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Quantitative PCR versus metagenomics for monitoring antibiotic resistance genes: balancing high sensitivity and broad coverage

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Abstract

The widespread occurrence of clinically relevant antibiotic resistance within humans, animals, and environment motivates the development of sensitive and accurate detection and quantification methods. Metagenomics and quantitative PCR (qPCR) are amongst the most used approaches. In this study, we aimed to evaluate and compare the performance of these methods to screen antibiotic resistance genes in animal faecal, wastewater, and water samples. Water and wastewater samples were from hospital effluent, different treatment stages of two treatment plants, and of the receiving river at the discharge point. The animal samples were from pig and chicken faeces. Antibiotic resistance gene coverage, sensitivity, and usefulness of the quantitative information were analyzed and discussed. While both methods were able to distinguish the resistome profiles and detect gradient stepwise mixtures of pig and chicken faeces, qPCR presented higher sensitivity for the detection of a few antibiotic resistance genes in water/wastewater. In addition, the comparison of predicted and observed antibiotic resistance gene quantifications unveiled the higher accuracy of qPCR. Metagenomics analyses, while less sensitive, provided a markedly higher coverage of antibiotic resistance genes compared to qPCR. The complementarity of both methods and the importance of selecting the best method according to the study purpose are discussed.

Keywords: qPCR, metagenomics sequencing, gene quantification, sensitivity, limit of detection, limit of quantification

Introduction

Antibiotic resistance is a major global human-health threat, lately addressed under the One-Health perspective, where the natural environment, animal wellbeing, and human- and animal health are interdependent. Culture-independent methods have been the gold standard to assess the environmental resistome, i.e. the whole set of antibiotic resistance genes in a specific niche and have been increasingly employed to characterize human and animal resistomes. The most commonly used culture-independent approaches have been quantitative PCR (qPCR) and high-throughput sequencing (whole genome sequencing and metagenomics). qPCR is a targeted method that permits the screening of specific genes, as long as their nucleotide sequences are known, and specific oligonucleotides can be designed to be used as primers. This approach has been used for several years to determine the prevalence of selected antibiotic resistance genes in different reservoirs (Manaia et al. 2016, Le et al. 2018, Rocha et al. 2019). Metagenomics is a nontargeted method that allows a broad overview of genetic determinants. This is done by assigning DNA sequence reads to databases containing all known genes, a procedure that generates profiles consisting of genes relative abundance that contribute to characterize genetic features in samples (e.g. resistomes or bacterial communities) (Munk et al. 2017, Lanza et al. 2018). The information provided by metagenomic analysis is influenced by the sequencing depth, the bioinformatics analysis methods, or the database against which antibiotic resistance genes are identified, among other (Cave et al. 2021). Regarding antibiotic resistance studies, metagenomics and qPCR are normally used with different aims, and although the use of both methods might be adequate in some cases, most studies rely and focus on only one of those. It has been argued that qPCR method is more sensitive and suitable for absolute quantification of target genes, while metagenomics has the potential to provide an overview of the genes as well as of the respective relative abundance of the antibiotic resistance genes.

This work aimed to compare the use of metagenomics and qPCR analyses to screen antibiotic resistance genes in animal faecal, wastewater, and water samples. We also compared the performance of both methods, specifically for genes coverage, sensitivity, and usefulness of the quantitative information produced. The experimental design included two sets of samples, one of hospital effluent, different stages of wastewater treatment of two plants, and the receiving river up- and downstream the discharge point, and another comprised of pig and chicken faeces mixed at different proportions to represent various pig and chicken microbiomes.

