J Antimicrob Chemother
https://doi.org/10.1093/jac/dkac002

Journal of Antimicrobial Chemotherapy

Risk factors for the abundance of antimicrobial resistance genes *aph(3′)-III*, *erm*(B), *sul2* and *tet*(W) in pig and broiler faeces in nine European countries

Dongsheng Yang [1]*, Dick J. J. Heederik[1], Dik J. Mevius[2,3], Peter Scherpenisse[1], Roosmarijn E. C. Luiken[1], Liese Van Gompel [1], Magdalena Skarżyńska[4], Katharina Wadepohl[5], Claire Chauvin[6], Eri Van Heijnsbergen[1], Inge M. Wouters[1], Gerdit D. Greve[1], Betty G. M. Jongerius-Gortemaker[1], Monique Tersteeg-Zijderveld[1], Magdalena Zając [4], Dariusz Wasyl[4], Katharina Juraschek[7], Jennie Fischer[7], Jaap A. Wagenaar[2], Lidwien A. M. Smit [1] and Heike Schmitt[1,8] on behalf of the EFFORT consortium†

[1]Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands; [2]Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands; [3]Department of Bacteriology and Epidemiology, Wageningen Bioveterinary Research, Lelystad, The Netherlands; [4]Department of Microbiology, National Veterinary Research Institute, Pulawy, Poland; [5]Außenstelle für Epidemiologie, Tierärztliche Hochschule Hannover, Hannover, Germany; [6]ANSES, Epidemiology, Health and Welfare Unit, Paris, France; [7]Department of Biological Safety, German Federal Institute for Risk Assessment, Berlin, Germany; [8]National Institute for Public Health and the Environment, Bilthoven, The Netherlands

*Corresponding author. E-mail: d.yang@uu.nl
†Members of the EFFORT group are listed in the Acknowledgements section.

Received 20 May 2021; accepted 26 December 2021

Objectives: The occurrence and zoonotic potential of antimicrobial resistance (AMR) in pigs and broilers has been studied intensively in past decades. Here, we describe AMR levels of European pig and broiler farms and determine the potential risk factors.

Methods: We collected faeces from 181 pig farms and 181 broiler farms in nine European countries. Real-time quantitative PCR (qPCR) was used to quantify the relative abundance of four antimicrobial resistance genes (ARGs) [*aph(3′)-III*, *erm*(B), *sul2* and *tet*(W)] in these faeces samples. Information on antimicrobial use (AMU) and other farm characteristics was collected through a questionnaire. A mixed model using country and farm as random effects was performed to evaluate the relationship of AMR with AMU and other farm characteristics. The correlation between individual qPCR data and previously published pooled metagenomic data was evaluated. Variance component analysis was conducted to assess the variance contribution of all factors.

Results: The highest abundance of ARG was for *tet*(W) in pig faeces and *erm*(B) in broiler faeces. In addition to the significant positive association between corresponding ARG and AMU levels, we also found on-farm biosecurity measures were associated with relative ARG abundance in both pigs and broilers. Between-country and between-farm variation can partially be explained by AMU. Different ARG targets may have different sample size requirements to represent the overall farm level precisely.

Conclusions: qPCR is an efficient tool for targeted assessment of AMR in livestock-related samples. The AMR variation between samples was mainly contributed to by between-country, between-farm and within-farm differences, and then by on-farm AMU.

Introduction

Antimicrobial resistance (AMR) in farm animals is of increasing concern as it may be linked to human AMR.[1,2] Identifying AMR determinants in farm animals may contribute to reducing AMR exposure at the animal–human interface and through the environment.

As the major food-producing animals in Europe, pigs and broilers are of special importance regarding the occurrence of AMR and related farm determinants.[3] On-farm antimicrobial use

(AMU) has been identified as a major determinant influencing AMR levels in farm animals.[4,5] Recently, Van Gompel *et al.*[6] reported significant positive associations between AMU and corresponding AMR abundance (macrolides and tetracyclines) in pigs. Similar associations were reported by Luiken *et al.*[7] between tetracycline use in broiler farms and tetracycline resistance in broiler faeces. In addition to on-farm AMU, other relevant farm characteristics may also influence AMR abundance in pigs and broilers. For example, biosecurity subcategories such as 'cleaning and disinfection', and 'measures between compartments and use of equipment' in pig farms were related to the significant increase in the relative abundance of all macrolide resistance genes [fragments per kb reference per million bacterial fragments (FPKM) were generated and aggregated] in the finisher faeces.[6] Moreover, a significant negative association was reported between manure storage at broiler farms and the prevalence of simultaneous resistance to amoxicillin/clavulanic acid, ceftiofur and cefoxitin in *Escherichia coli* isolates from broiler faeces.[8]

As part of the cross-sectional project 'Ecology from Farm to Fork Of microbial drug Resistance and Transmission' (EFFORT), we previously reported on risk factors for AMR in pig and broiler farms based on metagenomic analysis of DNA isolated from pooled faecal samples. In the present study, we investigated whether risk factors for AMR abundance can also be found using selected antimicrobial resistance gene (ARG) targets analysed by real-time quantitative PCR (qPCR) in individual faecal samples. In addition, we aimed to study the effects of a sampling depth of 5–7 individual samples per farm on risk factor analysis and variability in ARG abundances within and between farms.

Materials and methods

Study population and sampling procedure

Between May 2014 and June 2016, 181 pig farms and 181 broiler farms were visited in nine European countries (Belgium, Bulgaria, Germany, Denmark, Spain, France, Italy, the Netherlands and Poland). Countries were anonymized to letters 'A–I' in line with previous EFFORT publications.[6,7,9] In each country, 25 fresh faecal samples were randomly collected on each of 20 conventional farms (or 21 pig farms and 19 broiler farms in Country E or 21 broiler farms in Country A and Country B) complying with the previously described inclusion criteria (e.g. non-mixed, as close to slaughter as possible).[6,7,10] Data from 179 pig farms and 180 broiler farms remained for the present analysis, excluding 3 farms that cannot be linked to AMU data. In agreement with local farming organizations, farms were selected based on inclusion criteria and partly based on convenience (e.g. distance to the farm). Also considering the limited sample numbers per country, the selected farms cannot be regarded as representing the livestock sector of the participating countries. Faecal samples were collected without floor contact (sterile spoons were used). Within 24 h, all individual faecal samples were transported to the laboratory at 4°C and stored at −80°C until DNA extraction.[6,7]

Questionnaire, AMU and biosecurity measurement

General herd characteristics, AMU (group-treatment and purchased) and biosecurity information were retrieved from a standardized questionnaire (Table S1, available as Supplementary data at *JAC* Online) completed by farmers in each participating farm together with the visiting researchers.[6,7] Group-treatment AMU was defined as any treatment simultaneously applied to all animals present in, at least, the smallest housing unit (i.e. pen in pigs, barn in broilers) of each farm. Purchased AMU was defined as the antimicrobials purchased for the entire farm 1 year before sampling. AMU was expressed as treatment incidences [TIs, based on DDD (DDDvet)] as previously described.[6,7] While one TI was provided for broilers, TIs calculated for pigs included separate TIs for sucklers, weaners and fatteners and a TI adjusted for a lifespan of 200 days (TI 200). Biosecurity in this study was calculated using the Biocheck.UGent™ scoring system, based on 108 questions related to farm biosecurity.[11] The internal biosecurity subcategories (e.g. cleaning and disinfection) were gathered by questions related to counteracting the pathogen spread within the farm, while the external biosecurity subcategories (e.g. location of the farm) were gathered by questions related to preventing pathogens from entering the farm. The mean of internal and external biosecurity was defined as total biosecurity. More information and one example of the questionnaire can be found in the Supplementary methods and materials ('*Standardized questionnaire*').

