Use of synthesized double-stranded gene fragments as qPCR standards for the quantification of antibiotic resistance genes

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12 **Abstract**

13 Pollution of various environmental matrices by antibiotic resistance genes (ARGs) has
14 become a growing threat to human health. For the quantitative analysis of the presence of
15 ARGs, there is a need for sensitive and robust qPCR assays which can detect various genes
16 from different types of DNA extracts. Fourteen ARGs were selected as target genes in this
17 study including: bla_{TEM}, bla_{OXA-1} and bla_{CTX-M} coded for resistance to β-lactams; *ermB* coded
18 for macrolides; *tetA, tetG, tetM, tetQ, tetW* and *tetX* coded for tetracyclines; *sul I* and *sul II*
19 coded for sulfonamides; *drfA1* and *drfA12* coded for trimethoprim; integron gene *intI 1* and *intI*
20 *2*. Chemically synthesized double-stranded gene fragments were modified using molecular
21 biology methods and used as real-time PCR standards as well as to establish in-house qPCR
22 assays. The *ermB* gene from a naturally occurring plasmid was used to compare the
23 performance of qPCR assay with the chemically synthesized *ermB*. Additionally,
24 environmental water, soil and faeces samples were used to validate the established qPCR
25 assays. Importantly, the study proves the usefulness of rapidly synthesized oligonucleotides
26 serving as qPCR standards for ARG analysis and provides comparable sensitivity and
27 reliability to a traditional amplicon standard.

28

29 **Keywords:** antibiotic resistance genes; chemically synthesized gene fragments; gene cloning;
30 qPCR

1. Introduction

Antibiotic resistance genes (ARGs) are recognised as emerging environmental micropollutants (Pruden et al., 2006). These genes are diverse and ubiquitous in natural environments and an increasing number of reports have been published on the prevalence of ARGs in various environmental matrices, including surface water, drinking water, soil, aquaculture and agriculture (Cheng et al., 2016, Fernando et al., 2016, Wang et al., 2014, Xu et al., 2016). In order to address antimicrobial resistance (AMR), including antibiotic resistance, one of the multiple initiatives led by the WHO is to establish the Global Antimicrobial Resistance Surveillance System (GLASS), calling for more international participants and data to fully assess the resistance gene prevalence worldwide, which necessitates a standardised approach to the analysis and sharing of the data related to antibiotic resistance at a global level (World Health Organization, 2018a). It is reported that many of the same microbes affect both animals and humans via the environment they share and 60% of human infectious diseases are spread from animals (World Health Organization, 2018b). Despite the knowledge of environmental influences on AMR, current surveillance systems often neglect environmental sampling (Thakur and Gray, 2019). The role the environment plays as a reservoir of maintaining AMR genes is as equal important as AMR in human and animal populations. Hence, it is necessary to apply the One Health approach and study environmental reservoirs more closely (Thakur and Gray, 2019, World Health Organization, 2017), linking the health of people to the health of animals and the environment in order to establish effective surveillance systems to combat AMR.

An ARG is a specific gene which, when expressed, renders an otherwise susceptible host bacterium more resistant to a particular antibiotic (Sukumar et al., 2016). In general, classic molecular techniques such as PCR (polymerase chain reaction), are still of great importance for defining the dissemination of known ARGs in environmental samples (Allen, 2014). The absolute quantity of an ARG in a system is usually expressed as gene 'copy number', indicating the number of copies of a gene in the genome, including any mobile genetic elements (MGEs). For both quantitative and qualitative ARG analysis, standard templates are

59 needed to initially set up the assay and then for use as a positive control. Materials that can
60 be used as standards include PCR-amplified target sequences, plasmids containing the target
61 gene sequence, or commercially prepared DNA (Dhanasekaran et al., 2010). A known ARG
62 sequence can be amplified by PCR from genomic or mobile element DNA using gene specific
63 primers and visualised by gel electrophoresis. The amplified gene fragment can be ligated to
64 a known-sized vector and then transformed to competent cells for the reproduction of vector
65 containing target resistance gene. Positive clones carrying target ARG inserts are usually used
66 as standards for absolute quantification of ARG from various sample types (Calero-Caceres
67 et al., 2014, Chen and Zhang, 2013). This traditional method, however, is limited to the
68 availability of positive isolates, especially for those very rare or newly-discovered ARGs.
69 Additionally, when a research project has many ARGs of interest, it is costly to purchase the
70 commercially available strains or plasmids harbouring target ARGs. Synthetic oligonucleotides,
71 on the other hand, can be a useful alternative to obtain specific gene fragments. Relying on
72 well-developed synthesis platform, double-stranded DNA fragments with various lengths can
73 be easily purchased from biotechnology companies at an affordable price and used for a wide
74 range of applications, such as antibody research (Dickinson et al., 2013), genome engineering
75 (Cobb et al., 2015, Ghorbal et al., 2014), and qPCR standards (Greiman and Tkach, 2016,
76 Gunawardana et al., 2014). For instance, Greiman and Tkach utilised a 224-bp laboratory
77 synthesized fragment of the *Neorickettsia. risticii* GroEL gene to generate a standard curve
78 for the determination of the prevalence of *Neorickettsia* infection within multiple stages of the
79 digenean life cycle (Greiman and Tkach, 2016); According to Krüttgen et al., a chemically
80 synthesized $bla_{\text{NDM-1}}$ gene was introduced as a convenient positive control for the setup of in-
81 house assays for $bla_{\text{NDM-1}}$ detection (Krüttgen et al., 2011).
82 In this study, we aim to establish a convenient and cost-effective method for those laboratories
83 wishing to setup in-house assays for the quantitative analysis of ARGs in different
84 environmental matrices. The selection of the target ARGs was based on: 1) the antibiotic to
85 which they confer resistance; 2) the mechanism of resistance and, 3) the presence in different
86 environmental matrices. Fourteen ARGs, including bla_{TEM}, bla_{OXA-1} and bla_{CTX-M} coding for

87 resistance to β-lactams; *ermB* for macrolides; *tetA, tetG, tetM, tetQ, tetW* and *tetX* for
88 tetracyclines; *sul I* and *sul II* for sulfonamides; *drfA1* and *drfA12* for trimethoprim; and the
89 integron genes *intI 1* and *intI 2* were selected as target genes in this study. A traditional qPCR
90 assay for *ermB* was used for comparison using a naturally occurring plasmid encoding *emrB*.
91 Importantly, this study proves the usefulness of rapidly synthesized gene fragments serving
92 as qPCR standards for ARGs when biological isolates are not commonly available. To the
93 best of our knowledge, this is the first research using synthetic gene fragments as qPCR
94 standards for the quantitative analysis of multiple ARGs in various environmental samples.

