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Antimicrobial resistance genes *aph(3′)-III*, *erm*(B), *sul2* and *tet*(W) abundance in animal faeces, meat, production environments and human faeces in Europe

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Background: Real-time quantitative PCR (qPCR) is an affordable method to quantify antimicrobial resistance gene (ARG) targets, allowing comparisons of ARG abundance along animal production chains.

Objectives: We present a comparison of ARG abundance across various animal species, production environments and humans in Europe. AMR variation sources were quantified. The correlation of ARG abundance between qPCR data and previously published metagenomic data was assessed.

Methods: A cross-sectional study was conducted in nine European countries, comprising 9572 samples. qPCR was used to quantify abundance of ARGs [*aph(3′)-III*, *erm*(B), *sul2*, *tet*(W)] and 16S rRNA. Variance component analysis was conducted to explore AMR variation sources. Spearman's rank correlation of ARG abundance values was evaluated between pooled qPCR data and earlier published pooled metagenomic data.

Results: ARG abundance varied strongly among animal species, environments and humans. This variation was dominated by between-farm variation (pigs) or within-farm variation (broilers, veal calves and turkeys). A decrease in ARG abundance along pig and broiler production chains ('farm to fork') was observed. ARG abundance was higher in farmers than in slaughterhouse workers, and lowest in control subjects. ARG abundance showed a high correlation (Spearman's $\rho > 0.7$) between qPCR data and metagenomic data of pooled samples.

Conclusions: qPCR analysis is a valuable tool to assess ARG abundance in a large collection of livestock-associated samples. The between-country and between-farm variation of ARG abundance could partially be explained by antimicrobial use and farm biosecurity levels. ARG abundance in human faeces was related to livestock antimicrobial resistance exposure.

Introduction

Antimicrobial resistance (AMR) poses a threat not only to humans but also to animals worldwide.[1] High antimicrobial use (AMU) leads to the selection of resistant bacteria, which limits therapeutic options in animals and humans.

A variety of methods have been carried out to quantify AMR levels in faeces, such as conventional testing of phenotypes of antimicrobial susceptibility of selected organisms.[2,3] Besides, next-generation sequencing (NGS) is emerging as a new method to detect genetic determinants conferring AMR in selected isolates or metagenomically in DNA of bacterial communities.[4–6] However, due to the high costs and technological constraints of NGS methods, the number of samples per study is often limited, or animal samples are pooled together (e.g. at farm or herd level).[4] Consequently, in pooled samples, within-farm variation of antimicrobial resistance gene (ARG) abundance cannot be determined. Compared with NGS, real-time quantitative PCR (qPCR) is an affordable and widely applied method that can also provide precise quantification of certain ARG targets,[4] allowing comparisons of ARG abundance between and within sampling sites.

Direct and indirect exposure of humans to livestock, companion animals or animal products are known risk factors for AMR acquisition.[7–9] Intensity and frequency of contact with animals have been shown to represent risk factors for carrying resistant strains such as livestock-associated MRSA (LA-MRSA) or ESBL-producing bacteria.[10,11] Although the correlation of qPCR-quantified ARG abundance between animals and humans has been studied,[12–14] the insights are limited by geographical distribution or sample type variation.

As part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, we used qPCR to quantify four frequently occurring ARGs [*aph(3′)-III*, *erm*(B), *sul2* and *tet*(W)] in a total of 9572 samples collected in nine European countries. We analysed samples from animal faeces, meat, production environments and human faeces. The objectives of the current study were: (1) to describe ARG abundance among different sample types and countries; (2) to quantify AMR variation sources (between countries, between and within farms, and determinants) based on variance component analysis (VCA); and (3) to determine the correlation between ARG abundance quantified by qPCR and metagenomics.

Materials and methods

Study population and sampling procedure

Between 2014 and 2017, we collected a large set of samples (9572 samples) from various sources. The sampling procedures have been partially described before.[5,6,13,15–23] Faecal samples were taken from farm animals [pigs,[5,13,23] broilers,[6,23] veal calves,[15] turkeys,[16] fish (intestines were collected)], companion animals (cats, dogs)[17] and wild boars. Carcass samples were taken from pigs[13] and broilers at slaughterhouses. Raw meat (pork,[13] chicken, turkey, veal and trout) samples were purchased at food stores. We also collected environmental samples [electrostatic dustfall collector (EDC)[19,20] and gloves of slaughterhouse workers[13]] and human faeces (humans occupationally exposed to pigs or broilers at Dutch and German farms or slaughterhouses, and control subjects from the Dutch 'Lifelines' cohort).[13,18,21,22] Animal samples were collected from nine European countries (Belgium, Bulgaria, Germany, Denmark, Spain, France, Italy, the Netherlands and Poland). More details about faecal sampling are described in the Supplementary methods, available as Supplementary data at *JAC* Online (*Sampling procedure of faeces*).

Before DNA extraction, animal faecal samples were stored at 4°C, transported to the laboratory within 24 h and stored at −80°C.[4–6,15,16,23] Exposed EDC cloths were put into a small resealable bag with sterile tweezers and frozen at −80°C.[19,20] Carcass, meat and glove samples were collected in a stomacher bag (INTERSCIENCE, 400 mL, UK), transported and stored at 4°C, followed by further preparations.[13] Human faecal samples were refrigerated directly after collection, transported and stored on dry ice.[13,18,21,22]

ARG target selection

Within the EFFORT project, the ARG target selection was based on several criteria, including: (1) sufficient abundance to be measured in >25% of the samples estimated from the prevalence and relative abundance of ARG targets in previous metagenomic analyses of pig and broiler faeces, in order to enable large-scale statistical analyses of risk factors such as AMU;[4] (2) the inclusion of targets of unrelated antimicrobial classes; (3) limited correlation of the chosen gene concentrations to avoid redundancy; and (4) a PCR protocol should either exist or be achievable. More details about the selection process are described in the Supplementary methods (*ARG targets selection process*). Although genes of particular clinical relevance (such as genes encoding resistance mechanisms in WHO priority-resistant pathogens, i.e. ESBL, carbapenemase and/or vancomycin resistance genes) were initially prioritized, they were estimated to be detectable only in <10% of samples from at least one species (based on the strength of the respective metagenomic signal). These genes were deselected as such low prevalence would limit the power of the planned statistical analyses. The resulting genes represent resistance to the antimicrobial classes aminoglycosides, macrolides, sulphonamides and tetracyclines, all of which are used in animal practice to a varying extent.