DNA extraction, qPCR and sequencing

Individual faecal DNA (7 samples per pig farm and 5 samples per broiler farm) and pooled faecal DNA (25 samples pooled together per farm) were extracted in one central lab using the modified QIAamp Fast DNA Stool Mini Kit (Cat. No. 51604; QIAGEN, The Netherlands) as described before.[7,12] Following DNA extraction, qPCR was performed to quantify the abundance of four ARGs [*aph(3′)-III*, *erm*(B), *sul2* and *tet*(W)] along with the 16S rRNA gene used for the normalization of ARG copies. These gene targets represent four different antimicrobial classes and were chosen based on the results of metagenomic analyses[10] showing that these genes are of sufficient abundance to be detected in the majority of faecal samples, and these genes are only moderately correlated, hence different aspects of the total resistome can be captured by a limited number of assays (D. Yang, D. J. J. Heederik, P. Scherpenisse, L. Van Gompel, R. E. C. Luiken, K. Wadepohl, M. Skarżyńska, E. Van Heijnsbergen, I. M. Wouters, G. D. Greve, B. G. M. Jongerius-Gortemaker, M. Tersteeg-Zijderveld, L. Portengen, K. Juraschek, J. Fischer, M. Zając, D. Wasyl, J. A. Wagenaar, D. J. Mevius, L. A. M. Smit, H. Schmitt, unpublished data). The qPCR analysis of 16S, *erm*(B) and *tet*(W) was previously described by Van Gompel *et al.*[12] Briefly, the DNA template was diluted with TE buffer (1:100) (Thermo Fisher Scientific, USA) to overcome possible inhibition. For all DNA samples, the PCR reaction (10 μl) consisted of 5 μl of supermix [IQ SYBR Green Supermix for 16S, SsoAdvanced™ Universal Probes Supermix for *erm*(B) or IQ supermix for *tet*(W) (Bio-Rad, USA)], 3 μl of DNA template, primers [16S: 200 nM each; *tet*(W): 600 nM each; *erm*(B): 400 nM each] and a probe [*tet*(W): 200 nM; *erm*(B): 250 nM].

The qPCR process for *aph(3′)-III* and *sul2* was previously described by Yang *et al.*[13] Briefly, the DNA template was diluted with TE buffer (1:100). For all DNA samples, the PCR reaction (10 μl) consisted of 5 μl of supermix (SsoAdvanced™ Universal Probes Supermix), 3μl of DNA template, primers [*aph(3′)-III*: 400 nM; *sul2*: 100 nM] and a probe [*aph(3′)-III*: 250 nM; *sul2*: 100 nM].

In addition, synthetic DNA encoding blue fluorescence protein (bfp) was used as an internal amplification control (IAC). Bfp primers [*aph(3′)-III*: 400 nM; *erm*(B): 400 nM; *sul2*: 100 nM; *tet*(W): 600 nM] and probes [*aph(3′)-III*: 250 nM; *erm*(B): 250 nM; *sul2*: 100 nM; *tet*(W): 200 nM] were added. A total of four positive and eight negative control samples (TE buffer pH 8) were used per PCR plate. More details regarding the qPCR assays and quality control procedures have been described previously.[12,13]

DNA of pooled faecal samples from pigs and broilers was extracted at the Technical University of Denmark (DTU) and shipped on dry ice to the Oklahoma Medical Research Foundation (OMRF; Oklahoma City, OK, USA) for shotgun metagenomic sequencing. In total, faecal DNA from 181 pigs and 181 broilers was sequenced on the HiSeq 3000 platform (Illumina), yielding >18 billion paired-end reads. More details about the subsequent processing of metagenomic data were described in our previous research.[6,7,10]

Statistical analysis

The relative abundance of ARGs in this study was calculated by $\log_{10}$ (ARG copies/16S copies) to normalize for different amounts of bacterial DNA per sample. Overall, differences were compared by performing a classic or Welch's analysis of variance (ANOVA) depending on the variance homogeneity.[14,15] In the case of a significant difference, *post hoc* tests [i.e. respectively a Tukey's honest significant difference (Tukey HSD) test[16] or a Games–Howell *post hoc* test[17]] were carried out. Unless otherwise specified, appropriate *post hoc*-test *P* values are reported.

R version 4.0.3 was used for all statistical analyses.[18] Before running the mixed model, potential farm characteristics (age, weight, farm size etc.) were selected based on the opinions of experts in the EFFORT group and the published literature on farm animal risk factor analysis.[6,7,13,19,20] A linear mixed model with both country and farm as random effects was applied to take the between-country and between-farm variation into account. Changes in estimates and significance of associations with or without AMU in the model were determined.

Firstly, we ran the mixed model for AMR and selected farm characteristics other than AMU. Associations were selected by univariable analysis ($P<0.2$) and subsequently an automatic backward analysis was conducted using the 'step' function in the 'R' package *lmerTest*.[21] The multivariable model without AMU was adjusted after the fixed parts were eliminated step by step ($P>0.05$). Considering the high level and limited variance of biosecurity score in Country I, we performed a sensitivity analysis between ARG abundances and farm biosecurity without Country I. Secondly, the same procedure was applied, but with adjustment for AMU in all mixed models. Due to the right-skewed distribution, AMU was $\log_{10}$ transformed after adding a pseudocount of 1. Considering the number of broiler farms without AMU, a sensitivity analysis was performed between ARG abundances and binary AMU (1 or 0 meaning using antimicrobials or not, respectively).

After checking the distribution of datasets, Spearman's rank correlation was used to evaluate the correlation of relative ARG abundances between individual qPCR data and previously published pooled metagenomic data for pigs and broilers.[6,7,10] The median of 5–7 individual qPCR results per farm was calculated before correlation analysis. To match the ARG targets of qPCR, all downstream gene abundances of *aph(3′)-III*, *erm*(B), *sul2* and *tet*(W) were collected from the metagenomic data (FPKM) and summed per gene target. FPKM was $\log_{10}$ transformed after adding a pseudocount of 1.

To determine the variance contribution of all risk factors, variance component analysis (VCA) was conducted. First, we determined variance in the null model (AMR ~ country + farm) using the 'R' package *VCA*.[22] Subsequently, significant risk factors in the multivariable model with AMU determined in the previous step were included in the VCA null model. The variance components were inspected for each ARG target.

