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

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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.\textsuperscript{6,5} Recently, Van Gompel et al.\textsuperscript{5} reported significant positive associations between AMU and corresponding AMR abundance (macrolides and tetracyclines) in pigs. Similar associations were reported by Luiken et al.\textsuperscript{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.\textsuperscript{6} Moreover, a significant negative association was reported between manure storage at broiler farms and the prevalence of simultaneous resistance to amoxicillin/clavulanic acid, cefotiofur and cefoxitin in Escherichia coli isolates from broiler faeces.\textsuperscript{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.\textsuperscript{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).\textsuperscript{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.\textsuperscript{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.\textsuperscript{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.\textsuperscript{6,7} While one TI was provided for broilers, TIs calculated for pigs included separate TIs for suckers, 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\textsuperscript{11} scoring system, based on 108 questions related to farm biosecurity.\textsuperscript{11} The internal biosecurity subcategories (e.g. cleaning and disinfection) were gathered by questions related to countering 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.\textsuperscript{11,12} 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\textsuperscript{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 resistance 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. Wadepohli, M. Skarzyńska, E. Van Heijnsbergen, I. M. Wouters, G. D. Greve, B. G. M. Jongerius-Garntekerman, M. Tersteeg-Zijderveld, L. Portengen, K. Juroschek, J. Fischer, M. Zajac, D. Wasyl, J. A. Wagenaar, D. J. Mievius, 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.\textsuperscript{13}

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.\textsuperscript{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), 3 μl 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.\textsuperscript{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.\textsuperscript{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. In the case of a significant difference, post hoc tests (i.e., respectively a Tukey's honest significant difference (Tukey HSD) test or a Games–Howell post hoc test) 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. 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. 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 `iterTest`. 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 1, we performed a sensitivity analysis between ARG abundances and farm biosecurity without Country 1. 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. 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 \( \text{aph}(3')-\text{III}, \text{erm}(B), \text{sul2} \) and \( \text{tet}(W) \) 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. 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 \( \text{tet}(W) \) \((P < 0.01)\) in pigs and \( \text{erm}(B) \) \((P < 0.01)\) in broilers, while the lowest mean relative abundance was found in \( \text{sul2} \) \((P < 0.01)\) in pigs and \( \text{aph}(3')-\text{III} \) \((P < 0.01)\) 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, whileaminoglycoside 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.
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.
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 \( \text{aph}(3')-\text{III} \) 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

![Figure 3](image3.png)

**Figure 3.** Associations between cleaning and disinfection level and relative \( \text{erm}(B) \) 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](image4.png)

**Figure 4.** Associations between cleaning and disinfection level and relative \( \text{tet}(W) \) 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.
Table 1. Multivariable linear mixed model with AMU in pigs

| AMU                                                                 | 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                                                                |             |        |      |        |             |         |             |           |         |             |           |
| TLDVvet tetracyclines (log_{10}) suckler                           | 0.38        | 0.03   | 0.04-0.73 | 0.16 | 0.05 | 0-0.32 |
| TLDVvet lincosamide & macrolide (log_{10}) fatty                   | 0.40        | <0.01  | 0.19-0.6  | 0.23 | <0.01 | 0.07-0.39 |
| TLDVvet total (log_{10}) fatty                                      | 0.34        | <0.01  | 0.23-0.46 | 0.09 | 0.02  | 0.02-0.16 |
| Herd characteristics                                                |             |        |      |        |             |         |             |           |         |             |           |
| Weaning age of piglets (days)                                       | 0.00        | 0.01   | 0.04 to -0.01  | 0.004 | 0.03  | 0-0.008 |
| Current number of fatteners                                         | 7.70 × 10^{-5} | 0.02    | 0-0.0001 |        |        |         |
| Internal biosecurity                                                |             |        |      |        |             |         |             |           |         |             |           |
| Farrowing and suckling period                                       | 0.004       | 0.02   | 0-0.01 |        |        |         |
| Cleaning and disinfection                                           | 0.004       | 0.03   | 0-0.008 |        |        |         |

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. Only associations with a P value less than 0.05 are involved.