DNA extraction, qPCR and sequencing

DNA of animal and human faeces was extracted using the modified QIAamp Fast DNA Stool Mini Kit (Cat. No. 51604; QIAGEN, The Netherlands) as described before.[13,15,23] DNA of EDC and gloves was respectively extracted using the modified NucleoSpin® 8 Plant II Kit and NucleoSpin® 96 Food Kit (MACHEREY-NAGEL, Germany), while DNA of meat was extracted using the modified NucleoSpin® Food Kit (MACHEREY-NAGEL).[13,20] Following DNA extraction, qPCR was conducted to quantify the abundance of four ARGs [*aph(3′)-III*, *erm*(B), *sul2*, *tet*(W)] along with the 16S rRNA gene as a measure of total bacterial DNA (Table S1). More details on the qPCR process have been described in previous papers[13,15,23] and the Supplementary methods (*qPCR process*). qPCR quality control, comprising a number of elements, is described in the Supplementary methods (*Quality control and quantification of qPCR results*).

ARG copy number per unit of the sample was $\log_{10}$ transformed. Subsequently, relative ARG abundance was calculated using 16S rRNA as a general bacterial molecular marker to normalize the bacterial community size in samples.

DNA of pooled faecal samples from pigs and broilers was extracted at the Technical University of Denmark (DTU) with the same extraction method and shipped on dry ice for shotgun metagenomic sequencing at the Oklahoma Medical Research Foundation (OMRF; Oklahoma City, OK, USA). In total, pooled faecal DNA collected from 181 pig farms and 178 broiler farms were shotgun sequenced on the HiSeq 3000 platform (Illumina), resulting in >18 billion paired-end reads. More details on the subsequent processing of the metagenomic data were described in our previous study.[4–6]

Comparison of ARG abundance across sample types, farms and countries

We compared ARG loads between samples from nine countries, between samples collected along Dutch pig and German broiler production lines, and between different human populations.

Principle component analyses (PCA) were performed to evaluate the similarities and differences in the distributions of relative ARG abundance across sample sources and species. The package *vegan*[24] was used on R version 4.0.3.[25] Data were $\log_{10}$ transformed before PCA due to the right-skewed distribution.[26]

Except for PCA results, all comparisons of ARG abundance in this study were conducted using a classic or Welch's analysis of variance (ANOVA), depending on the variance homogeneity.[27,28] In the case of a significant difference in ANOVA ($P<0.05$), *post hoc* tests [Games–Howell *post hoc* test[29] or Tukey's honest significant difference test (Tukey HSD)[30]] were carried out to test differences between groups. Unless otherwise specified, appropriate *post hoc* test *P* values are reported.

VCA

VCA was conducted per ARG target with a null model (AMR ~ country + farm) to evaluate the between-country, between-farm and within-farm variation of relative ARG abundance in faecal samples of four animal species (pigs, broilers, veal calves and turkeys) using the package *VCA*[31] on R version 4.0.3.[25] In addition, to be consistent with our study in pigs and broilers,[23] we also determined the contribution of farm characteristics (e.g. AMU, biosecurity measures) in veal calves and turkeys to the variance by adjusting these factors into the null model. More details are described in the Supplementary methods (*Variance component analyses in veal calves and turkeys*).

Correlation between pooled qPCR and pooled metagenomic data

As data were not normally distributed, Spearman's rank correlation of relative ARG abundance was evaluated between pooled qPCR data and earlier published pooled metagenomic data.[4] To match the ARG targets of qPCR, all downstream gene abundance values for *aph(3′)-III*, *erm*(B), *sul2* and *tet*(W) were collected from the metagenomic data [fragments per kb reference per million bacterial fragments (FPKM)] and summed per gene target. FPKM was $\log_{10}$ transformed after adding a pseudocount of 1.

Ethics

'Lifelines' research was conducted according to the protocols approved by the Medical Ethics Review Board of the Medical Center Groningen (NL) (Protocol METc2007/152). Written consent was received from all participants. The Medical Ethical Committee of the University Medical Centre Utrecht (NL) confirmed that the Dutch 'Medical Research Involving Human Subjects Act' did not apply for the study of the EFFORT research (Protocols 14–346/C, 14–403/C). Participants in EFFORT were compensated financially (100 Euros per farm family, 25 Euros per slaughterhouse employee).[4,21]

Results

Table 1 describes the 9572 samples analysed by qPCR. Human stool samples were collected in the Netherlands and Germany, including pig and broiler farmworkers and their families ($n=127$), pig and broiler slaughterhouse workers ($n=669$) and a healthy control population ($n=46$) (Table 1 and Table S2). After a quality check involving the limit of detection (LOD) and limit of quantification (LOQ), 7084 (74%) samples had detectable gene levels for *erm*(B) and 6700 (70%) for *tet*(W), while 4892 (80%) samples for *aph(3′)-III* and 4543 (74%) samples for *sul2* could be detected (Table 1). Only a small number of ARG targets (generally less than 10% of the total sample size) were detected in fish faeces.