Results

The relative abundance of four ARGs varied highly between countries and farms in pigs and broilers (Figures 1 and 2). Among the four ARGs, the highest mean relative abundance was observed in *tet*(W) ($P<0.01$) in pigs and *erm*(B) ($P<0.01$) in broilers, while the lowest mean relative abundance was found in *sul2* ($P<0.01$) in pigs and *aph(3′)-III* ($P<0.01$) in broilers (Tables S2 and S3). Similar variation was found in AMU data (Figures S1–S3), which showed that tetracyclines were most frequently used among all antimicrobial classes in pigs, while aminoglycoside use was generally lower than the use of other antimicrobial classes in broilers. In addition, we found that the main biosecurity scores (external, internal, total) showed large between- and within-country variation (Figures S4 and S5).

Association of AMR and farm characteristics other than AMU

The results of univariable analysis are presented in the Supplementary results ('*Univariable analysis between AMR and farm characteristics other than AMU*'). In pigs, all significant ($P<0.05$) farm characteristics (weaning age of piglets, current age of

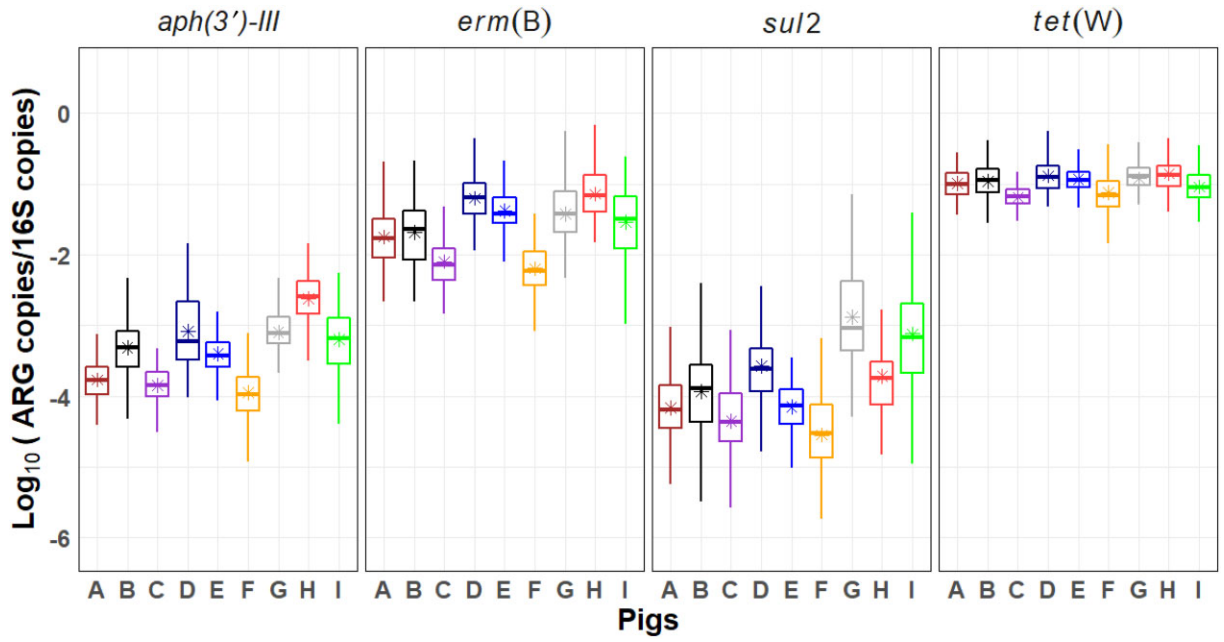

Figure 1. Relative abundance of four ARGs per country in pigs. The whiskers represent the IQR and the centre line represents the median. The asterisks show the mean in each country. Letters A–I represent the nine countries.

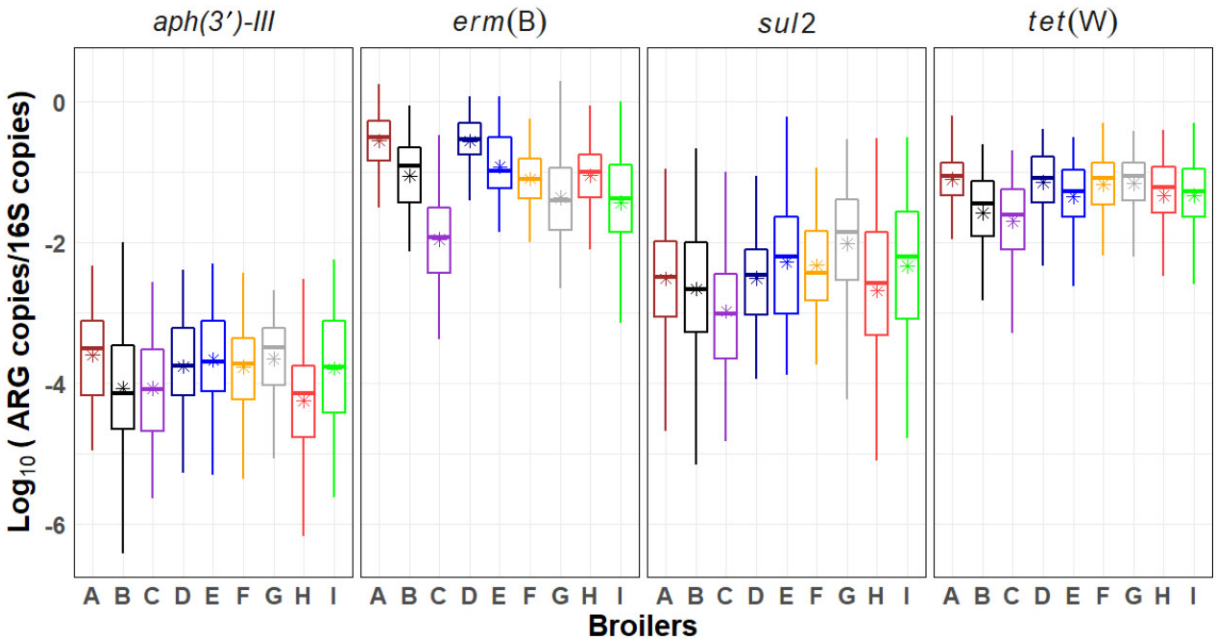

Figure 2. Relative abundance of four ARGs per country in broilers. The whiskers represent the IQR and the centre line represent the median. The asterisks show the mean in each country. Letters A–I represent the nine countries.

fatteners, biosecurity subcategories 'feeding and equipment supply' and 'location of the farm') in the univariable analysis were significant ($P<0.05$) in the multivariable model without AMU (Tables S4 and S5), except for *erm*(B), where only the biosecurity subcategory 'cleaning and disinfection' remained significant ($\beta=0.004$; $P=0.02$) (Figure 3, Table S5). In both univariable analysis and the multivariable model without AMU of broilers, we found significant ($P<0.05$) associations between relative ARG abundances and the number of farmworkers [*erm*(B), *tet*(W)], weight of broilers at set-up [*erm*(B)], average number of rounds per year (*sul2*) and the biosecurity subcategories 'disease management' [*tet*(W)] and 'removal of manure and carcasses' [*aph(3′)-III*, *erm*(B), *tet*(W)] (Tables S6 and S7). The sensitivity analysis without Country I gave the same results as the analysis including Country I.