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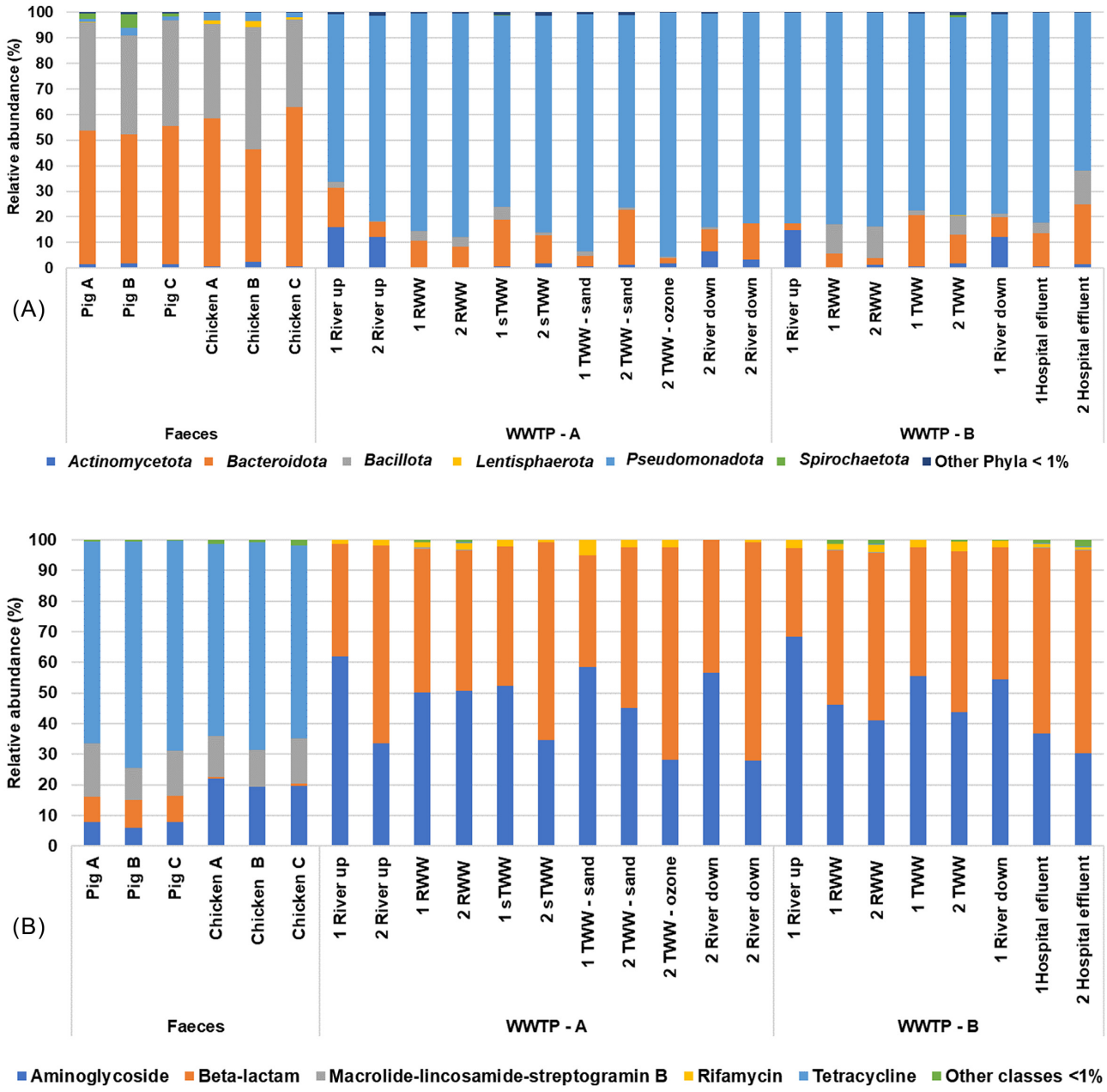

Figure 1. Bacterial community (A) and resistome (B) composition at the phylum and antibiotic family levels, respectively, based on metagenome analysis. Pig and chicken faecal material were collected from three animals from the same farm (A, B, and C). Water samples were collected in two sampling campaigns (1 and 2) from two WWTPs (A and B), of raw (RWW) and treated (TWW) at different treatment stages, the corresponding receiving river (upstream and downstream the discharge point) and hospital effluent that enters the WWTP-B.

Methods

Samples and DNA extraction

The study involved wastewater, water, pig, and chicken faecal samples. Wastewater and water samples were collected at different treatment stages from two wastewater treatment plants (WWTPs), surface water was collected upstream and downstream the WWTP discharge points in the river, and the effluent of a hospital discharging into one of the WWTPs was collected in the hospital. These samples corresponded to two campaigns as described before (Ferreira et al. 2022). Pig and chicken faeces DNA extracts of samples collected and processed as described before (Munk et al. 2018), were mixed in nine different proportions—0:1, 1:10, 1:100, 1:1000 and 1:0, 10:1, 100:1, 1000:1 and 1:1, pig:chicken, respectively. The two sets of samples will be referred to as water and animal faeces, unless a specific designation is required.