Comparison of relative ARG abundance across sample types and countries

Among animal faeces, the highest mean relative abundance of *tet*(W) was generally seen in farm animals, especially in pigs (Figure 1). Among all sample types (Figure S1 and Table S3), the mean relative *aph(3′)-III* abundance was highest in broiler slaughterhouse faeces and lowest in wild boar faeces. For *sul2*, the mean relative abundance was lowest in wild boar faeces. The mean relative *sul2* abundance was highest in veal calf faeces, which was significantly higher than all the other sample types ($P<0.01$) (Figure S1 and Table S3). For *erm*(B) and *tet*(W), the mean relative abundance was highest in broiler farm dust samples and lowest in retail pork. The mean relative abundance of *tet*(W) in broiler farm dust samples was significantly higher than *tet*(W) abundance in all the other sample types ($P<0.05$). For all ARGs, cats showed higher mean relative abundance than dogs, but differences were not statistically significant ($P>0.05$) (Figure S1 and Table S3).

Between-country variation of relative ARG abundance in dust samples and in pig and broiler faeces is described elsewhere.[20,23] For other animal species (cat, dog, veal calves), the Netherlands generally showed the lowest relative abundance of all ARGs (Figure S2). For veal calf faecal samples, we observed significantly lower relative abundance of *aph(3′)-III* ($P<0.05$) and *tet*(W) ($P<0.01$) in the Netherlands than in all the other countries (Figure S2).

Relative ARG abundance across the pig and broiler production chain

We found an overall decreasing trend of the mean relative ARG abundances along the production chain of pigs in the Netherlands and broilers in Germany, i.e. from primary production to slaughterhouse to retail meat (Figures 2 and 3). Farm dust showed higher ($P<0.01$) mean relative ARG abundances than faeces recovered from the same farms, except for *tet*(W) in pigs. In addition, we found a significantly higher ($P<0.01$) mean relative *aph(3′)-III* abundance in faeces of pig farmers than in faeces of pig slaughterhouse employees. A significantly higher ($P<0.05$) mean relative *sul2* abundance was observed in faeces of pig slaughterhouse employees than in faeces of control subjects (Figure 2). Meanwhile, faeces from broiler farmers showed a higher mean relative abundance of all ARGs than faeces from broiler slaughterhouse employees, which was significant for *aph(3′)-III* ($P<0.01$) (Figure 3). Furthermore, we found higher mean relative *erm*(B) and *tet*(W) abundance in pig/broiler slaughterhouse carcasses than in slaughterhouse meat and retail meat, which was significant for *erm*(B) ($P<0.01$) (Figures 2 and 3).

Table 1. Overview of all sample types and numbers of samples with detectable qPCR targets

	Final sample country					Final sample number, *n* (%)					Total sample number
Description	*16S*	*aph(3′)-III*	*erm*(B)	*sul2*	*tet*(W)	*16S*	*aph(3′)-III*	*erm*(B)	*sul2*	*tet*(W)	
Pig faeces (farms)	All nine	All nine	All nine	All nine	All nine	1230 (97)	1084 (86)	1259 (100)	1042 (82)	1262 (100)	1265
Broiler faeces (farms)	All nine	All nine	All nine	All nine	All nine	884 (97)	847 (93)	902 (99)	840 (93)	898 (99)	908
EDC (pig farms)	All nine	All nine	All nine	All nine	All nine	462 (92)	431 (86)	477 (95)	436 (87)	475 (94)	503
EDC (broiler farms)	All nine	All nine	All nine	All nine	All nine	501 (90)	430 (77)	530 (95)	341 (61)	526 (94)	559
Pooled pig faeces (farms)	All nine	All nine	All nine	All nine	All nine	165 (92)	168 (94)	176 (98)	168 (94)	175 (98)	179
Pooled broiler faeces (farms)	All nine	All nine	All nine	All nine	All nine	169 (95)	170 (96)	177 (99)	170 (96)	176 (99)	178
Retail pork	All nine	—	All nine	—	BG, DK, ES, PL	848 (98)	—	140 (16)	—	8 (1)	862
Retail chicken	All nine	—	All nine	—	All nine	875 (99)	—	592 (67)	—	383 (43)	882
Turkey faeces (farms)	DE, ES, FR	DE, ES, FR	DE, ES, FR	DE, ES, FR	DE, ES, FR	290 (95)	263 (87)	299 (98)	264 (87)	301 (99)	304
Veal calf faeces (farms)	DE, FR, NL	DE, FR, NL	DE, FR, NL	DE, FR, NL	DE, FR, NL	405 (96)	339 (81)	415 (99)	387 (92)	420 (100)	420
Fish faeces (farms)	ES, FR, PL	FR, PL	ES, FR, PL	ES, FR, PL	ES, PL	270 (77)	5 (1)	27 (8)	140 (40)	6 (2)	352
Wild boar faeces	IT, PL	IT, PL	IT, PL	IT, PL	IT, PL	156 (78)	43 (22)	83 (42)	30 (15)	197 (98)	200
Cat faeces	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	141 (95)	117 (79)	121 (81)	43 (29)	148 (99)	149
Dog faeces	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	BE, IT, NL	148 (99)	104 (69)	92 (61)	82 (55)	146 (97)	150
Retail turkey	DE, ES, FR	—	DE, ES, FR	—	DE, ES, FR	143 (99)	—	112 (77)	—	59 (41)	145
Retail veal	DE, ES, FR	—	DE, ES, FR	—	ES	130 (100)	—	38 (29)	—	2 (2)	130
Retail fish	FR, NL, PL	—	FP, PL	—	FR	122 (99)	—	6 (5)	—	1 (1)	123
Human faeces (pig farms)	NL, DE	NL, DE	NL, DE	NL, DE	NL	93 (99)	90 (96)	93 (99)	57 (61)	93 (99)	94
Human faeces (poultry farms)	NL, DE	NL, DE	NL, DE	NL, DE	DE	33 (100)	33 (100)	32 (97)	26 (79)	33 (100)	33
Pig faeces (slaughterhouses)	NL	NL	NL	NL	NL	59 (98)	58 (97)	60 (100)	20 (33)	60 (100)	60
Pig carcass (slaughterhouses)	NL	—	NL	—	NL	166 (84)	—	28 (14)	—	21 (11)	198
Gloves (pig slaughterhouses)	NL	—	NL	—	NL	310 (96)	—	178 (55)	—	148 (46)	324
Pig meat (slaughterhouses)	NL	—	.	—	.	190 (96)	—	—	—	—	198
Human faeces (pig slaughterhouses)	NL	NL	NL	NL	NL	479 (99)	441 (91)	480 (99)	296 (61)	480 (99)	483
Human faeces (control)	NL	NL	NL	NL	NL	46 (100)	46 (100)	46 (100)	17 (37)	46 (100)	46
Broiler faeces (slaughterhouses)	DE	DE	DE	DE	DE	60 (97)	47 (76)	62 (100)	55 (89)	62 (100)	62
Broiler carcass (slaughterhouses)	DE	—	DE	—	DE	198 (98)	—	159 (79)	—	146 (72)	202
Gloves (broiler slaughterhouses)	DE	—	DE	—	DE	231 (94)	—	208 (84)	—	190 (77)	247
Broiler meat (slaughterhouses)	DE	—	DE	—	DE	127 (98)	—	109 (84)	—	54 (42)	130
Human faeces (broiler slaughterhouses)	DE	DE	DE	DE	DE	180 (97)	176 (95)	183 (98)	129 (69)	184 (99)	186
Overall	All nine	All nine	All nine	All nine	All nine	9111 (95)	4892 (80)	7084 (74)	4543 (74)	6700 (70)	9572