In contrast, several significant ($P<0.05$) farm characteristics in the univariable models dropped out from the multivariable model without AMU. For example, in pigs, biosecurity subcategories 'vermin and bird control' and 'materials between compartments and equipment use' were non-significant ($P>0.05$) with relative *erm*(B) abundance in the multivariable model without AMU (Tables S4 and S5). Similarly, in broilers, we found fewer variables (biosecurity subcategories 'cleaning and disinfection' and 'visitors and farmworkers') were left for *aph(3′)-III* in the multivariable model without AMU compared with the univariable model (Tables S6 and S7). Furthermore, we found a significant negative association ($\beta=-0.004$; $P=0.02$) between relative *tet*(W) abundance and biosecurity score of 'cleaning and disinfection' in the multivariable model without AMU (Figure 4, Table S7).

Association of AMR, AMU and other farm characteristics

We found a significant positive association between lincosamide and macrolide use with relative *erm*(B) abundance in both pigs and broilers ($P<0.01$) (Tables 1 and 2, Figures S6 and S7) and between tetracycline use during the suckler period with relative *tet*(W) abundance in pigs ($\beta=0.16$; $P=0.05$) (Table 1). Total AMU showed a significant association with relative *tet*(W) abundance in both pigs ($\beta=0.09$; $P=0.02$) and broilers ($\beta=0.17$; $P<0.01$) (Tables 1 and 2). The sensitivity analysis using dichotomized AMU data gave the same results as the analysis using continuous AMU data.

Adjustment for AMU led to some changes in risk factor analysis outcomes. In the multivariable model with AMU of pigs, for example, 'current age of fattener' and 'location of the farm' were omitted for *aph(3′)-III*; 'farrowing and suckling period' ($\beta=0.004$; $P=0.03$) was added for *sul2*; (Table 1). In the multivariable model with AMU of broilers, almost all farm characteristics with significant ($P<0.05$) associations with ARG abundances remained present, except for *tet*(W), in which 'weight of broilers at set-up' was no longer significant ($P>0.05$). (Table 2, Table S7).

Correlation analysis between the median individual qPCR data and pooled metagenomic data

In the correlation assessment of median individual qPCR data and pooled metagenomic data from pigs and broilers, we only found a moderate correlation (Figure 5). *erm*(B) always showed a high correlation ($\rho>0.7$; $P<0.05$) of the four ARG targets.

VCA of multivariable linear mixed model

In the VCA null model of pigs (Table S8), variance contributions from country and farm were higher than the other variables, while in broilers (Table S9) most variances were due to within-farm variation. In both pigs and broilers, the within-farm variance was lowest for *erm*(B) compared with other components.

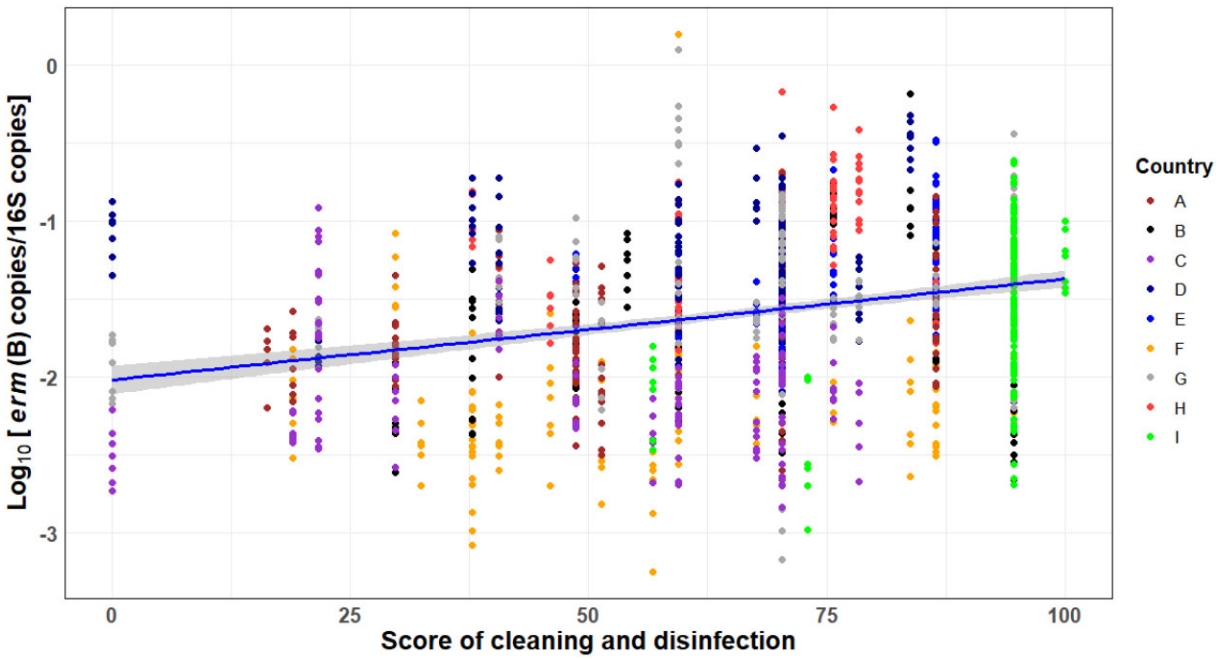

Figure 3. Associations between cleaning and disinfection level and relative *erm*(B) abundance in pigs in nine countries. Cleaning and disinfection: one of the subcategories of internal biosecurity. The blue line represents the linear relationship between ARG abundance and the score of cleaning and disinfection; the grey area around the line demonstrates the 95% CI. Letters A–I represent the nine countries.