95 **2. Materials and methods**

96 2.1 ARG fragment design and cloning

97 Nucleic acid sequence for individual ARG were downloaded from the NCBI website
98 (https://www.ncbi.nlm.nih.gov/nucleotide). Specific pair of primers for each ARG (Table 1) was
99 used to trim both sides of the sequence that obtained from NCBI website. ARG sequences
100 selected in this study were supplied in Table S1. Chemically synthesized double-stranded
101 ARGs (refer to hereafter as 'gBlocks' gene fragments) were obtained from Integrated DNA
102 Technology (UK) in dry form, ranging from 103 to 516 bp in length. Once received, gBlocks
103 gene products were re-suspended in Tris-EDTA buffer (10 mM Tris-HCl, 1 mM disodium EDTA,
104 pH 8.0, Sigma-Aldrich, UK) according to the manufacturers' instructions to reach a final
105 concentration of 10 or 20 ng/μL based on the length of gene fragment and stored at -20°C for
106 further process.

107 2.2 gBlocks Cloning

108 As gBlocks are blunt-end DNA fragments, it is necessary to add adenosine (A) overhangs to
109 gBlocks for the compatibility with T/A cloning vectors. The gBlocks DNA suspensions were
110 incubated at 50°C for 20 minutes prior to use. The A-tailing experiment was conducted at room
111 temperature. 0.6 μL Taq DNA polymerase (5 units/μL), 1.5 μL 10× PCR buffer (Taq PCR Core
112 Kit, QIAGEN, UK), 0.05 mM dATP (BIOLINE, UK), 50 ng gBlocks DNA fragments, and PCR
113 grade water were combined to a final volume of 15 μL. A reaction tube adding PCR grade
114 water instead of gBlocks was used as a negative control. After 30 minutes' incubation at 70°C,
115 the A-tailing products were ready for T/A cloning.

116 In order to compare the performance of gene fragment originated from both chemically
117 synthesis and resistance plasmid, a plasmid pMTL9301 DNA carrying *ermB* was also used for
118 gene cloning. Fresh PCR product with the confirmed presence of *ermB* gene was excised and
119 purified using QIAquick Gel Extraction Kit (QIAGEN, UK).

120 1 μL purified PCR product or A-tailed gBlocks was ligated into pGEM Easy Vector and then
121 transformed into *Escherichia coli* JM109 competent cells using pGEM Easy Vector Systems

122 (Promega, UK) according to the manufacturers' instructions. Successful recombinant cells
123 (blue colonies) were picked from LB agar plate containing 100 mg/L Ampicillin (Sigma-Aldrich,
124 UK) and Blue/White Select Screening reagent (Sigma-Aldrich, UK) and screened by PCR
125 (TECHNE, UK) using the primers listed in Table 1 to evaluate cloning of the target genes.
126 Details about PCR conditions can be found in SI. 6 μL of each PCR product were verified by
127 1.5% agarose gel electrophoresis. All PCR products were sequenced for the verification of the
128 presence of ARGs. The sequence results were compared with existing sequences using
129 BLAST alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Plasmid DNA were extracted
130 from vector containing the insert using the QIAprep Spin Miniprep Kit (QIAGEN, UK) and the
131 concentration of the vector was measured by Qubit 3.0 Fluorometer (Invitrogen, UK) using
132 dsDNA Broad Range Assay Kit (Invitrogen, UK).

133 2.3 qPCR Procedures

134 Plasmid DNA containing target genes were used to generate standard curves. The numbers
135 of copies of plasmid DNA per microliter were calculated using the following formula (Zhang et
136 al., 2009).

137
138

$$\frac{Copies}{\mu L} = \frac{DNA\ mass\ concentration\ (ng/\mu L) \times 10^{-9} \times 6.022 \times 10^{23}}{(3015\ bp^{*} + amplicon\ size\ bp) \times 660}$$

139 * The length of the pGEM Easy vector is 3015 bp.

140 Primer sequences and amplicon size of individual ARGs are described in Table 1. Seven-point
141 standard curves with copy numbers ranging from 10^2 to 10^8 for qPCR were generated using
142 10-fold serial dilutions of the plasmid DNA carrying target ARGs. A final volume of 20 μL
143 reaction mixture was used, consisting of 10 μL of Luna Universal qPCR Master Mix (New
144 England Biolabs, UK), 0.5 μL of each primer (10 μM), 1 μL DNA template, and 8 μL of PCR
145 grade water. The PCR protocol was as follows: 1 min at 95°C, followed by 40 cycles of 15 s
146 at 95°C, 30 s at 60°C, and then a final melt curve stage with temperature ramping from 60 to
147 95°C. Each reaction was run in triplicate and a non-template control was included. All the
148 qPCR assays were performed in 96-well plates under standard conditions, as per the
149 instructions of the manufacturer, in a 7500 Real-Time PCR system (Applied Biosystems). The

150 qPCR efficiencies were generated by the software (Applied Biosystems 7500 v2.3) on the
151 basis of the standard curves.