Final sample number was all samples left after a quality check (technical standard, LOD and LOQ). Missing values (—) were due to lack of information. BE, Belgium; BG, Bulgaria; DE, Germany; DK, Denmark; ES, Spain; FR, France; IT, Italy; NL, the Netherlands; PL, Poland. Some results have been reported or partly reported in previous papers: veal calf faeces (farms);[15] pig faeces (slaughterhouses), pig carcass (slaughterhouses), gloves (pig slaughterhouses), pig meat (slaughterhouses) and human faeces (pig slaughterhouses);[13] turkey faeces (farms);[16] EDC (pig farms), EDC (broiler farms);[20] and pig faeces (farms), broiler faeces (farms).[23]

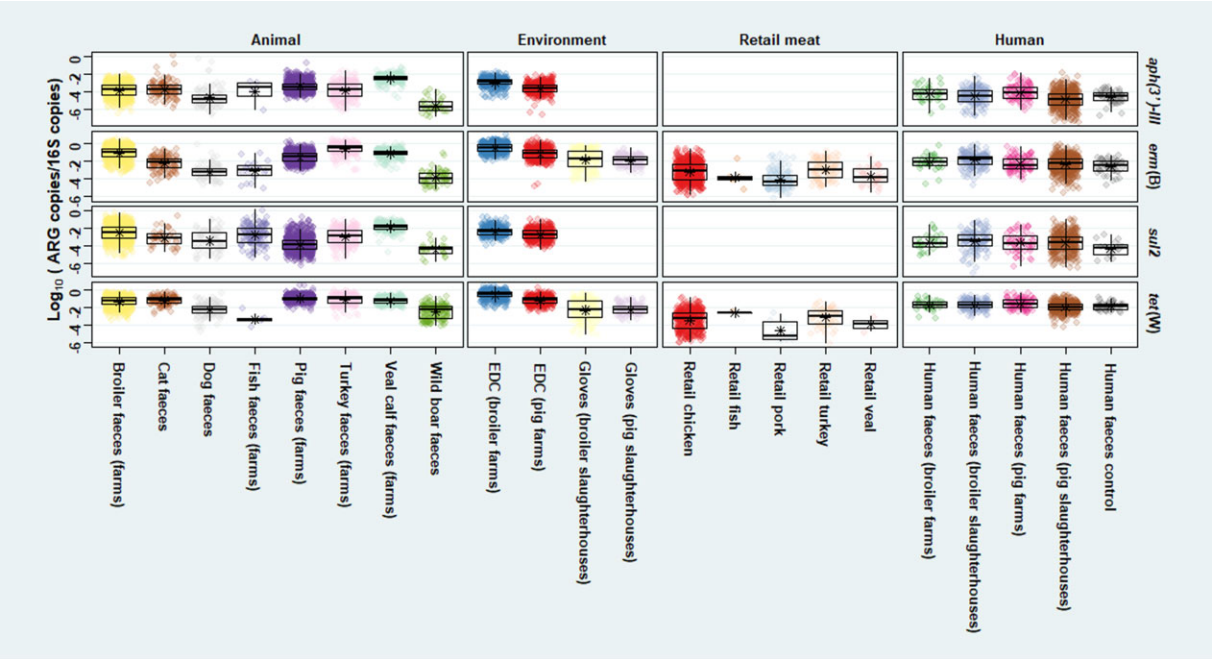

Figure 1. Relative abundance of four targets [*aph(3′)-III*, *erm*(B), *sul2*, *tet*(W)] in all samples. Relative abundance of gene target was calculated by $\log_{10}$ (gene copies/16S copies). Asterisk shows the mean by sample type. Pooled faeces, slaughterhouse pig and broiler faeces, and slaughterhouse carcass samples were not included in this figure.