Water samples were processed and analyzed in triplicate, through filtration and DNA extraction (filtration of 50 ml of raw wastewater, 200–250 ml of treated wastewater after secondary treatment or after sand filtration or after ozonation, 100 ml of hospital effluent, 250–300 ml of river water through polycarbonate membranes, 0.22 μm porosity, Whatman, England) (Ferreira et al. 2022). The DNA extracts of animal faecal samples were obtained from 0.2 g of biomass by using a modified QIAamp Fast DNA Stool Mini Kit protocol (51604, Qiagen) as described before (Munk et al. 2018). DNA yields from pig and chicken faeces were combined in gradient proportions. The individual DNA extracts and the mixtures corresponded to a total of 50 extracts that were analyzed

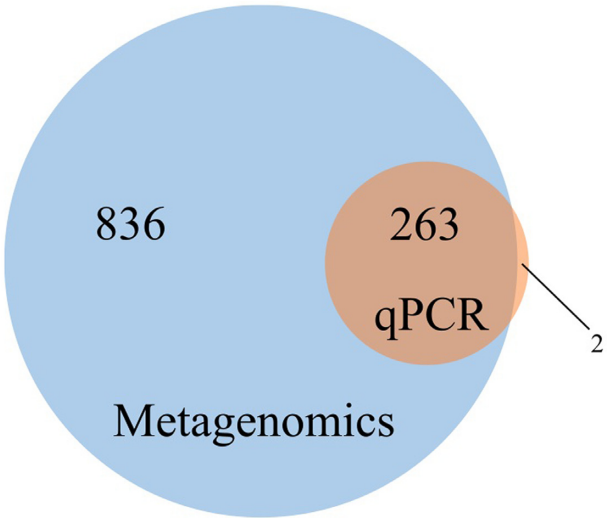

Figure 2. Venn diagram of the number of antibiotic genes that are detected in all faecal and sewage samples using metagenomics and qPCR methods. The numbers represent presence of a gene only and do not indicate the abundance of such genes in the sample pool.

in parallel with metagenomics sequencing and qPCR to quantify the relative abundance of genes encoding for antibiotic resistance and the bacterial biomarker 16S rRNA gene.

Quantitative qPCR and metagenomics

For the comparative qPCR analysis, the genes 16S rRNA and bla_{CTX}, bla_{TEM}, bla_{OXA-1}, bla_{SHV}, bla_{OXA-48}, bla_{OXA-58}, bla_{KPC}, bla_{VIM}, *aadA1*, *qnrS*, *sul1*, *sul2*, and *tet*(O) that confer resistance to β-lactams, aminoglycosides, sulfonamides, quinolones, and glycopeptides were selected. The selection was based on the frequent occurrence and high abundance in wastewater samples, where the measurement may inform about wastewater treatment efficiency or impacts. The qPCR procedures followed the MIQE and other quality control guidelines (Bustin et al. 2009, Borchardt et al. 2021). Acceptable quantifications followed specific criteria, such as the calibration curves for each gene presented reaction efficiencies that ranged from 90% to 110% and R^2 values >0.99; the authenticity of each amplicon was verified based on the expected melting temperature (single peak); the values quantified in samples were within the range defined in the calibration curve (Rocha et al. 2020). The conditions used for each gene quantification were in accordance to previous studies (Narciso-da-Rocha et al. 2018, Iakovides et al. 2019, Ferreira et al. 2022) and are described in Table S1 (Supporting Information).

For metagenome analyses, DNA libraries were constructed using Nextera XT DNA library preparation kit (Illumina) according to the manufacturer's instructions. The libraries were subjected to sequencing using Illumina NextSeq sequencing platform. The raw sequence reads were quality checked (FastQC v.0.11.15 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and trimmed (BBduk2, Bushnell 2014). Read assignments to reference databases was done using KMA 1.2.21 (Clausen et al. 2018). ResFinder database (2020–08-28) and SILVA 16S rRNA database were used to assign antimicrobial resistance gene and bacterial taxa, respectively, in the metagenomic samples. All abundance values from the metagenomic sequencing were based on FPKM (fragments per kilobase of exon per million). Those were calculated by accounting for differences in sequencing depth and the size of the antibiotic resistance genes by dividing the number of reads mapped to a gene by the length of the gene in kilobases (Trapnell et al. 2010). Rarefaction analyses to estimate bacterial taxa and antibiotic resistance gene richness and correlate them to sequence depth were also done. These analyses indicated that all samples were sequenced deep enough to capture an even number of bacteria and antibiotic resistance genes, without influencing the output due to variations in sequence depth (Figure S1, Supporting Information).