152 2.4 Method validation

153 Different types of environmental samples were used to validate the qPCR assays. Water
154 samples were collected from the River Thames, ponds in Regent's Park and Hyde Park in
155 London; soil and duck faeces samples were collected from Regent's Park. The geographical
156 location of sampling sites is provided in Figure S1. All samples were collected in triplicate in
157 pre-autoclaved amber glass bottles or sterile tubes and kept refrigerated at 4 °C without
158 preservatives until they were processed within 24 h of sampling. DNA was extracted in
159 triplicate using the FastDNA SPIN Kit for Soil (MP Bio, UK) according to the manufacturers'
160 instructions. Water samples (500 mL) were filtered using a vacuum filtration apparatus through
161 a 0.22 μm mixed cellulose esters membrane filters (Millipore, UK) and DNA was extracted
162 from the membrane. For soil and faeces samples, DNA was extracted directly from 0.5 g (wet)
163 of the raw samples. The quality and concentration of the extracted DNA were determined by
164 NanoDrop and 1.5% agarose gel electrophoresis and stored at -20 °C until further analysis.
165 qPCR settings for environmental DNA samples were the same as for qPCR standards as
166 described about.

167 To assess qPCR inhibitions, dilutions of the standards were spiked with environmental DNA
168 and the threshold cycle (Ct) and copy numbers were compared to the known copy numbers
169 of the target genes in the standards (Colomer-Lluch et al., 2011). No inhibition of qPCR by
170 environmental DNA was detected.

171 Table 1. Primers and amplicon size of target ARGs in this study.

Target Gene	Primer	Sequence	Amplicon Size (bp)	Reference
bla_{CTX-M}	bla_{CTX-M}-F	CTATGGCACCACCAACGATA	103	(Marti et al., 2013)
	bla_{CTX-M}-R	ACGGCTTTCTGCCTTAGGTT		
bla_{OXA-1}	bla_{OXA-1}-F	ACCAAAGACGTGGATGCAAT	325	(Tennstedt et al., 2005)
	bla_{OXA-1}-R	TGCACCAGTTTTCCCATACA		
bla_{TEM}	bla_{TEM}-F	CCCCGAAGAACGTTTTC	516	(Mabilat and Courvalin, 1990)
	bla_{TEM}-R	ATCAGCAATAAACCAGC		
ermB	ermB-F	ACGACGAAACTGGCTAAAATAAGT	412	This study
	ermB-R	CTGTGGTATGGCGGGTAAGT		
tetA	tetA-F	GCTACATCCTGCTTGCCTTC	210	(Ng et al., 2001)
	tetA-R	CATAGATCGCCGTGAAGAGG		
tetG	tetG-F	GCTCGGTGGTATCTCTGCTC	468	(Ng et al., 2001)
	tetG-R	AGCAACAGAATCGGGAACAC		
tetM	tetM-F	ACAGAAAGCTTATTATATAAC	171	(Aminov et al., 2001)
	tetM-R	TGGCGTGTCTATGATGTTCAC		
tetQ	tetQ-F	AGAATCTGCTGTTTGCCAGTG	169	(Aminov et al., 2001)
	tetQ-R	CGGAGTGTCAATGATATTGCA		
tetW	tetW-F	GAGAGCCTGCTATATGCCAGC	168	(Aminov et al., 2001)
	tetW-R	GGGCGTATCCACAATGTTAAC		
tetX	tetX-F	CAATAATTGGTGGTGGACCC	468	(Ng et al., 2001)
	tetX-R	TTCTTACCTTGGACATCCCG		
sul I	sul I-F	CACCGGAAACATCGCTGCA	158	(Luo et al., 2010)
	sul I-R	AAGTTCCGCCGCAAGGCT		
sul II	sul II-F	CTCCGATGGAGGCCGGTAT	190	(Luo et al., 2010)
	sul II-R	GGGAATGCCATCTGCCTTGA		
dfrA1	dfrA1-F	TGGTAGCTATATCGAAGAATGGAGT	425	(Grape et al., 2007)
	dfrA1-R	TATGTTAGAGGCGAAGTCTTGGGTA		
dfrA12	dfrA12-F	GAGCTGAGATATACACTCTGGCACT	155	(Grape et al., 2007
	dfrA12-R	GTACGGAATTACAGCTTGAATGGT		
intI 1	intI 1-F	CCTCCCGCACGATGATC	280	(Goldstein et al., 2001)
	intI 1-R	TCCACGCATCGTCAGGC		
intI 2	intI 2-F	TTATTGCTGGGATTAGGC	233	(Goldstein et al., 2001)
	intI 2-R	ACGGCTACCCTCTGTTATC		
16S	1369F	CGGTGAATACGTTCYCGG	143	(Gaze et al., 2011)
	1492R	GGWTACCTTGTTACGACTT		

172 2.5 Statistical Analysis

173 In this study, the absolute abundance of ARG was defined as the ARG copies per litre aqueous
174 samples (copies/L) or per gram soil/faeces samples (copies/g). The relative abundance of
175 ARG was defined as the normalised ARG copies to the 16S rRNA copies. Average and
176 standard deviations calculation of all data were done with Microsoft Excel 2016. The results
177 of qPCR were analysed using 7500 software v2.3 (Applied Biosystems, UK). One-way
178 analysis of variation (ANOVA) test was used to evaluate the differences between ARGs
179 detected in environmental DNA samples with significance level of 5% ($P < 0.05$). ANOVA and
180 Pearson correlation analysis were carried out using OriginPro 2018. All figures were generated
181 by OriginPro 2018. Only samples with three replicates that had been amplified were regarded
182 as positive.

3. Results and discussion

3.1 PCR and gel electrophoresis

Recombinant cells were selected and subjected to colony PCR to confirm insertion of the correct DNA fragment. The PCR products were run on the gel to visualise the specific band for each ARG. As can be seen from Figure 1, all of the target genes were amplified and formed a single band. The size of each ARG band as it appeared on the gel was in accordance with the amplicon size specified in Table 1, ranging from 103 bp for $bla_{CTX\text{-}M}$ to 516 bp for bla_{TEM}. Both *ermB* gene bands were the same size and intensity.