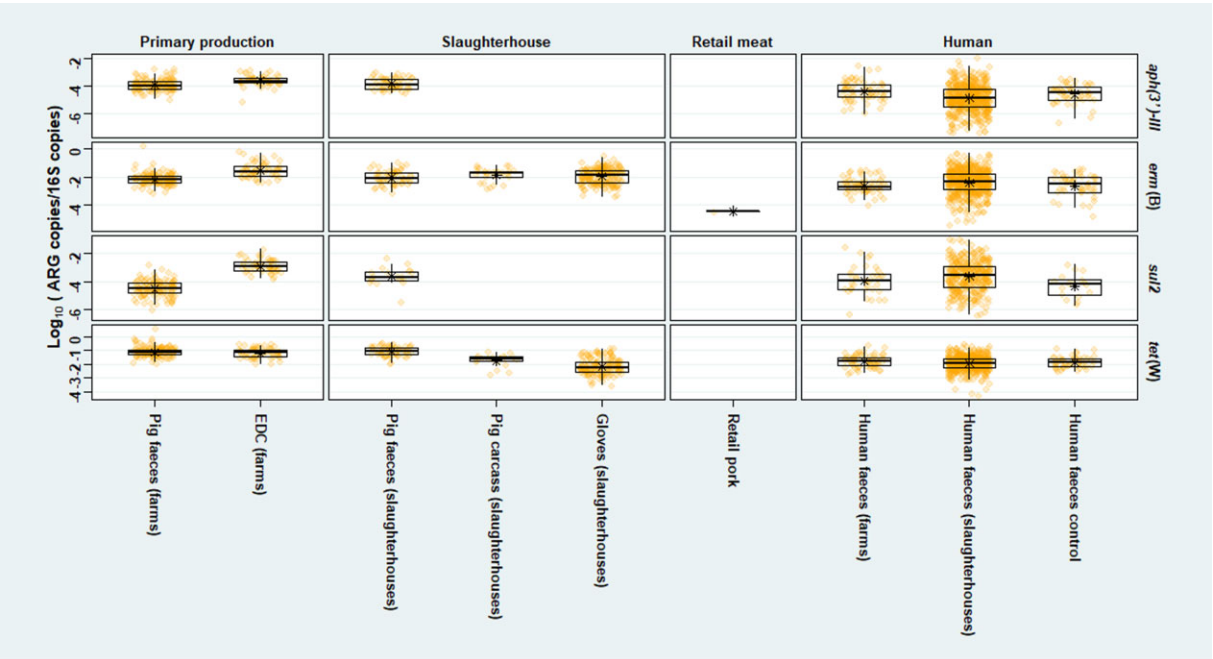

Figure 2. Relative abundance of four targets [*aph(3′)-III*, *erm*(B), *sul2*, *tet*(W)] in samples related to pig production in the Netherlands. Relative abundance of gene target was calculated by $\log_{10}$ (gene copies/16S copies). Only pig farms and slaughterhouses in the Netherlands were involved. Human faeces of control subjects were collected from the 'Lifelines' cohort in the Netherlands.[18] Asterisk shows the mean by sample type in the Netherlands.

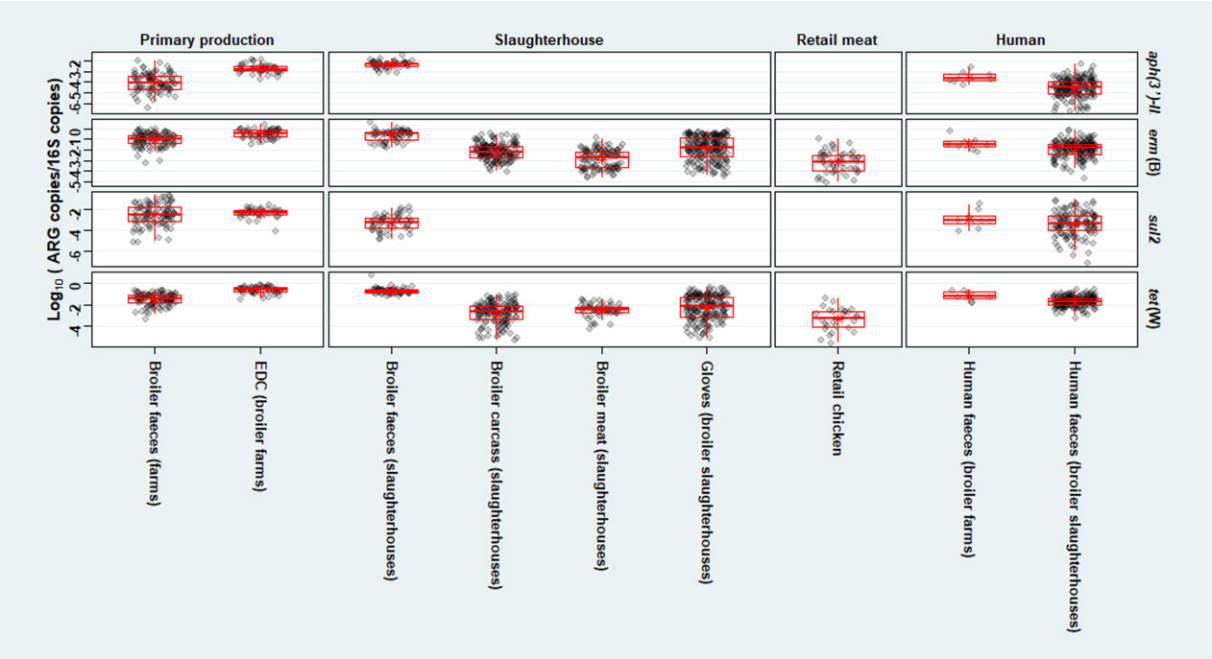

Figure 3. Relative abundance of four targets [*aph(3′)-III*, *erm*(B), *sul2*, *tet*(W)] in samples related to broiler production in Germany. Relative abundance of gene target was calculated by $\log_{10}$ (gene copies/16S copies). Only broiler farms and slaughterhouses in Germany were involved. Asterisk shows the mean by sample type in Germany.

Determination of differences in ARG patterns by animal species

The faecal resistome, here measured as the frequency distributions of faecal ARGs, differed between animal species (Figure 4). A high distribution overlap of ARG abundance was found among farm animals and differed from wild boars and dogs. The results of fish are not shown in Figure 4 as there were too many missing values per gene target. In the production chain of pigs and broilers, faeces and production environment samples showed higher relative ARG abundance than human faeces in both farms and slaughterhouses (Figures S3–S6). ARG distribution overlapped obviously between carcass and gloves and differed from human stool samples (Figure S4 and Figure S6).