Matching qPCR and metagenomics outputs

To compare both method outputs, the metagenomes were screened for the sequences that were amplified by the qPCR primers using Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Only sequences showing no mismatches (100% identity) with the forward and reverse primer of a specific gene were considered for the comparison of the qPCR and metagenomics results. The qPCR quantifications were expressed as relative abundance of gene copy number/16S rRNA gene copy number aiming at comparing with the corresponding results obtained based on metagenomics analysis, expressed as the number of reads of specific gene in relation to the total number of reads in the same run assigned to the 16S rRNA gene sequence.

Predicted values for stepwise dilutions of pig and chicken

The preparation of stepwise dilutions of pig and chicken DNA extracts aimed to assess the sensitivity of both methods. Sensitivity was assessed based on the comparison of predicted and observed values for the different proportions of pig and chicken faecal DNA, and whether the gradient stepwise dilutions between pig and chicken microbiomes can be proportionally observed in antibiotic resistance prediction using metagenomics sequencing or qPCR. Predicted quantifications were estimated based on values obtained for pig and chicken DNA, corresponding to the mixtures 0:1 (entirely chicken faeces) and 1:0 (entirely pig faeces), based on qPCR or metagenomics, and further increase or decrease to 1:10, 1:100, 1:1000, 1:1, 10:1, 100:1, and 1000:1.

Results and discussion

Metagenomics: overview of the bacterial community and resistome composition

Water and animal faecal samples were selected for this comparative study because are expected to have different bacterial community and resistome composition. The metagenomics analysis of the bacterial communities revealed that although the same phyla could be found in water and animal faecal material, the structure was different, and it was possible to infer the sample type based on bacterial community composition. A total of 18 bacterial phyla were found in the water and faecal samples, each of which accounted for more than 1000 reads per phylum. The remainder of the bacterial phyla had low abundance values (less than 1000 reads). While the phyla *Bacteroidota* and *Bacillota* predominated in animal faecal samples (~90% relative abundance), *Pseudomonadota* predominated in water samples (>60% relative abundance) (Fig. 1A). Surface water samples (river) harboured a higher relative abundance of *Actinomycetota* (3%–16%) compared to wastewater (<2%). These results reveal a distinct bacterial community composition in animal faecal and water samples that, as expected, would yield a distinct pool of antibiotic resistance genes (resistome) (Fig. 1B). Accordingly, the metagenomic analysis showed a different resistome in water and in animal faecal