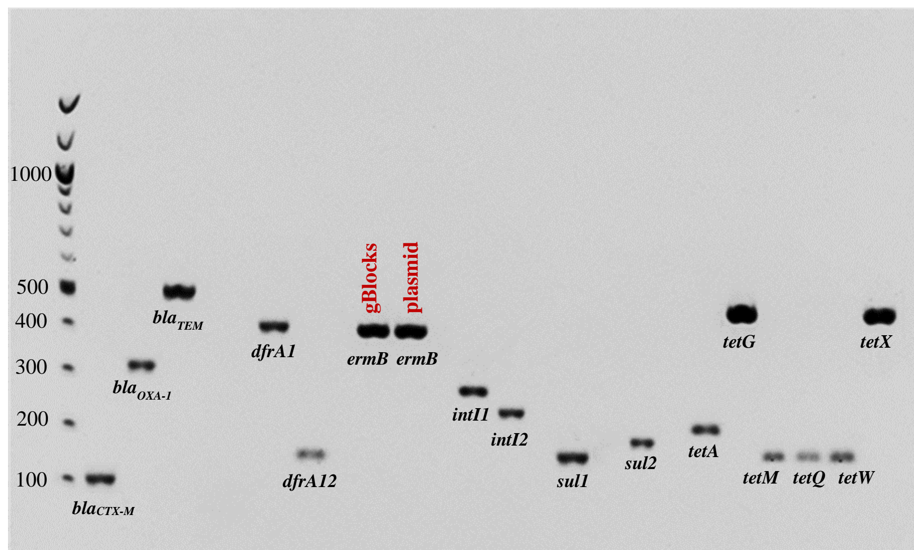


Figure 1. Electrophoresis bands of target genes. (*ermB* - left: originated from gBlocks; right: originated from plasmid)

3.2 Specificity and sensitivity of the qPCR assays

In this study, amplification efficiencies for all target genes ranged from 80.9% to 107.4% with good linearity (Table 2), indicating the reliability of synthetic gene fragments as qPCR standards. Ideally, the qPCR efficiency should be 1.0, however, if consistent, lower efficiency value is also acceptable due to the potential PCR inhibitors in DNA extracts (Luby et al., 2016). The calculation of limit of detection followed previous research by performing eight replicates of each dilution of the standard for each gene, and the lower gene copies gave results that

210 were accurately reproducible (coefficient of variation less than 10%) was considered as limit

211 of detection (Calero-Caceres et al., 2014). Only runs resulting in gene copies higher than the

212 detection limit were applied for the calculation of resistance gene concentrations. The limit of

213 detection of each qPCR assay was shown in Table 2.

214 Table 2. Standard curves, amplification efficiency, R^2 value of each qPCR array.

Target Gene	**Standard curve**	**R^2**	**Amplification efficiency**	**LOD (GC/μL)**
bla_{CTX-M}	Y = -3.44X + 39.19	0.999	95.3%	9.6
bla_{OXA-1}	Y = -3.53X + 38.05	0.999	92.1%	7.3
bla_{TEM}	Y = -3.16X + 36.66	0.993	107.4%	6.1
**ermB* (gBlocks)	Y = -3.30X + 41.55	0.997	100.9%	9.2
**ermB* (plasmid)	Y = -3.55X + 36.30	0.995	91.3%	4.6
tetA	Y = -3.44X + 36.72	0.999	95.4%	3.2
tetG	Y = -3.84X + 40.25	0.983	82.0%	6.8
tetM	Y = -3.91X + 38.88	0.997	80.9%	11.2
tetQ	Y = -3.26X + 35.25	0.999	102.8%	2.0
tetW	Y = -3.31X + 35.89	0.998	100.3%	7.1
tetX	Y = -3.38X + 40.51	0.997	97.6%	6.3
sul I	Y = -3.43X + 38.11	0.997	95.8%	5.1
sul II	Y = -3.81X + 39.01	0.999	83.0%	7.3
dfrA1	Y = -3.63X + 36.45	0.997	88.5%	4.5
dfrA12	Y = -3.34X + 39.42	0.997	99.4%	6.1
intI 1	Y = -3.51X + 43.04	0.995	92.8%	3.1
intI 2	Y = -3.10X + 39.65	0.998	96.8%	3.8
16S	Y = -3.39X + 36.34	1.000	97.3%	3.9

215 **ermB* (gBlocks): chemically-synthesized *ermB*; *ermB* (plasmid): plasmid-carrying *ermB*.

216 LOD: Limit of Detection; GC/μL: gene copies/ μL

217

The stability of the standards is a critical issue as they are typically used for long-term studies, with different experimental designs where multiple samples need to be compared over time with great accuracy as well as for short term studies (Dhanasekaran et al., 2010). Once extracted, the highly-purified plasmids incorporating gBlocks fragments can be stored in the freezer and used as qPCR standards for extended periods of time. Alternatively, positive clones can also be stored in ampicillin-containing media and plasmid DNA can be extracted sustainably with sufficient quantity and high purity when needed.

In order to compare the performance of synthetic *ermB* (gBlocks) and plasmid-harbouring *ermB* serving as qPCR standards, same downstream gene cloning experiments were conducted. Both gBlocks-*ermB* and plasmid-*ermB* achieved good amplification efficiency and linearity (100.9%, 0.997 and 91.3%, 0.995, respectively). Better *ermB* assay efficiency was observed with the gBlocks fragment standard. Sequence alignment results also showed 100% similarity for both *ermB* gene fragments, indicating the performance of gBlocks-*ermB* qPCR assay was comparable with plasmid-harbouring *ermB*. In general, gBlocks standards enable more independent qPCR assay development which is not limited to the availability of the positive isolates or plasmids, especially when a research project has several ARGs of interest.