VCA of relative ARG abundance

In the null model for pigs, broilers, veal calves and turkeys, we generally found the within-farm variation of AMR was low (absolutely and relatively) in pig faeces but high in faeces of the other three animal species. (Figure 5 and Table S4). In pig faeces, we found high between-country and between-farm variation. In broiler faeces, within-farm variation dominated the relative ARG abundance, while the smallest variation was found between countries (Figure 5 and Table S4).

In the adjusted VCA model for veal calves and turkeys (Tables S5 and S6), we found that between-country and between-farm variation could partly be explained by AMU and biosecurity measures. For example, in the adjusted model for *sul2* in veal calves, the use of trimethoprim and sulphonamide accounted for 6.78% of the total variation, while the contribution of between-country variation decreased from 11.49% to 6.07%. In the adjusted model for *erm*(B) in turkeys, the biosecurity measure 'Visitor access more than once a month' accounted for 13.70% of the total variation, while the contribution of between-farm variation decreased from 46.56% to 30.66%. For pigs and broilers, the adjusted VCA models are described elsewhere.[23]

Comparison of ARG abundance between qPCR data and metagenomic data

In total, 4, 15, 19 and 5 gene variants in the metagenomic data were allocated to *aph(3′)-III*, *erm*(B), *sul2* and *tet*(W), respectively. For pigs and broilers, we found a high correlation ($\rho > 0.7$; $P < 0.01$) between qPCR data and metagenomic data of pooled faecal samples for the four ARG targets, except for *tet*(W) ($\rho < 0.7$; $P < 0.01$) in pigs (Figure 6).

Discussion

We applied qPCR on a large scale to explore the association between ARG abundance from different sources, animal species and humans in nine European countries in more than 9500 samples. A significant decrease in relative ARG abundance was observed along both pig and broiler production chains. In addition, the between-country and between-farm variation in pigs, broilers, veal calves and turkeys could partially be explained by AMU and biosecurity levels. Furthermore, a high correlation between qPCR and metagenomically assessed ARGs in pooled faecal samples was found in pigs and broilers.

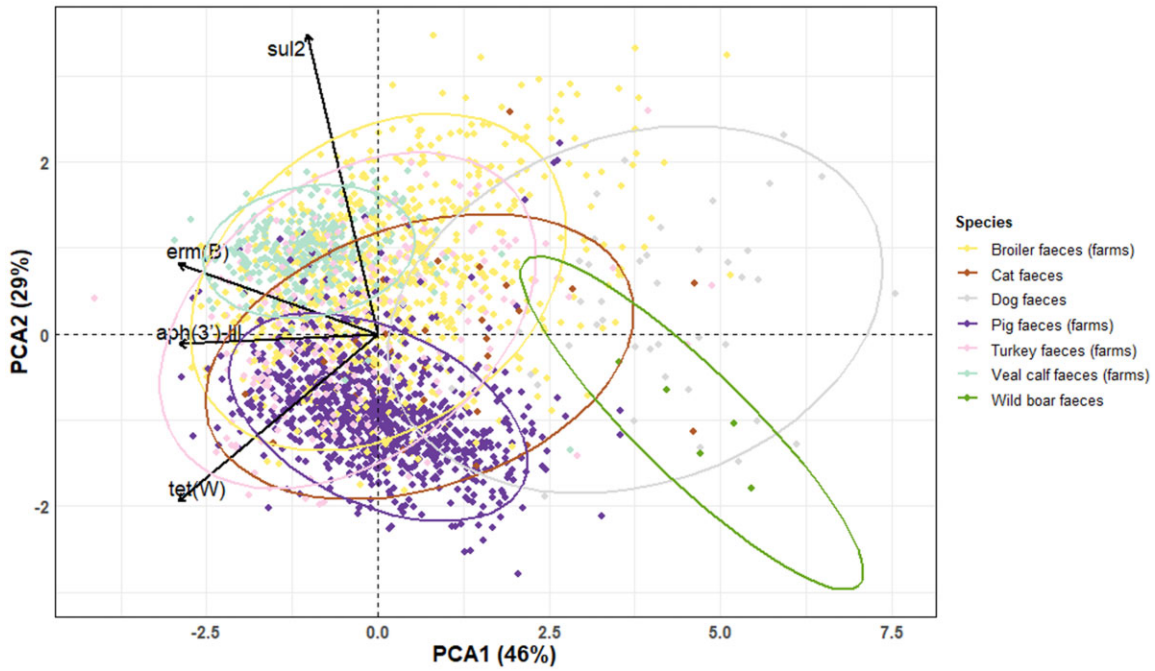

Figure 4. PCA biplot of relative ARG abundance in animal faecal samples. ARG targets: *aph(3′)-III*, *erm*(B), *sul2*, *tet*(W). Relative abundance of gene target was calculated by $\log_{10}$ (gene copies/16S copies). Symmetric scaling was used. Circles indicate 95% confidence ellipses that were computed with the assumption of multivariate normal distribution of the data.