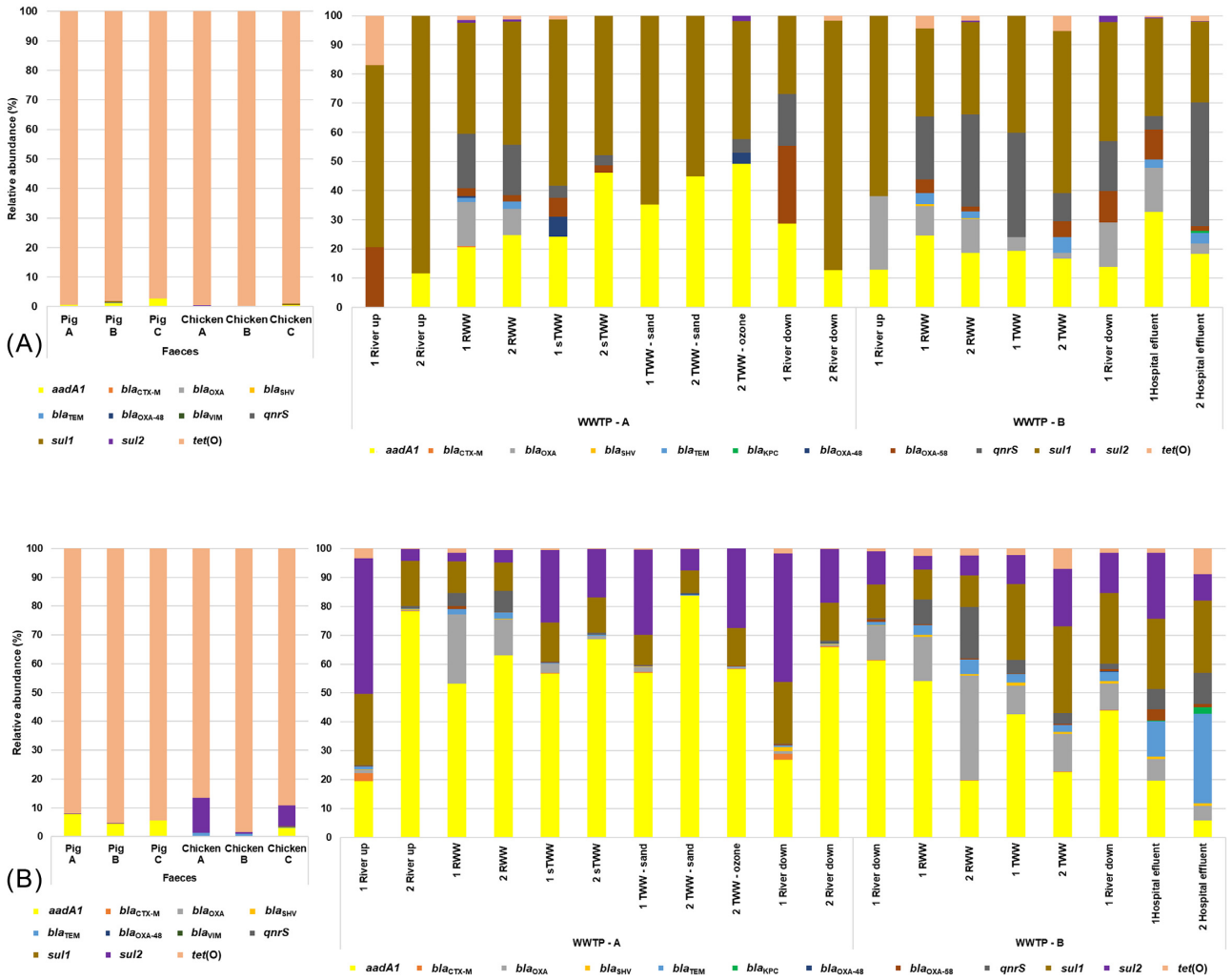

Figure 3. Relative abundance of antibiotic resistance genes measured based on metagenomes screening (gene number of reads/sum number of reads of all genes) and with *in silico* detection with primers used in qPCR assay (A) and qPCR (gene copy number/sum all gene copy number) (B). Pig and chicken faecal material were collected from three animals from the same farm (A, B, and C). Water samples were collected in two sampling campaigns (1 and 2) from two WWTPs (A and B), of raw (RWW) and treated (TWW) wastewater at different treatment stages, the corresponding receiving river (upstream and downstream the discharge point) and hospital effluent that enters the WWTP-B.

samples. Although pig and chicken presented distinct profiles, animal faeces were dominated by genes conferring resistance to tetracycline, macrolide–lincosamide–streptogramin B, that accounted to ~80%, ranging 63%–74% and 10%–17% of each, respectively. These gene categories presented relative abundance values below 1% in water samples, where β-lactam and aminoglycoside resistance genes, ranging 37%–61% and 28%–68%, respectively, accounted for more than 90% of the detected antibiotic resistance genes (Fig. 1B). The β-lactam resistance genes presented relative abundance values ranging 8%–9% in pig, and of 0.1%–0.8% in chicken, being the resistance class that most differentiated both types of animal resistome. However, this distinction may vary with the origin of the animals, as Munk et al. (2018) observed some poultry samples that yielded relative abundance values of β-lactam resistance genes identical to those reported in pigs. The contrasts between the resistome of water and animal samples suggest that the bacterial community is a major factor determining the resistome in a specific environment. The association between resistome and bacterial community was demonstrated by Li et al. (2018), who analyzed 656 metagenomic datasets of different types of environment (human/animal gut, wastewater, ocean, and soil) and with a wide geographic representation in Asia, Europe, and North- and South-America). The authors concluded that the abundance of antibiotic resistance genes was significantly correlated ($P < .001$, based on 9999 permutations) with community structure at phylum level (Li et al. 2018).