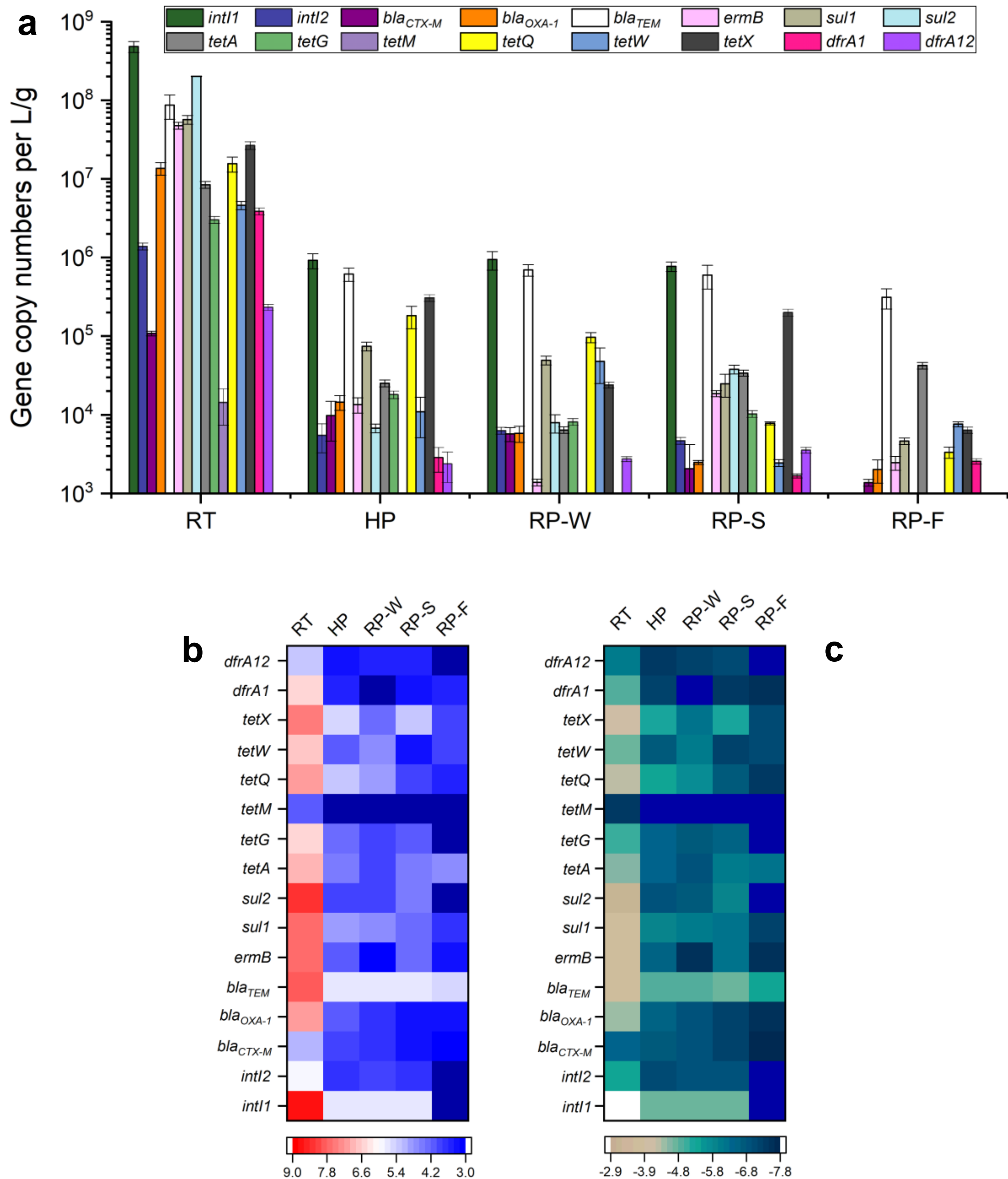
3.3 Validation of qPCR assays

Water, soil and duck faeces samples from the natural environment were used to validate the established qPCR assays based on gBlocks modification. For the consistency of data analysis, only the results of *ermB* originated from gBlocks were present in the figures and table in the following context. For comparisons, raw qPCR results of gBlocks-*ermB* and plasmid-*ermB* can be found in Table S2.

3.3.1 ARG abundance

An overview of the absolute abundance of ARGs and integron genes *intI 1* and *intI 2* in different environmental samples is shown in Figure 2a. The concentration of ARGs among all the

245 samples was between 10^3 ~ 10^8 copies/L, with the detection frequencies ranging from 71.42%
246 to 100%.



247 Figure 2. a: Concentrations of ARGs and integron genes in environmental samples; A
248 heatmap of b: absolute abundance; c: relative abundance (normalised to the corresponding
249 16S rRNA) of ARGs showing distinct pattern between River Thames (RT), Hyde Park (HP),
250 Regent's Park water (RP-W), soil (RP-S) and faeces (RP-F) sample.

