+CLA: ceftazidime/ clavulanic acid, IMI:imipenem, TZP4: piperacillin /
21 tazobactam constant 4, FEP: cefepime.

22 **Table 3. AMR gene detected in metagenomic data with different methods.**

Reservoir ID	Resfinder									
	Raw reads (KMA)					Accessbled contigs (Blast)				
	Beta-lactam	Aminoglycoside	Sulfonamides	Fosfomycin	Macrolides	Multi-drug efflux pump	Beta-lactam	Fosfomycin	Sulfonamides	Multi-drug efflux pump
Punggol	<i>bla</i> _{MIR-5} , <i>bla</i> _{OKP-B-3} , <i>bla</i> _{OXA-10} , <i>bla</i> _{OXA-19} , <i>bla</i> _{SHV-204}		<i>sul1</i>	<i>fosA</i> , <i>fosA5</i>		<i>OqxA</i> , <i>OqxB</i> , <i>qacE</i>	<i>bla</i> _{OKP-B-12} *, <i>bla</i> _{SHV-11} *	<i>fosA5</i>	<i>sul1</i> *	<i>oqxA</i> *
Chinese Garden	<i>bla</i> _{OXA-10} , <i>bla</i> _{GES-5}	<i>aph(6)-Id</i>	<i>sul1</i>		<i>erm(C)</i>		<i>bla</i> _{OXA-10}			
Serangoon	<i>bla</i> _{CMG}			<i>fosA</i>		<i>OqxA</i> , <i>OqxB</i> , <i>OqxA</i>	<i>bla</i> _{CMG} *	<i>fosA</i> *		
Bedok										
Lower Seletar										
Marina	<i>bla</i> _{OXA-10} , <i>bla</i> _{OXA-19}		<i>sul1</i>			<i>qacE</i>	<i>bla</i> _{OXA-10} *, <i>bla</i> _{OXA-35} *			

23

24 AMR genes were detected based on the ResFinder database with different methods. The raw reads were mapped to the database with KMA, and the AMR genes on
 25 assembled contigs were detected with Blast mode. *: without 100% identify.

26