Detection and measurement of antibiotic resistance genes: metagenomics vs. QPCR-based

A major goal of this study was to assess and compare metagenomics and qPCR sensitivity to quantify antibiotic resistance genes. A group of 14 genes (11 in animal faecal samples and 12 water samples, nine of which were tested in both type of sample) was monitored based on qPCR. With metagenomics, it was possible to identify 1099 antibiotic resistance genes (Fig. 2), including gene alleles. Therefore, the comparison of sensitivity of both methods relied on the evaluation of relative abundance values obtained for the genes that were quantified with both methods. Those quantifications were normalized by the 16S rRNA gene abundance in the case of qPCR or the total number of reads that were assigned to 16S rRNA gene in the case of metagenomics. Both methods showed dissimilarities between water and animal faeces

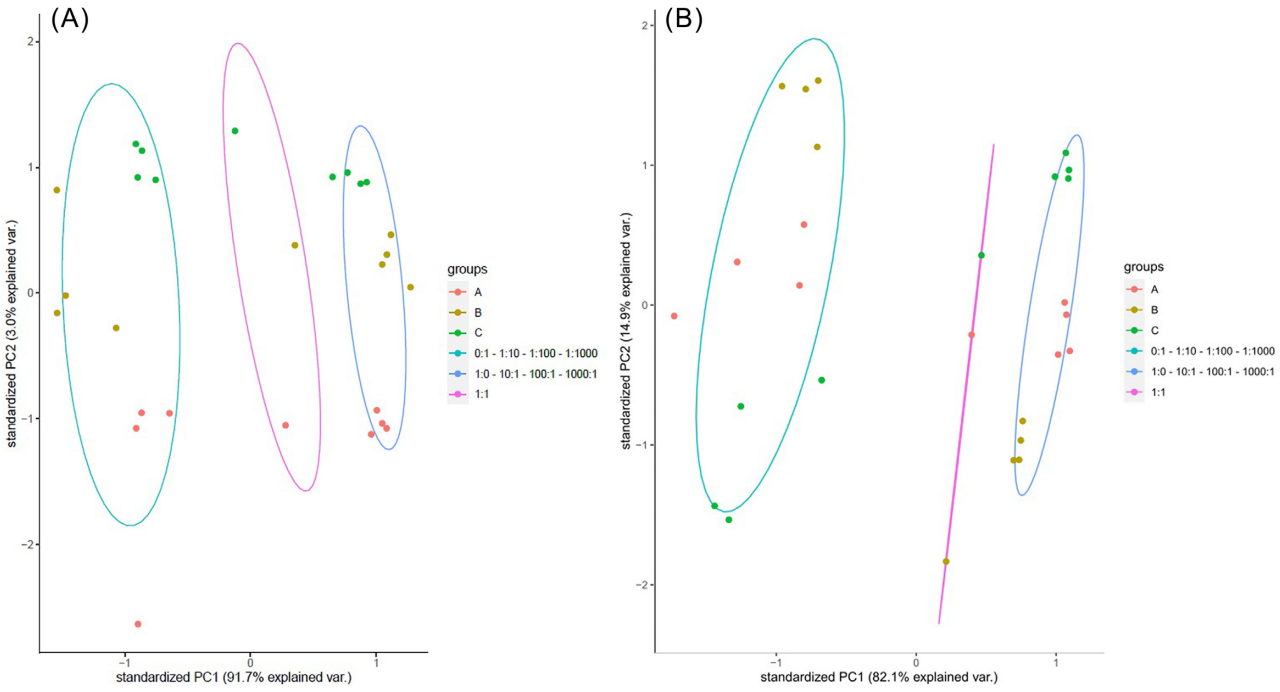

Figure 4. Antibiotic resistance gene profile dissimilarity analysis of three pig: chicken faecal mixtures (A: red dots, B: brown dots, and C: green dots). (A) Metagenomics analysis and (B) qPCR analysis. Each mixture is a series of nine stepwise proportions of pig:chicken faeces organized in distinct groups (0:1–1:1000–1:100–1:10, cyan-blue; 1:1, pink; 1000:1–100:1–10:1–1:0, blue). Ordination matrix is visualized using principal components analysis—PCA based on Bray–Curtis distances, and antibiotic resistance gene measurements were recorded with (A) metagenomics and (B) qPCR method.

antibiotic resistance gene composition and structure. However, in both types of samples, genes suggested to be abundant according to qPCR analysis, such as those encoding resistance to aminoglycosides (*aadA1*), β-lactams (bla_{TEM}), sulfonamides (*sul1*, *sul2*), or tetracycline (*tet*(O)) were not detected or presented lower relative abundance based on metagenomics analyses. While the qPCR method permitted the detection of the 12 genes screened in water samples, with bla_{KPC} being measured only in five of the 19 samples, the genes with the lowest abundance values were frequently not detected based on the metagenomics analysis. For instance, the genes bla_{CTX}, bla_{SHV}, bla_{TEM}, and *tet*(O), detected in the 19 water samples by qPCR, were only detected in one, three, five, and seven samples, respectively, with the metagenomics analysis. The most divergent result was, however, observed for *sul2* gene, which was among the most abundant based on qPCR analysis and detected in only five of the 19 samples with the metagenomics analysis (Fig. 3).