Table 2. Multivariable linear mixed model with AMU in broilers

| AMU                                                                 | 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 TLDVvet lincosamide & macrolide (log_{10})                     | 0.47        | <0.01  | 0.27-0.67 | 0.17 | <0.01 | 0.08-0.26 |
| Group TLDVvet trimethoprim & sulphonamide (log_{10})                 | -0.24       | 0.03   | -0.46 to -0.02 | 0.08 | 0.01  | 0.18-1.41 |
| Purchase TLDVvet aminoglycosides (log_{10})                          | 0.00        | 0.04   | 0.001-0.06 | -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. Only associations with a P value less than 0.05 are involved.
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. 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 and therefore these animals require less antimicrobial treatment, which results in lower AMR levels later in the rearing process.

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 farm-workers act as a source of ARGs for farm animals, as was described for MRSA CC398 in pig farms in Norway. 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, good management and good feeding practices) 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

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. 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.
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’. Similar results were reported in previous veal calf studies. 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 management on farms was shown to be negatively associated with the prevalence of β-lactam resistance in flocks. 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 and ARG transmission 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, 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, 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. 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 3. VCA of the multivariable model with AMU in pigs

| Herd characteristics | AMU | aph(3′)-III | erm(B) | sul2 | tet(W) |
|----------------------|-----|-------------|--------|------|--------|
|                      | VC  | %Total      | SD     | VC   | %Total | SD |
| TiDDDvet tetracyclines (log_{10}) | 0.003 | 0.45 | 0.05 | 0.01 | 6.56 | 0.07 |
| TiDDDvet lincosamide & macrolide (log_{10}) | 0.02 | 5.24 | 0.13 | 0.00 | 0.00 | 0.00 |
| TiDDDvet total (log_{10}) | 0.04 | 12.60 | 0.21 | NA  | 0.00 | 0.00 |
| TiDDDvet total (log_{10}) 200 | NA  | 0.00 | 0.00 | NA  | 0.00 | 0.00 |
| Others | Country | 0.17 | 46.73 | 0.41 | 0.10 | 32.53 | 0.32 |
| | Farm | 0.08 | 24.55 | 0.29 | 0.15 | 49.32 | 0.39 |
| | Residual | 0.05 | 14.05 | 0.22 | 0.04 | 12.91 | 0.20 |

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

| AMU                                                                 | aph(3’)-III | erm(B) | sul2 | tet(W) |
|---------------------------------------------------------------------|-------------|--------|------|--------|
| AMU                                                                 | VC | %Total | SD | VC | %Total | SD | VC | %Total | SD | VC | %Total | SD |
| Group TI<sub>DDVvet</sub> lincosamide & macrolide (log<sub>10</sub>) | 0.05 | 10.59 | 0.23 |     |       |     |     |       |     |     |       |     |
| Group TI<sub>DDVvet</sub> trimethoprim & sulphonamide (log<sub>10</sub>) | 0.02 | 3.02 | 0.13 |     |       |     |     |       |     |     |       |     |
| Group TI<sub>DDVvet</sub> total (log<sub>10</sub>)                  |     |       |     | 0.04 | 14.23 | 0.20 |     |       |     |     |       |     |
| Purchase TI<sub>DDVvet</sub> aminoglycosides (log<sub>10</sub>)      |     |       |     | 0.08 | 9.19  | 0.29 |     |       |     |     |       |     |
| 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                                                   |     |       |     |     |       |     |     |       |     |     |       |     |
| Cleaning and disinfection                                            |     |       |     |     |       |     |     |       |     |     |       |     |
| External biosecurity                                                 |     |       |     | 0.01 | 0.95  | 0.07 |     |       |     |     |       |     |
| Removal of manure and carcasses                                      |     |       |     | 0.04 | 7.51  | 0.21 |     |       |     |     |       |     |
| Others                                                              |     |       |     | 0.11 | 18.15 | 0.32 |     |       |     |     |       |     |
| Country                                                              |     |       |     | 0.17 | 33.68 | 0.42 |     |       |     |     |       |     |
| Farm                                                                |     |       |     | 0.17 | 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. 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.

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).

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Haiitse GRAVELAND (UJVM), 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 (DU), Andzej HOSZOWSKI (NVRI), Agnieszka PŁKALASA-FINSKA (UPP), Ewa PAZDZIOR (NVRI), Hristo DASKALOV (NDRVI), Helmut W. SAATKAMP (BEC) and Katharina D. C. STARK (SAFOSO), as well as Dick J. J. HEEDERIK, Dirk 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.

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