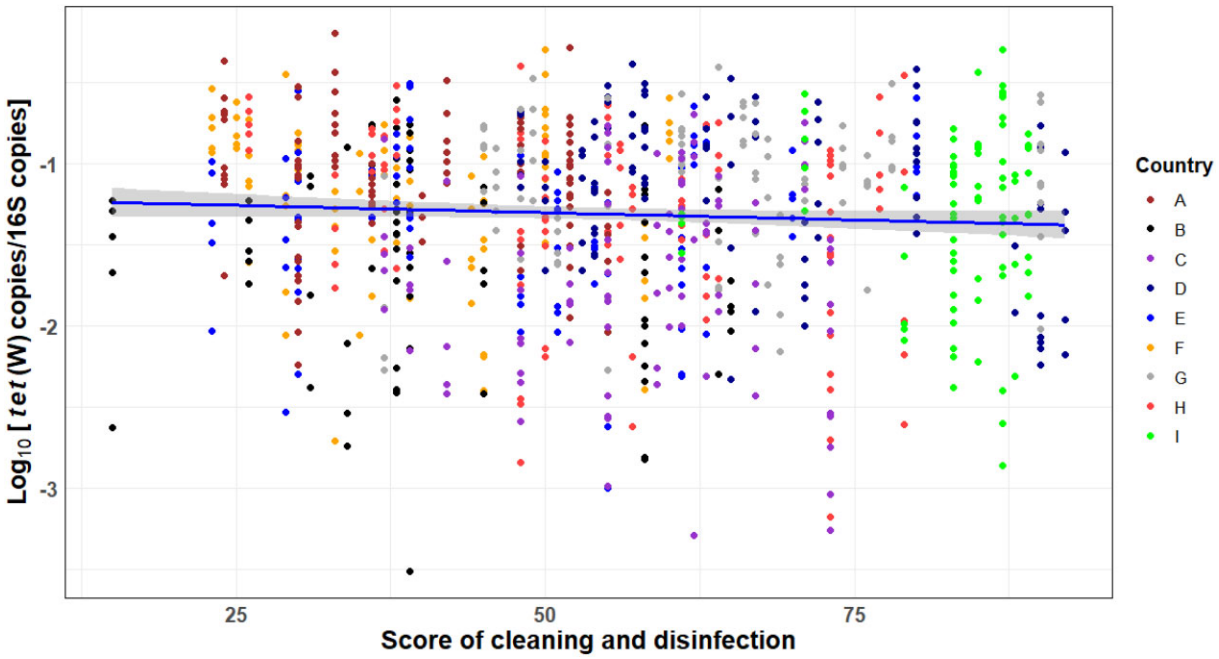

Figure 4. Associations between cleaning and disinfection level and relative *tet*(W) abundance in broilers in nine countries. Cleaning and disinfection: one of the subcategories of internal biosecurity. The blue line represents the linear relationship between ARG abundance and the score of cleaning and disinfection; the grey area around the line demonstrates the 95% CI. Letters A–I represent the nine countries.

After farm characteristics were adjusted in the VCA null model, we found a shift of variance contribution from between-country or between-farm variation to farm characteristics, especially to AMU. In pigs, after farm characteristics were adjusted, we found AMU contributed 12.60% to the total variation of relative *aph(3′)-III* abundance, while the variance contribution percentages of country (53.83% to 46.73%) and farm (31.99% to 24.55%) decreased (Table 3, Table S8). In broilers, between-farm

Table 1. Multivariable linear mixed model with AMU in pigs

	aph(3′)-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	*P* value	95% CI	Beta	*P* value	95% CI	Beta	*P* value	95% CI	Beta	*P* value	95% CI
AMU												
TI_{DDDvet} tetracyclines (log_{10}) suckler							0.38	0.03	0.04–0.73	0.16	0.05	0–0.32
TI_{DDDvet} lincosamide & macrolide (log_{10}) fattener				0.40	<0.01	0.19–0.6						
TI_{DDDvet} total (log_{10}) fattener	0.34	<0.01	0.23–0.46				0.23	<0.01	0.07–0.39			
TI_{DDDvet} total (log_{10}) 200										0.09	0.02	0.02–0.16
Herd characteristics												
Weaning age of piglets (days)	−0.02	0.01	−0.04 to −0.01									
Current number of fatteners	7.70×10^{-5}	0.02	0–0.0001									
Internal biosecurity												
Farrowing and suckling period							0.004	0.03	0–0.008			
Cleaning and disinfection				0.004	0.02	0–0.01						

Lincosamide & macrolide, use of macrolide + lincosamide + lincomycin/spectinomycin. The multivariable model with AMU was automatically adjusted using the 'step' function in the 'R' package *lmerTest*.[21] Only associations with a *P* value less than 0.05 are involved.

Table 2. Multivariable linear mixed model with AMU in broilers

	aph(3′)-III			*erm*(B)			*sul2*			*tet*(W)		
	Beta	*P* value	95% CI	Beta	*P* value	95% CI	Beta	*P* value	95% CI	Beta	*P* value	95% CI
AMU												
Group TI_{DDDvet} lincosamide & macrolide (log_{10})				0.47	<0.01	0.27–0.67						
Group TI_{DDDvet} trimethoprim & sulphonamide (log_{10})	−0.24	0.03	−0.46 to −0.02									
Group TI_{DDDvet} total (log_{10})										0.17	<0.01	0.08–0.26
Purchase TI_{DDDvet} aminoglycosides (log_{10})							0.80	0.01	0.18–1.41	−0.34	0.04	−0.68 to −0.01
Herd characteristics												
Number of farmworkers				0.03	0.04	0.001–0.06						
Weight of broilers at set-up (g)				0.02	0.01	0.004–0.04						
Average number of rounds/year							0.17	<0.01	0.07–0.26			
Internal biosecurity												
Disease management										−0.006	<0.01	−0.01 to −0.003
Cleaning and disinfection										−0.004	0.03	−0.008 to −0.001
External biosecurity												
Removal of manure and carcasses	0.008	<0.01	0–0.01	0.006	0.01	0.001–0.012				0.007	<0.01	0.003–0.01

Lincosamide & macrolide, use of macrolide + lincosamide + spectinomycin; trimethoprim & sulphonamide, use of sulphonamide + trimethoprim/sulphonamide. The multivariable model with AMU was automatically adjusted using the 'step' function in the 'R' package *lmerTest*.[21] Only associations with a *P* value less than 0.05 are involved.