Sensitivity: metagenomics vs. QPCR

A major goal of this study was to evaluate and compare the sensitivity of metagenomics and conventional qPCR in detecting antibiotic resistance genes. The approach used to assess sensitivity was the analysis of mixed samples that contained different proportions of DNA extracted from chicken or from pig faeces, and the results were expressed for each gene normalized by the 16S rRNA gene abundance in the case of qPCR or the total number of reads that were mapped to SILVA 16S rRNA gene in the case of metagenomics. Both methods managed to detect the gradual changes in the resistome genes, following the variable proportions of pig and chicken faecal DNA (0:1, 1:10, 1:100, 1:1000 and 1:0, 10:1, 100:1, 1000:1 and 1:1, pig:chicken). The gradient dilution of both microbiomes might be mirrored on the antibiotic resistance gene profiles (Fig. 4). The ability of both methods to detect those minor variations, due to the experimental gradient mixing of pig and chicken microbiomes, was also apparent with the dissimilarity analyses carried out on the antibiotic genes using Bray–Curtis dissimilarity matrix in our ordination analyses (principal components analysis—PCA) (Fig. 4). The samples that contained larger proportion of chicken faeces (1:10, 1:100, and 1:1000) were more similar to each other, than to the sample that was entirely chicken faeces (0:1). Also, those samples harboured more similar antibiotic resistance gene profiles collectively, compared to the samples that contained larger proportions of pig faeces (1:0, 10:1, 100:1, and 1000:1). Finally, the samples that had equal portions of pig and chicken faces (1:1) were closely related, as they harboured identical proportions of similar antibiotic resistance genes from pigs and chicken, yet different from the samples that were biased towards one microbiome than the other (Fig. 4). These observations suggest that both methods were able to detect the minor changes in the antibiotic resistance genes in complex communities such as pig and chicken faeces with gradient and stepwise changes. However, qPCR quantifications were more accurate at recording the stepwise gradual changes in the resistomes of mixtures of the pig and chicken microbiomes. This conclusion is based on the fact that the observed results showed more similarities to the calculated predicted measurements separated farther in the PCA space (Figs 4 and 5). Also, clinically relevant antibiotic resistance genes present in raw and treated wastewater and in surface water were only detected and quantified based on the targeted method (qPCR), suggesting the suitableness of this method to monitor water quality and safety.

Conclusions

When comparing qPCR and metagenomics high throughput sequence analyses to detect and quantify bacterial communities