251 ARGs are ubiquitous in the environment. All of the fourteen selected ARGs were detected in
252 River Thames water samples. In general, the overall abundance of ARGs in the River Thames
253 was two to three orders of magnitude higher than in the parks' water samples. The order of
254 the average gene copies from low to high was: RP-F (3.86×10^4 copies/g), RP-S (7.28×10^4
255 copies/g), RP-W (7.97×10^4 copies/L), HP (9.91×10^4 copies/L), and RT (3.37×10^7 copies/L).
256 The result is consistent with the initial hypothesis as the River Thames is much affected by
257 anthropogenic activities. There are many residential and commercial areas along the River
258 Thames, and it is also the receiving water body to the municipal wastewater treatment works.
259 Previous research in Huangpu River has shown that the levels of ARGs in areas with
260 anthropogenic activities was much higher than in areas that were less affected by human
261 activities (Jiang et al., 2013). Considering the River Thames is used as a drinking water source,
262 the high detection frequency and concentration of ARGs in river water may imply a potential
263 health threat to the public.
264 The parks selected in this study are located in Central London and have been open to public
265 for decades. Not surprisingly, ten out of fourteen ARGs were detected in wild duck faeces
266 samples. It was very common for park visitors to walk dogs along the pond pathways, however,
267 pet animals that live with humans, including cats and dogs, are reservoirs of antibiotic-resistant
268 bacteria due to the antibiotic treatment for diseases and the transfer of resistant bacteria from
269 humans (Guardabassi et al., 2004). Upon release into the soil and pond through surface runoff,
270 those resistant bacteria could have acted as donors of genes encoding antibiotic resistance,
271 or their presence could have been favoured as a result of selection pressure exerted by the
272 presence of antibiotic residues in animal excreta, contributing to the dissemination of ARGs to
273 the wild animals such as ducks and geese (Petersen et al., 2002).
274 Among all the ARGs targeted in this study, sulfonamide resistance genes, *sul1* and *sul2* had
275 the highest abundance with an average concentration of 3.11×10^7 copies L^{-1}/g^{-1}, followed by
276 *ermB* gene (9.58×10^6 copies L^{-1}/g^{-1} on average) encoding resistance to macrolides. β-
277 lactams resistance genes were the third most abundant resistance gene family, among which
278 bla_{TEM} gene had the highest concentration (1.79×10^7 copies L^{-1}/g^{-1} in average). *tetQ* and *tetX*

279 were the most abundant tetracycline resistance genes, with the average concentration ranging
280 from 3.18×10^6 to 4.57×10^6 copies L^{-1}/g^{-1}. Trimethoprim resistance genes, *dfrA1* and *dfrA12*,
281 were the least abundance (5.19×10^5 copies L^{-1}/g^{-1} in average) among the environmental
282 samples. Heatmaps illustrating distinct patterns of the absolute and relative abundance of
283 ARGs between environmental samples are shown in Figure 2 b & c. The trend for the relative
284 abundance of ARGs (normalised to the corresponding 16S rRNA gene copy number in the
285 sample) was similar to the absolute gene copies.
286
287 3.3.2 Relationship between ARGs and integron genes
288 Two mobile element genes, class I and class II integrons (*intI 1, intI 2*) were targeted in this
289 study. The River Thames water samples had the highest integron gene copy numbers (4.83
290 $\times 10^8$ copies/L for *intI 1* and 1.40×10^6 copies/L for *intI 2*). As an indicator of horizontal gene
291 transfer (HGT) potential, *intI 1* was reported to integrate and express more than 100 types of
292 resistance genes by gene cassettes, most of which were aminoglycoside and trimethoprim
293 resistance genes and β-lactamases (Gillings et al., 2008, Gillings et al., 2015). In this study,
294 *intI 1* showed significant positive correlations with all of the ARGs families ($p < 0.05$),
295 particularly with sulfonamides ($R^2 = 0.999$, $p < 0.001$), macrolides ($R^2 = 0.998$, $p < 0.001$), and
296 trimethoprim ($R^2 = 0.924$, $p < 0.001$). In contrast with class I integrons, class II integrons were
297 less commonly found in environmental samples. Previous research has shown that the *dfrA*
298 genes encoding resistance to trimethoprim can be found in class II integrons (Antunes et al.,
299 2006). This was consistent with our result as both *dfrA1* and *dfrA12* were found to have
300 significant positive correlations ($p < 0.001$) with *intI 2*. ARGs that are associated with mobile
301 genetic elements can propagate among species through horizontal gene transfer mechanisms,
302 contributing to the persistence and spread of ARGs in different environmental matrix (Chen
303 and Zhang, 2013).

304 3.4 The applicability of gBlocks

305 In this study, we demonstrate that using gBlocks gene fragments as qPCR standards provides
306 comparable assay performance to a traditional amplicon standard. It allows routine, reliable
307 identification or profiling of ARGs from samples in any research laboratory with access to a
308 real-time PCR instrument. The established in-house qPCR assays for ARGs were applicable
309 to different environmental samples, including surface water, soil and animal faeces.
310 Considering the complexity of DNA extracts from soil and animal faeces, this method can also
311 be applied to a wider range of sample types, for example wastewater, manure, sediment,
312 slurry and sludge. Apart from using as qPCR standards, gBlocks gene fragment can also be
313 used as positive control of conventional PCR due to the unavailability of biological isolates
314 (Krüttgen et al., 2011).

315 Culture-independent approaches have been successfully developed, among which qPCR, is
316 of great importance to provide an approximation of the dissemination of known ARGs in
317 environmental samples (Berendonk et al., 2015). In accordance with WHO's initiatives for the
318 surveillance of antibiotic resistance, more data across countries are needed to fully assess
319 the prevalence of ARGs worldwide (World Health Organization, 2018). gBlocks-based qPCR
320 standard method developed in this study provides a potential standardised approach for the
321 comparison of resistance prevalence in different sampling locations to acquire a temporal
322 perspective on resistance dynamics and to assess possible correlations between antibiotic
323 resistance and anthropogenic activities. Furthermore, the simplicity of the gBlocks product
324 format and operating procedure allow easy gene construction or modification, and it saves the
325 cost on commercially available strains/plasmids, or the time needed for lab-exchange
326 strains/plasmids.