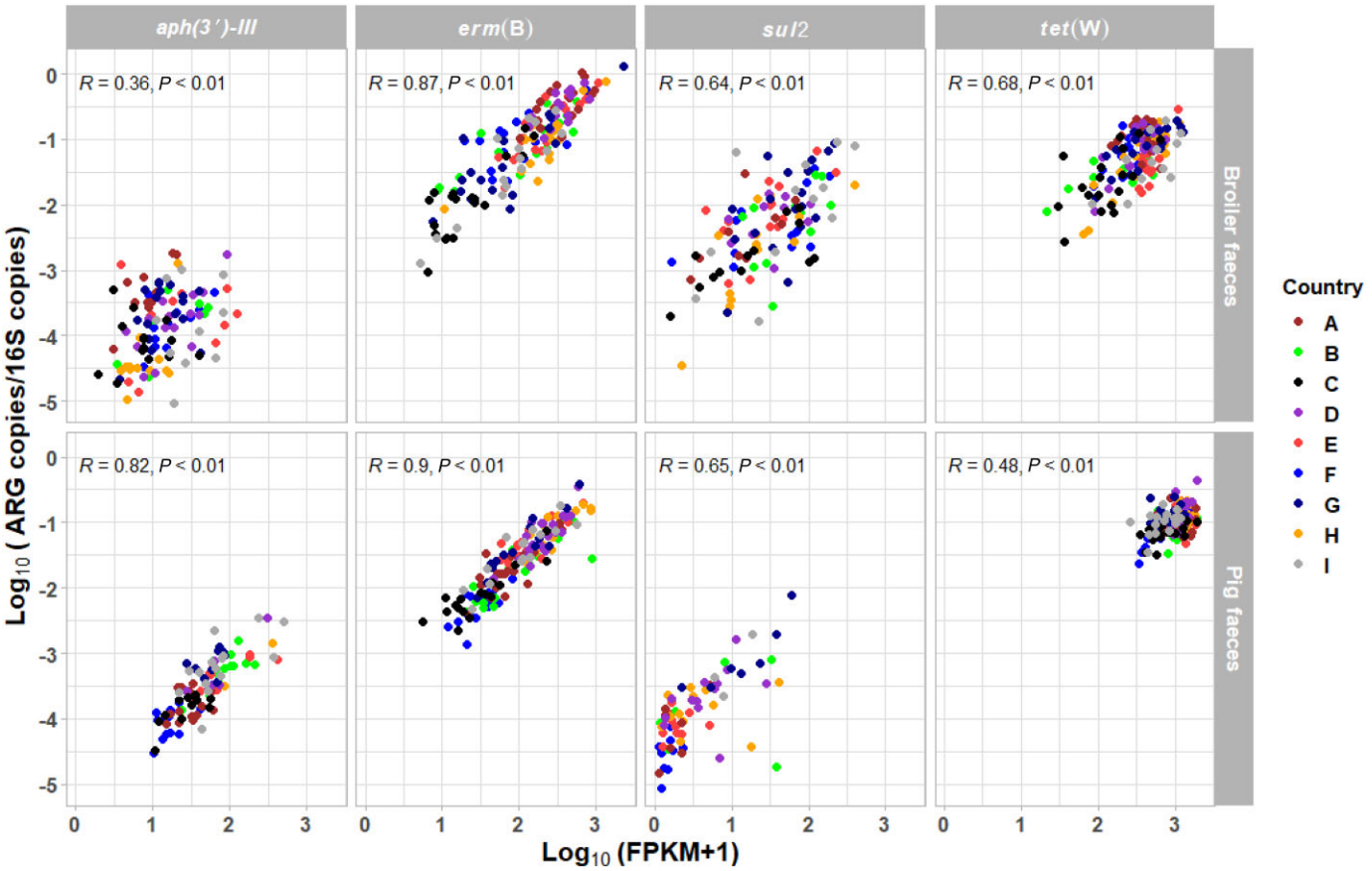

Figure 5. Correlation of AMR between median individual qPCR data and pooled metagenomic data in pigs and broilers. FPKM, fragments per kilobase reference per million bacterial fragments.[10] ARG targets: *aph(3′)-III*, *erm*(B), *sul2*, *tet*(W). The median of 5–7 individual qPCR results was calculated per farm before correlation analysis. Letters A–I represent the nine countries.

variation changed for all ARG targets. For example, after adjustment the contribution percentage of between-farm variation changed from 42.79% to 28.95%, while lincosamide and macrolide use contributed 10.59% to the total variation of relative *erm*(B) abundance (Table 4, Table S9).

Discussion

To find potential risk factors that contributed to AMR abundance in pigs and broilers, we assessed the relationship between on-farm AMR levels and AMU and other farm characteristics using a linear mixed model. The results showed that in addition to AMU, risk factors such as age and weight of animals, and biosecurity measures, were also significantly associated with AMR levels in pig and broiler faeces. A moderate correlation was observed between median individual qPCR data and previously published pooled metagenomic data. The between-country and between-farm variation could partially be explained by AMU. Different ARG targets seem to have different sample size requirements to accurately represent their overall farm-level abundance.

It has been well documented that many farm factors other than AMU can affect the AMR levels in farm animals.[6,8,13,20] In our study, different ARG targets were associated with different risk factors. This could be explained by the ARG target selection criterion that only a moderate correlation with other ARGs is allowed. In pigs, we found that piglets weaned at an older age have significantly lower *aph(3′)-III* levels in their faeces before slaughter. We assume that the immune system of piglets weaned at an older age is more mature[23,24] and therefore these animals require less antimicrobial treatment,[25] which results in lower AMR levels later in the rearing process.[26] The negative association ($P > 0.05$) we found between the weaning age of pigs and total AMU in the fattening period (data not shown) can also provide evidence for this. In broilers, we found a significant positive association between farm staff numbers and relative abundance of *erm*(B) and *tet*(W). This may suggest that farmworkers act as a source of ARGs for farm animals, as was described for MRSA CC398 in pig farms in Norway.[27] However, there have been only occasional reports on the introduction of specific resistant bacteria into animal farms—mostly, the transmission was documented from animals to workers.

Biosecurity is increasingly valued by farmers; several studies have shown that a high biosecurity index (high hygiene level,[28,29] good management[30] and good feeding practices[31]) has a positive effect on the control of disease and AMR levels in farm animals. In the present study, we found similar results in broiler farms, where 'disease management' and 'cleaning and

Table 3. VCA of the multivariable model with AMU in pigs

	aph(3′)-III			*erm*(B)			*sul2*			*tet*(W)		
	VC	%Total	SD	VC	%Total	SD	VC	%Total	SD	VC	%Total	SD
AMU												
TI_{DDDvet} tetracyclines (log_{10}) suckler							0.003	0.45	0.05	0.01	6.56	0.07
TI_{DDDvet} lincosamide & macrolide (log_{10}) fattener				0.02	5.24	0.13						
TI_{DDDvet} total (log_{10}) fattener	0.04	12.60	0.21				NA	0.00	0.00			
TI_{DDDvet} total (log_{10}) 200										NA	0.00	0.00
Herd characteristics												
Weaning age of piglets (days)	0.01	2.07										
Current number of fatteners	0.00	0.00	0.00									
Internal biosecurity												
Farrowing and suckling period							0.01	1.64	0.10			
Cleaning and disinfection				NA	0.00	0.00						
Others												
Country	0.17	**46.73**	0.41	0.10	32.53	0.32	0.30	**44.03**	0.54	0.01	9.33	0.09
Farm	0.08	24.55	0.29	0.15	**49.32**	0.39	0.18	26.77	0.42	0.04	**47.46**	0.20
Residual	0.05	14.05	0.22	0.04	12.91	0.20	0.18	27.11	0.43	0.03	36.65	0.17

VC, variance component; %Total, the percentage of the VC; SD, standard deviation; lincosamide & macrolide, macrolide + lincosamide + spectinomycin use; NA, too small a value that was automatically displayed as NA by the *VCA* package.[22] Values in bold indicate the highest VC percentage for each model. All values are rounded to two decimal places unless rounding would lead to the misinterpretation of an effect.

disinfection' as biosecurity subcategories were significantly associated with a lower relative *tet*(W) abundance. In contrast, biosecurity was also reported to be positively associated with AMR levels in pig faeces from the same farms as described here, particularly for the biosecurity subcategory 'cleaning and disinfection'.[6] Similar results were reported in previous veal calf studies.[13] Furthermore, in this study we found that broiler farms with higher biosecurity scores of 'transfer of faeces and carcasses' have higher AMR levels than other broiler farms. This is probably related to one biosecurity measure (removal of farm manure) included for this biosecurity subcategory. Similar results were reported in a previous study in broilers, in which manure storage on farms was shown to be negatively associated with the prevalence of β-lactam resistance in flocks.[8] These results indicate a complex relationship between on-farm biosecurity and AMR levels in farm animals. More in-depth and specific analyses of AMR and farm biosecurity are necessary in the future to understand the impact of possible interventions to reduce AMR in farm animals.