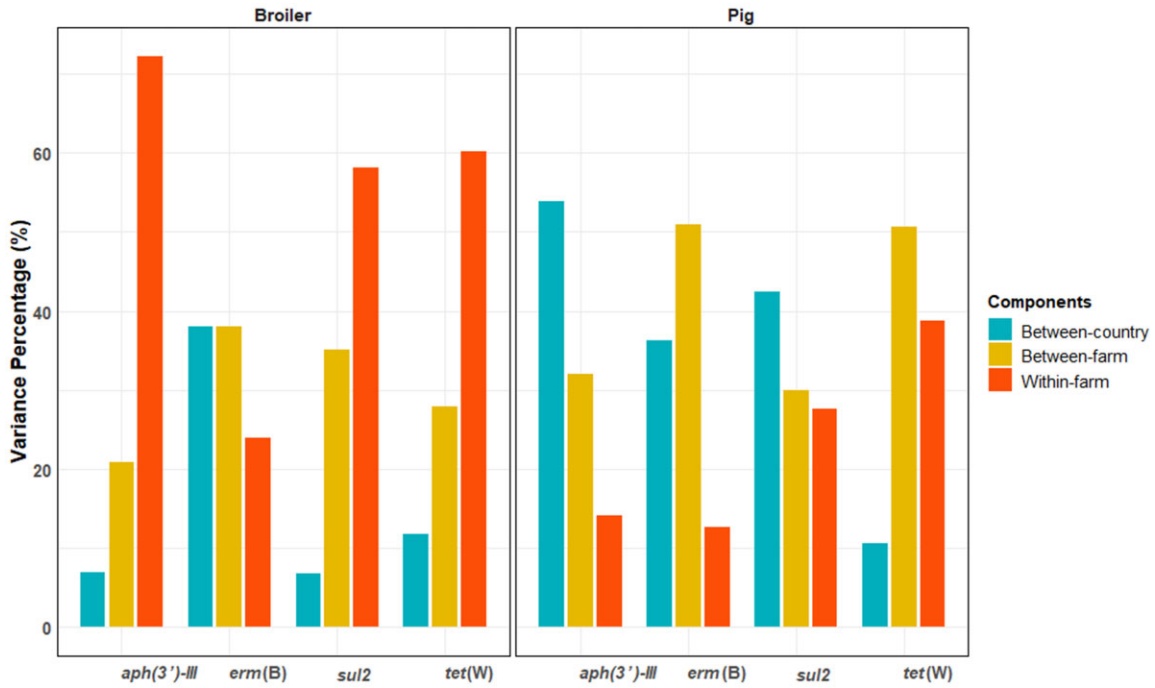

Figure 5. Variance component percentages of relative ARG abundance in faecal samples from pigs and broilers. Relative abundance of gene target was calculated by $\log_{10}$ (gene copies/16S copies). Variance percentages of three components (between-country variance, between-farm variance and within-farm variance) were calculated using VCA.

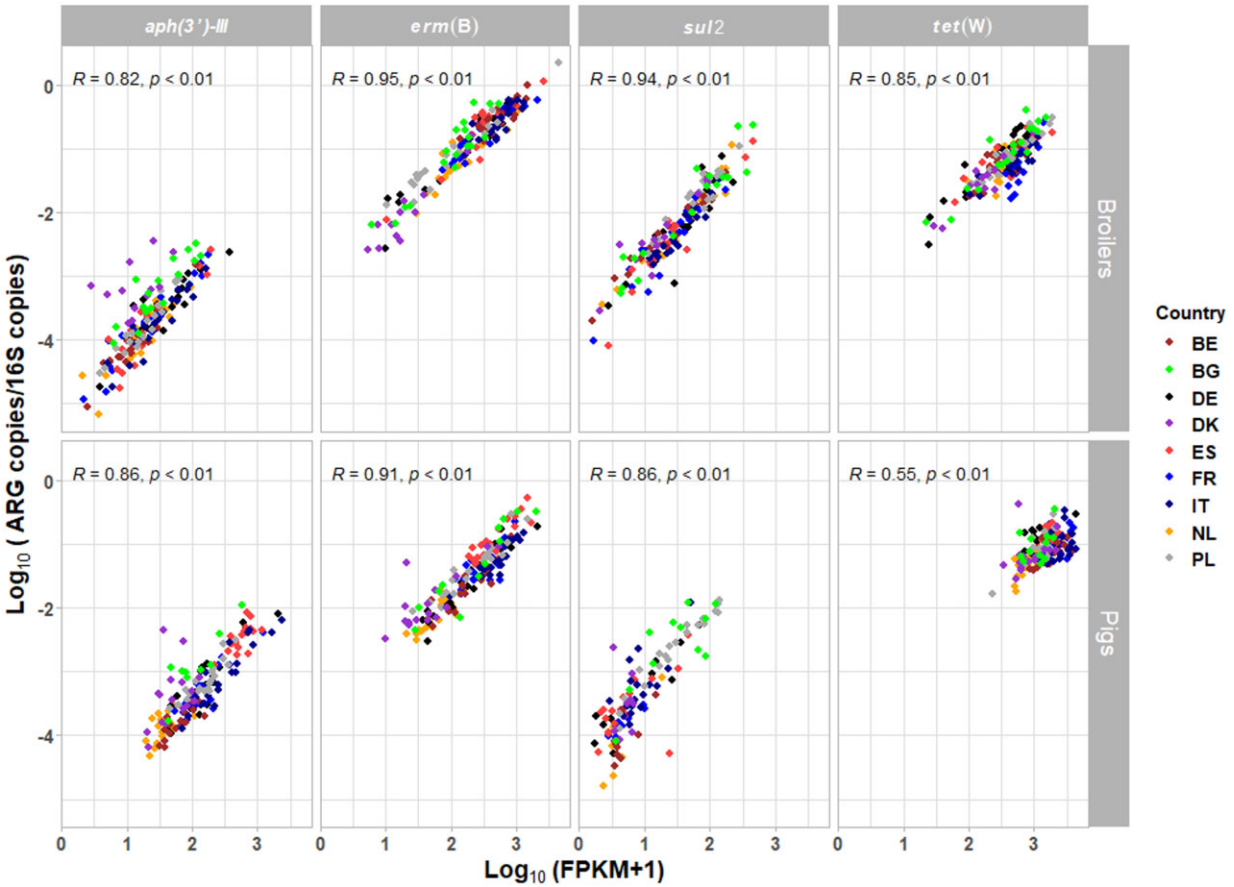

Figure 6. Spearman's rank correlation of relative ARG abundance and ARG FPKM abundance in pooled faecal samples from pig and broiler farms. ARG targets: *aph(3')-III*, *erm*(B), *sul2*, *tet*(W). Relative abundance of gene target was calculated by $\log_{10}$ (gene copies/16S copies). FPKM was $\log_{10}$ transformed after adding a pseudocount of 1.[4] BE, Belgium; BG, Bulgaria; DE, Germany; DK, Denmark; ES, Spain; FR, France; IT, Italy; NL, the Netherlands; PL, Poland.