327 There are challenges and limitations to the application of gBlocks-based qPCR standards for
328 environmental ARGs analysis. One drawback is that, similar to conventional qPCR method,
329 the numbers of targeted ARGs in a research project is limited. Theoretically, synthetic ARG
330 fragments can be obtained and constructed as many as needed, but this may come at
331 significant cost and labor. In general, up to 20 ARGs target numbers would be appropriate for

332 a given study. Although the raw ARGs fragments are easily to obtain, the regions selected for
333 individual ARG may vary, and may occasionally not fit the production criteria due to the
334 sequence complexity. The operational procedures of preparing gBlocks standards are
335 straightforward, however, specialised training will be needed to avoid the potential cross-
336 contamination that might occur during the gene cloning process. Furthermore, qPCR is highly
337 based on the quality and purity of extracted DNA, which will vary in efficiency across
338 environmental matrices and is likely to carry through inhibitors depending on DNA extraction
339 methods that can interfere with qPCR (Luby et al., 2016). Harmonised guidelines regarding
340 the DNA extraction methods; the target resistance genotypes or primer sets will be useful for
341 the direct comparisons between different environmental compartments (Berendonk et al.,
342 2015).
343
344 **4. Conclusion**

345 In summary, we established in-house qPCR assays using chemically synthesized
346 oligonucleotides (gBlocks) as standards for the quantification of fourteen ARGs and two
347 integron genes. The performance of gBlocks-*ermB* standard was comparable to traditional
348 *ermB* standard from a naturally occurring plasmid with similar sensitivity and amplification
349 efficiency. The qPCR assays have been successfully applied to surface water, soil and animal
350 faeces samples to assess the ARGs prevalence in the environment. Our study provides a
351 routine and reliable method for the identification or profiling of ARGs, especially suitable for a
352 research project that has several ARGs of interest or for those very rare or newly-discovered
353 ARGs.
354
355 **Conflict of interest**

356 No conflict of interest declared.

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487 # Supporting Information:

488 # Use of synthesized double-stranded gene fragments as qPCR standards

489 # for the quantification of antibiotic resistance genes

490

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500 Figure S1. Geographic map of sampling sites. 1: Regent's Park; 2: Hyde Park; 3: River Thames

501 **PCR Procedures**

502 A 25 µL PCR reaction system was performed for all genes. The reaction mixture consisted of
503 12.5 µL BioMix^{TM} Red (BIOLINE, UK), 1 µL of each primer (10 µM), 1 µL of DNA template, and
504 9.5 µL of PCR grade water. The PCR programme used was as follows: 95 °C for 3 min, followed
505 by 35 cycles consisting of 95 °C for 15 s, 55 °C for 30 s, 75 °C for 30 s, and a final extension
506 step at 72 °C for 7 min. PCR grade water was used as the negative control in every run.