After the multivariable model was adjusted for AMU, we expected that fewer farm characteristics would be associated with AMR (compared with the model without AMU), because of assumed interlinkages between AMU and other farm characteristics. Generally, our results were in agreement with these expectations. However, compared with the model without AMU, there was one additional factor (biosecurity subcategory 'farrowing and suckling period') that showed a significant positive association with relative *sul2* abundance in the AMU-adjusted model in pigs. One explanation is that at the same AMU level, pigs with a longer farrowing and suckling period are at a higher risk of acquiring resistance. Several studies have reported possible bacterial spread[32] and ARG transmission[33,34] between sows and piglets, especially around parturition. Therefore, it is necessary to take co-varying factors into account when establishing potential risk factors of AMR.

Consistent with previous risk factor analysis reports of metagenomic data in pigs,[6] we found significant positive associations between relative *erm*(B) abundance with lincosamide and macrolide use, and between total AMU during the fattening phase and relative abundance of *aph(3′)-III* and *sul2*. When comparing our results with the meta-analysis of metagenomic data in broilers,[7] we did not find comparable significant ARG/AMU associations in this study, which may be due to the fact that the selected specific gene targets for the qPCR approach do not necessarily represent the whole ARG group linked with a specific antimicrobial class.

In the correlation analysis, we only found a moderate correlation of AMR between the median of 5–7 individual qPCR readouts and the previously published metagenomic data assessed in pooled samples.[10] In addition to the incompletely reproduced risk factor analysis results of these two datasets, we speculate that collecting 5 or 7 individual faecal samples per farm probably does not represent the farm-level AMR as accurately as pooling 25 individual faecal samples together per farm. Meanwhile, we found consistent results with previous metagenomic data in risk factor analysis of *erm*(B), and we observed a high correlation of *erm*(B) abundance between median individual qPCR data and pooled metagenomic data in both pigs and broilers. This may indicate that ARGs have different sample size requirements per farm to accurately represent their overall farm level. The low within-farm variance for *erm*(B) in VCA results of both pigs and broilers may further provide evidence for our speculation.

In addition, the VCA results in the multivariable model with AMU showed that AMU is the most important variance

Table 4. VCA of the multivariable model with AMU in broilers

	aph(3′)-III			*erm*(B)			*sul2*			*tet*(W)		
	VC	%Total	SD	VC	%Total	SD	VC	%Total	SD	VC	%Total	SD
AMU												
Group TI_{DDDvet} lincosamide & macrolide (log_{10})				0.05	10.59	0.23						
Group TI_{DDDvet} trimethoprim & sulphonamide (log_{10})	0.02	3.02	0.13									
Group TI_{DDDvet} total (log_{10})										0.04	14.23	0.20
Purchase TI_{DDDvet} aminoglycosides (log_{10})							0.08	9.19	0.29	0.04	13.37	0.20
Herd characteristics												
Number of farmworkers				0.02	3.64	0.14						
Weight of broilers at set-up (g)				0.00	0.00	0.00						
Average number of rounds/year							0.02	2.17	0.14			
Internal biosecurity												
Disease management										NA	0.00	0.00
Cleaning and disinfection										NA	0.00	0.00
External biosecurity												
Removal of manure and carcasses	0.01	0.95	0.07	0.004	0.87	0.07				0.01	4.04	0.11
Others												
Country	0.04	7.51	0.21	0.17	**33.68**	0.42	0.07	8.03	0.27	0.01	2.37	0.08
Farm	0.11	18.15	0.32	0.15	28.95	0.39	0.25	27.77	0.50	0.03	11.17	0.18
Residual	0.41	**70.38**	0.64	0.11	22.28	0.34	0.47	**52.84**	0.69	0.16	**54.82**	0.40

VC, variance component; %Total, the percentage of the VC; SD, standard deviation; lincosamide & macrolide, macrolide + lincosamide + spectinomycin use; trimethoprim & sulphonamide, sulphonamide + trimethoprim/sulphonamide use; NA, too small a value that was automatically displayed as NA by the '*VCA*' package.[22] Values in bold indicate the highest VC percentage for each model. All values are rounded to two decimal places unless rounding would lead to the misinterpretation of an effect.

component in comparison with other farm characteristics. Compared with the null VCA model, the between-country and between-farm variation in pigs [*aph(3′)-III*] and the between-farm variation in broilers (all ARG targets) decreased considerably, mainly shifted to AMU. This suggests that the between-country and between-farm variation can partially be explained by AMU. Furthermore, it appeared that the farm characteristics included in our study only explained a limited part of the observed total AMR variation. This indicates that there are likely unidentified/unstudied determinants (e.g. historical AMU, farm management factors) that need to be evaluated and considered in future studies.

Conclusions

This study shows that qPCR is an efficient tool for targeted assessment of AMR in livestock-related samples. The AMR variation between samples was first and foremost caused by between-country, between-farm and within-farm differences, and secondly by AMU. In addition, there are other farm characteristics that have a low but significant impact on AMR levels in farm animals, which requires further research. More attention needs to be paid to sample size in future epidemiological studies of ARGs.

Acknowledgements

We would like to thank all the farmers and field workers from Belgium, Bulgaria, Denmark, France, Germany, Italy, Poland, Spain and the Netherlands. We also wish to thank all the laboratory analysts and data analysts at the Institute for Risk Assessment Sciences, the Netherlands (Daisy de Vries, Nynke Jansen, Janne Heederik).

Members of the EFFORT consortium

Haitske GRAVELAND (UUVM), Philip JOOSTEN (UGENT), Steven SARRAZIN (UGENT), Jeroen DEWULF (UGENT), Alieda VAN ESSEN (WBVR), Bruno GONZALEZ-ZORN (UCM), Gabriel MOYANO (UCM), Pascal SANDERS (ANSES), Julie DAVID (ANSES), Christophe SOUMET (ANSES), Antonio BATTISTI (IZSLT), Andrea CAPRIOLI (IZSLT), Thomas BLAHA (TIHO), Maximiliane BRANDT (TIHO), Frank AARESTRUP (DTU), Tine HALD (DTU), Ana Sofia Ribeiro DUARTE (DTU), Andrzej HOSZOWSKI (NVRI), Agnieszka PĘKALA-SAFIŃSKA (NVRI), Ewa PAŻDZIOR (NVRI), Hristo DASKALOV (NDRVI), Helmut W. SAATKAMP (BEC) and Katharina D. C. STÄRK (SAFOSO), as well as Dick J. J. HEEDERIK, Dik J. MEVIUS, Jaap A. WAGENAAR, Lidwien A. M. SMIT and Heike SCHMITT (authors of this paper).