Comparison of ARG abundance among animal species

We speculate that the observed variation of ARGs across animals is partly a result of different AMU exposure. For example, the highest mean relative *tet*(W) abundance was observed in farm animals, especially in pigs. This could be explained by previous findings in EFFORT,[32,33] in which a higher proportion of tetracycline use was found in pig farms (15.3%)[33] than in broiler farms (11%)[32] among nine countries. Still, as there is a strong association between faecal microbiome and resistome,[4,19,21] the difference in ARG abundance among species can also be related to differences in microbiome compositions. In fish samples, we only detected a low concentration of ARGs. This may be due to the fact that fish faecal samples in our study were collected from frozen guts, which made it difficult to separate fish intestines and faecal contents, leading to a high proportion of host DNA. As a result, AMR levels of fish faeces may have been underestimated. In the future, more appropriate approaches for collecting fish faecal samples are worthy of studying.

Wild boars showed the lowest mean ARG abundance among all animal species, which is likely the result of negligible antimicrobial exposure of wild animals.[34,35] This is consistent with previous results of European wild-animal AMR studies.[36–38] In companion animals, we saw higher relative ARG abundance in cat faeces than in dog faeces. Since the previous EFFORT study showed no AMU–AMR association in companion animals,[17] this higher ARG abundance in cat faeces may be related to differences in the gut microbiota, or may be due to the fact that cats roam more freely than dogs,[39] through which cats are probably exposed to more environmental sources than dogs.

ARG abundance declines along the pig and broiler production chain

In pig and broiler production chains, a decline in relative ARG abundance was seen from primary production to slaughterhouse and retail meat. The main explanation is that livestock gut bacteria are enriched in AMR genes through AMU, but also through exposure to AMR through animal faeces and dust in farms. We found higher relative ARG abundance in farm dust than in farm animal faeces in pig and broiler farms, which is consistent with previous findings using metagenomic data.[19] The explanation may be that microorganisms in dust sources such as animal

faeces[19,20] and constituents (skin, feather)[40–42] are richer in ARGs. The relative ARG abundance in retail pork and chicken meat was found to be significantly lower than that of carcasses and meat samples in pig and broiler slaughterhouses. One potential explanation is that the production steps along the slaughterhouse line, including the cooling process, reduce bacterial loads and related AMR.[13,43] Additionally, location of ARGs in bacterial hosts that are unable to survive the production process may lead to a reduction in AMR levels from farm to fork. More research is needed in the future to link these changes to specific production steps.

Sources of AMR variation in pigs, broilers, veal calves and turkeys

For pigs and broilers, substantial between-country variation in ARG levels was previously reported in faecal samples.[4–6,23] For veal calves and turkeys, we found high within-farm variation. Considering the large variation in individual characteristics,[44,45] it is not surprising that the AMR abundance varies highly among animals per farm. The other explanation is the limited sample size [sample numbers per farm (7/5) and country numbers (3)] in veal calves and turkeys. The differences in farm management practices may also be associated with the distribution of AMR variation.

In our previous adjusted VCA models of pigs and broilers,[23] we observed that among farm characteristics, AMU contributed most to total AMR variation. In the present study on veal calves and turkeys, we found that in addition to AMU, farm biosecurity measures also played an important role in AMR variation. Although the small sample size of veal calves and turkeys reduced statistical power in our study, our results provide evidence that AMU and farm biosecurity contributed to both variation sources (country and farm).

Correlation of ARG abundance between qPCR data and metagenomic data

To the best of our knowledge, this study is the first to compare ARG abundance using qPCR and metagenomics in the same animal samples in parallel. In this context, we found a high correlation between pooled metagenomic data[46] and pooled qPCR data. This indicates that qPCR with pooled data can be an alternative cost-effective approach for the quantitative analysis of ARG targets when a project budget is limited.

ARG abundance in humans

Previous studies reported that farmers had higher nasal MRSA prevalence than slaughterhouse workers,[47] and AMR exposure in pig farms was higher than in pig slaughterhouses.[48] Similar results were found in this study where pig/broiler farmers showed significantly higher mean relative *aph(3′)-III* abundance than pig/broiler slaughterhouse workers. Although the exposure difference, as well as other determinants (working hours, life history etc.) within the production chain, may affect workers' ARG carriage,[13] we argue that people working in pig/broiler farms are more likely to be exposed to ARGs than people working in pig/broiler slaughterhouses, especially considering the high dust levels in farms.[19] There is also a possibility that antimicrobial residues in farm dust could impact human gut microbiota by selecting for resistance.[49,50] In the future, more in-depth studies are needed to reproduce and confirm these findings.

Conclusions

This study shows that qPCR analysis is a valuable tool to assess the abundances of selected ARGs in a large amount of livestock-associated samples collected across Europe. High variation of ARG abundance assessed using qPCR was found across animal species, environmental samples and humans. A 'farm to fork' decreasing trend in ARG abundance was found for both pigs and broilers. The between-country and between-farm variation could be partially attributed to AMU and farm biosecurity levels. Occupational livestock AMR exposure is related to the ARG abundance in human faeces.

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Transparency declarations

None to declare.

Supplementary data

Supplementary methods, references, Tables S1 to S6 and Figures S1 to S6 are available as Supplementary data at *JAC* Online.

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