507 Table S1. Antibiotic Resistance Genes (ARGs) Oligonucleotides Used in This Study

Gene	Amplicon Size (bp)	GeneBank Accession No.	Sequence used in this study
bla $bla_{CTX\text{-}M}$	103	KT867021.1	CTATGGCACCACCAACGATATCGCGGTGATCTGGCCAAAAGATCGTGCGCCGCTGATTCTGGTCA CTTACTTCACCCAGCCTCAACCTAAGGCAGAAAGCCGT
bla_{TEM}	516	KT867019.1	CCCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGTGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGT ACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCTGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGC TAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGC GCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGAT
$bla_{OXA\text{-}1}$	325	KR338947.1	ACCAAAGACGTGGATGCAATTTTCTGTTGTTTGGGTTTCGCAAGAAATAACCCAAAAAATTGGATTAAATAAAATCAAGAATTATCTCAAAGATTTTGATTATGGAAATCAAGACTTCTCTGGAGATAAAG AAAGAAACAACGGATTAACAGAAGCATGGCTCGAAAGTAGCTTAAAAATTTCACCAGAAGAACAAATTCAATTCCTGCGTAAAATTATTAATCACAATCTCCCAGTTAAAAACTCAGCCATAGAAAACAC CATAGAGAACATGTATCTACAAGATCTGGATAATAGTACAAAACTGTATGGGAAAACTGGTGCA
ermB	412	EU595407.1	ACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTCACTTTAATTCACCAAGATATTCTACAGTTT CAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAGGATTCTAC AAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTGCTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACT TACCCGCCATACCACAG
tetA	210	KF240812.1	GCTACATCCTGCTTGCCTTCGCGACACGGGGATGGATGGCGTTCCCGATCATGGTCCTGCTTGCTTCGGGTGGCATCGGAATGCCGGCGCTGCAAGCAATGTTGTCCAGGCAGGTGGATGAGGAACGTC AGGGGCAGCTGCAAGGCTCACTGGCGGCGCTCACCAGCCTGACCTCGATCGTCGGACCCCTCCTCTTCACGGCGATCTATG
tetG	468	KJ603219.1	GCTCGGTGGTATCTCTGCTCATGCCCCGTTTATCGCCGCCGCCCTTCTCAACGGGTTCGCGTTCCTGCTTGCCTGCATTTTCCTCAAGGAGACTCATCACAGCCATGGCGGGACCGGAAAGCCGGTTCGCA TCAAACCATTCGTTCTGTTACGGCTGGATGATGCATTGCGCGGGCTAGGTGCGCTTTTCGCAGTTTTCTTCATTATTCAACTGATCGGCCAAGTGCCTGCAGCCCTATGGGTCATATATGGCGAGGACCGT TTTCAGTGGAACACCGCGACCGTTGGTTTGTCGCTCGCGGCGTTTGGGGCAACACATGCGATCTTCCAAGCGTTTGTTACCGGCCCGCTTTCAAGCCGGCTTGGAGAGCGGCGCACGCTGCTGTTTGGCA TGGCTGCGGATGCGACTGGCTTCGTTCTTCTGGCTTTTGCCACGCAGGGATGGATGGTGTTCCCGATTCTGTTGCT
tetM	171	KR270482.1	ACAGAAAGCTTATTATATAACAGTGGAGCGATTACAGAATTAGGAAGCGTGGACAAAGGTACAACGAGGACGGATAATACGCTTTTAGAACGTCAGAGAGGAATTACAATTCAGACAGGAATAACCTC TTTTCAGTGGGAAAATACGAAGGTGAACATCATAGACACGCCA
tetQ	169	KX034803.1	AGAATCTGCTGTTTGCCAGTGGAGCAACGGAAAAGTGCGGCCGTGTGGATAATGGTGACACCATAACGGACTCTATGGATATAGAGAAACGTAGAGGAATTACTGTCCGGGCTTCTACGACATCTATTA TCTGGAATGGAGTGAAATGCAATATCATTGACACTCCG
tetW	168	EF489472.1	GAGAGCCTGCTATATGCCAGCGGAGCCATTTCAGAACCGGGGAGCGTCGAAAAAGGGACAACGAGGACGGACACCATGTTTTTGGAGCGGCAGCGTGGGATTACCATTCAAGCGGCAGTCACTTCCTT CCAGTGGCACAGATGTAAAGTTAACATTGTGGATACGCCC
tetX	468	KF905572.1	CAATAATTGGTGGTGGACCCGTTGGACTGACTATGGCAAAATTATTACAGCAAAACGGCATAGACGTTTCAGTTTACGAAAGAGACAACGACCGAGAGGCAAGAATTTTTGGTGGAACCCTTGACCTAC ACAAAGGTTCAGGTCAGGAAGCAATGAAAAAAGCGGGATTGTTACAAACTTATTATGACTTAGCCTTACCAATGGGTGTAAATATTGCTGATGAAAAAGGCAATATTTTATCCACAAAAAATGTAAAGCC CGAAAATCGATTTGACAATCCTGAAATAAACAGAAATGACTTAAGGGCTATCTTGTTGAATAGTTTAGAAAACGACACGGTTATTTGGGATAGAAAACTTGTTATGCTTGAACCTGGTAAGAAGAAGTGG ACACTAACTTTTGAGAATAAACCGAGTGAAACAGCAGATTTGGTTATTCTTGCCAATGGCGGGATGTCCAAGGTAAGAA
sul I	158	KJ801663.1	CACCGGAAACATCGCTGCACGTGCTGTCGAACCTTCAAAAGCTGAAGTCGGCGTTGGGGCTTCCGCTATTGGTCTCGGTGTCGCGGAAATCCTTCTTGGGCGCCACCGTTGGCCTTCCTGTAAAGGATCT GGGTCCAGCGAGCCTTGCGGCGGAACTT
sul II	190	KC898873.1	CTCCGATGGAGGCCGGTATCTGGCGCCAGACGCAGCCATTGCGCAGGCGCGTAAGCTGATGGCCGAGGGGGCAGATGTGATCGACCTCGGTCCGGCATCCAGCAATCCCGACGCCGCGCCTGTTTCGT CCGACACAGAAATCGCGCGTATCGCGCCGGTGCTGGACGCGCTCAAGGCAGATGGCATTCCC
dfrA1	425	KC862256.1	TGGTAGCTATATCGAAGAATGGAGTTATCGGGAATGGCCCTGATATTCCATGGAGTGCCAAAGGTGAACAGCTCCTGTTTAAAGCTATTACCTATAACCAATGGCTGTTGGTTGGACGCAAGACTTTTGA ATCAATGGGAGCATTACCCAACCGAAAGTATGCGGTCGTAACACGTTCAAGTTTTACATCTGACAATGAGAACGTAGTGATCTTTCCATCAATTAAAGATGCTTTAACCAACCTAAAGAAAATAACGGATC ATGTCATTGTTTCAGGTGGTGGGGAGATATACAAAAGCCTGATCGATCAAGTAGATACACTACATATATCTACAATAGACATCGAGCCGGAAGGTGATGTTTACTTTCCTGAAATCCCCAGCAATTTTAG GCCAGTTTTTACCCAAGACTTCGCCTCTAACATA
dfrA12	155	GU944735.1	GAGCTGAGATATACACTCTGGCACTACCTCACGCCCACGGCGTGTTTCTATCTGAGGTACATCAAACCTTCGAGGGTGACGCCTTCTTCCCAATGCTCAACGAAACAGAATTCGAGCTTGTCTCAACCGAA ACCATTCAAGCTGTAATTCCGTAC
intI 1	280	JN837682.1	CCTCCCGCACGATGATCGTGCCGTGATCGAAATCCAGATCCTTGACCCGCAGTTGCAAACCCTCACTGATCCGCATGCCCGTTCCATACAGAAGCTGGGCGAACAAACGATGCTCGCCTTCCAGAAAACC GAGGATGCGAACCACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGACGGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGATCCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCCG CCAATGCCTGACGATGCGTGGA
intI 2	233	FJ785524.1	TTATTGCTGGGATTAGGCGCGTGGGCAGTAGGCTGTTTCTGCTTTTCCCACCCTTACCGTCATGCACAGTGATGCAGCCATTATCAAAATCAAAATCTTTAACCCGCAAACGCAAGCATTCATTAATGCGC AAACCTGCACCATACAGCAGCGTAAAAATAACTTGGTTGCGAGTATCCATAACCTGCAAAATGCGTTGCACTTCATTTGCAGAGATAACAGAGGGTAGCCGT

508 Table S2. qPCR results of *ermB* in environmental samples

Samples	*ermB*-gBlocks (copies L^{-1}/g^{-1})	*ermB*-plasmid (copies L^{-1}/g^{-1})
RT	4.79×10^7	3.64×10^7
HP	1.35×10^4	1.67×10^4
RP-W	1.39×10^3	2.07×10^3
RP-S	1.87×10^4	2.81×10^4
RP-F	2.47×10^3	1.92×10^3

509 *ermB* (gBlocks): chemically-synthesized *ermB*; *ermB* (plasmid): plasmid-carrying *ermB*.

510 RT: River Thames; HP: Hyde Park; RP-W: Regent's Park water; RP-S: Regent's Park soil; RP-F:

511 Regent's Park faeces samples.

512 * No statistical difference ($P > 0.05$) was found between the results.

513