Funding

This work was part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, which is co-funded by the European Commission, 7th Framework Programme for Research and Innovation (FP7-KBBE-2013-7; grant agreement: 613754). Research at the National Veterinary Research Institute (PIWet), Poland, was supported by the Polish Ministry of Science: No. 3173/7PR/2014/2. D.Y. was also funded by the China Scholarships Council (No. 201709110149).

Transparency declarations

None to declare.

Supplementary data

Supplementary methods and materials, results, Tables S1 to S9 and Figures S1 to S7 are available as Supplementary data at *JAC* Online.

References

1 Dorado-Garcia A, Bos MEH, Graveland H *et al.* Risk factors for persistence of livestock-associated MRSA and environmental exposure in veal calf farmers and their family members: an observational longitudinal study. *BMJ Open* 2013; **3:** e003272.

2 Dohmen W, Schmitt H, Bonten M *et al.* Air exposure as a possible route for ESBL in pig farmers. *Environ Res* 2017; **155**: 359–64.

3 Notarnicola B, Tassielli G, Renzulli PA *et al.* Environmental impacts of food consumption in Europe. *J Clean Prod* 2017; **140**: 753–65.

4 Holmes AH, Moore LS, Sundsfjord A *et al.* Understanding the mechanisms and drivers of antimicrobial resistance. *Lancet* 2016; **387**: 176–87.

5 Murphy CP, Carson C, Smith BA *et al.* Factors potentially linked with the occurrence of antimicrobial resistance in selected bacteria from cattle, chickens and pigs: a scoping review of publications for use in modelling of antimicrobial resistance (IAM. AMR Project). *Zoonoses Public Health* 2018; **65**: 957–71.

6 Van Gompel L, Luiken RE, Sarrazin S *et al.* The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries. *J Antimicrob Chemother* 2019; **74**: 865–76.

7 Luiken REC, Van Gompel L, Munk P *et al.* Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries. *J Antimicrob Chemother* 2019; **74**: 2596–604.

8 Caffrey N, Nekouei O, Gow S *et al.* Risk factors associated with the A2C resistance pattern among *E. coli* isolates from broiler flocks in Canada. *Prev Vet Med* 2017; **148**: 115–20.

9 Sarrazin S, Joosten P, Van Gompel L *et al.* Quantitative and qualitative analysis of antimicrobial usage patterns in 180 selected farrow-to-finish pig farms from nine European countries based on single batch and purchase data. *J Antimicrob Chemother* 2019; **74**: 807–16.

10 Munk P, Knudsen BE, Lukjancenko O *et al.* Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat Microbiol* 2018; **3**: 898–908.

11 Biocheck.UGent™. https://biocheck.ugent.be/en.

12 Van Gompel L, Dohmen W, Luiken RE *et al.* Occupational exposure and carriage of antimicrobial resistance genes (tetW, ermB) in pig slaughterhouse workers. *Ann Work Expo Health* 2020; **64**: 125–37.

13 Yang D, Van Gompel L, Luiken REC *et al.* Association of antimicrobial usage with faecal abundance of *aph (3')-III*, *ermB*, *sul2* and *tetW* resistance genes in veal calves in three European countries. *Int J Antimicrob Agents* 2020; **56**: 106131.

14 Fox J. *Applied Regression Analysis and Generalized Linear Models*. Sage Publications, 2015.

15 Welch BL. The generalization of 'STUDENT'S' problem when several different population variances are involved. *Biometrika* 1947; **34**: 28–35.

16 Yandell BS. *Practical Data Analysis for Designed Experiments*. CRC Press, 1997.

17 Ruxton GD, Beauchamp G. Time for some *a priori* thinking about *post hoc* testing. *Behav Ecol* 2008; **19**: 690–3.

18 R Core Team. R: a language and environment for statistical computing (R Version 4.0. 3, R Foundation for Statistical Computing, Vienna, Austria, 2020). 2021. https://cran.r-project.org/bin/windows/base/old/4.0.3/.

19 Mo SS, Kristoffersen AB, Sunde M *et al.* Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks. *Prev Vet Med* 2016; **130**: 112–8.

20 AbuOun M, O'Connor HM, Stubberfield EJ *et al.* Characterizing antimicrobial resistant *Escherichia coli* and associated risk factors in a cross-sectional study of pig farms in great Britain. *Front Microbiol* 2020; **11:** 861.

21 Harrison XA, Donaldson L, Correa-Cano ME *et al.* A brief introduction to mixed effects modelling and multi-model inference in ecology. *PeerJ* 2018; **6**: e4794.

22 Schuetzenmeister A, Dufey F. VCA: variance component analysis. R package version 1.4. 3. 2021. https://cran.r-project.org/web/packages/VCA/VCA.pdf.

23 Blecha F, Pollman D, Nichols D. Weaning pigs at an early age decreases cellular immunity. *J Anim Sci* 1983; **56**: 396–400.

24 Main R, Dritz SS, Tokach M *et al.* Increasing weaning age improves pig performance in a multisite production system. *J Anim Sci* 2004; **82**: 1499–507.

25 Postma M, Backhans A, Collineau L *et al.* Evaluation of the relationship between the biosecurity status, production parameters, herd characteristics and antimicrobial usage in farrow-to-finish pig production in four EU countries. *Porcine Health Manag* 2016; **2**: 9.

26 Duse A, Waller KP, Emanuelson U *et al.* Risk factors for antimicrobial resistance in fecal *Escherichia coli* from preweaned dairy calves. *J Dairy Sci* 2015; **98**: 500–16.

27 Grøntvedt CA, Elstrøm P, Stegger M *et al.* Methicillin-resistant *Staphylococcus aureus* CC398 in humans and pigs in Norway: a 'One Health' perspective on introduction and transmission. *Clin Infect Dis* 2016; **63**: 1431–8.

28 Damiaans B, Renault V, Sarrazin S *et al.* Biosecurity practices in Belgian veal calf farming: level of implementation, attitudes, strengths, weaknesses and constraints. *Prev Vet Med* 2019; **172**: 104768.

29 Jones E, Snow L, Carrique-Mas JJ *et al.* Risk factors for antimicrobial resistance in *Escherichia coli* found in GB turkey flocks. *Vet Rec* 2013; **173**: 422.

30 Pholwat S, Pongpan T, Chinli R *et al.* Antimicrobial resistance in swine fecal specimens across different farm management systems. *Front Microbiol* 2020; **11**: 1238.

31 Davies RH, Wales AD. *Salmonella* contamination of cereal ingredients for animal feeds. *Vet Microbiol* 2013; **166**: 543–9.

32 Devriese LA, Hommez J, Pot B *et al.* Identification and composition of the streptococcal and enterococcal flora of tonsils, intestines and faeces of pigs. *J Appl Bacteriol* 1994; **77**: 31–6.

33 Mathew A, Upchurch W, Chattin S. Incidence of antibiotic resistance in fecal *Escherichia coli* isolated from commercial swine farms. *J Anim Sci* 1998; **76**: 429–34.

34 Stannarius C, Bürgi E, Regula G *et al.* Antimicrobial resistance in *Escherichia coli* strains isolated from Swiss weaned pigs and sows. *Schweiz Arch Tierheilkd* 2009; **151**: 119–25.