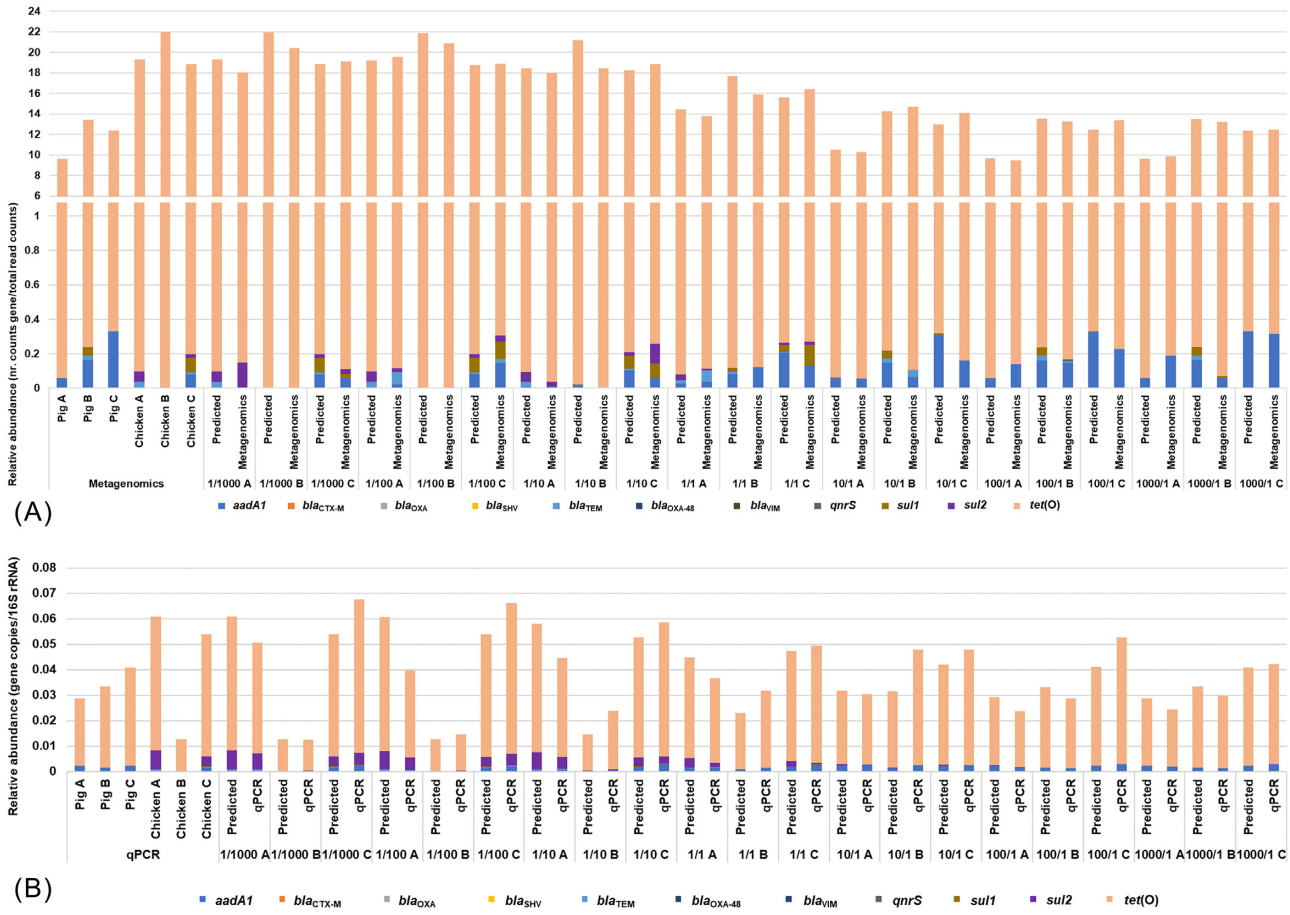

Figure 5. Relative abundance of antibiotic resistance genes in stepwise mixtures of pig and chicken faeces obtained by (A) metagenomics and (B) qPCR. Predicted bars refer to the assumed and calculated proportions of antibiotic resistance genes based on the metagenomics analysis from only pig and only chicken faecal microbiomes (the first six bars—without mixing).

and antimicrobial resistance genes, both methods supported the variations of antibiotic resistance gene profiles in the different types of samples. Quantifications of selected antibiotic resistance genes in river and in WWTPs after different treatment stages gave a quantitative value with qPCR and were nondetected by metagenomics analyses. Metagenomics sequencing of the microbiomes showed a higher coverage of the antibiotic resistance gene detection, while the qPCR showed higher sensitivity to measure the minor changes in such communities. While metagenomics is adequate for nontargeted surveys, qPCR can be customized for a reduced number of genes, representing a suitable approach for specific monitoring schemes. For the quality and safety assessment or to measure wastewater treatment efficiency by field operators, qPCR can be advantageous, as it can be readily interpreted based on a value, rather than on a complex pattern as is provided by metagenomics.

Author contributions

Catarina Ferreira (Methodology, Formal analysis, Data curation, Writing—original draft, Writing—review & editing), Saria Otani (Methodology, Formal analysis, Data curation, Writing—original draft, Writing—review & editing), Frank Møller Aarestrup (Conceptualization, Methodology, Resources, Writing—original draft, Writing—review & editing, Supervision, Funding acquisition), and Célia M. Manaia (Conceptualization, Methodology, Resources, Writing—original draft, Writing—review & editing, Supervision, Funding acquisition)

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Supplementary data

Supplementary data is available at FEMSMC online.

Conflict of interest . None declared.

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Data availability

The raw sequencing data (FASTQ) generated in this study have been deposited in the European Nucleotide Archive and can be accessed without restrictions. The data has the following study accession number: PRJEB59119. All exact sample ID's, experiment, and run accessions are under the study accession number. Source data are also provided with this paper.

This study utilized the publicly available database of ResFinder for AMR gene detection and annotation.

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