J Antimicrob Chemother 2019; **74**: 865–876
doi:10.1093/jac/dky518 Advance Access publication 14 January 2019

Journal of Antimicrobial Chemotherapy

The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries

Liese Van Gompel [1]*, Roosmarijn E. C. Luiken[1], Steven Sarrazin[2], Patrick Munk [3], Berith E. Knudsen[3], Rasmus B. Hansen[4] and Alex Bossers [1,5], Frank M. Aarestrup [3], Jeroen Dewulf [2], Jaap A. Wagenaar[5,6], Dik J. Mevius[5,6], Heike Schmitt[1,7], Dick J. J. Heederik[1], Alejandro Dorado-García[1] and Lidwien A. M. Smit[1] on behalf of the EFFORT consortium†

[1]Institute for Risk Assessment Sciences, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 2, 3584 CM Utrecht, The Netherlands; [2]Veterinary Epidemiology Unit, Department of Reproduction, Obstetrics and Herd Health, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, 9820 Merelbeke, Belgium; [3]Research Group for Genomic Epidemiology, National Food Institute, Technical University of Denmark, Kemitorvet 204, 2800 Kongens Lyngby, Denmark; [4]Intomics A/S, Lottenborgvej 26, 2800 Kongens Lyngby, Denmark; [5]Wageningen Bioveterinary Research, Houtribweg 39, 8221 RA, Lelystad, The Netherlands; [6]Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, The Netherlands; [7]Centre of Infectious Disease Control, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA, Bilthoven, The Netherlands

*Corresponding author. Tel: +31 (0) 30 253 6839; E-mail: l.vangompel@uu.nl orcid.org/0000-0002-6185-219X
†Members of the EFFORT group are listed in the Acknowledgements.

Received 10 April 2018; returned 7 July 2018; revised 30 October 2018; accepted 15 November 2018

Objectives: Previous studies in food-producing animals have shown associations between antimicrobial use (AMU) and resistance (AMR) in specifically isolated bacterial species. Multi-country data are scarce and only describe between-country differences. Here we investigate associations between the pig faecal mobile resistome and characteristics at the farm-level across Europe.

Methods: A cross-sectional study was conducted among 176 conventional pig farms from nine European countries. Twenty-five faecal samples from fattening pigs were pooled per farm and acquired resistomes were determined using shotgun metagenomics and the Resfinder reference database, i.e. the full collection of horizontally acquired AMR genes (ARGs). Normalized fragments resistance genes per kilobase reference per million bacterial fragments (FPKM) were calculated. Specific farm-level data (AMU, biosecurity) were collected. Random-effects meta-analyses were performed by country, relating farm-level data to relative ARG abundances (FPKM).

Results: Total AMU during fattening was positively associated with total ARG (total FPKM). Positive associations were particularly observed between widely used macrolides and tetracyclines, and ARGs corresponding to the respective antimicrobial classes. Significant AMU-ARG associations were not found for β-lactams and only few colistin ARGs were found, despite high use of these antimicrobial classes in younger pigs. Increased internal biosecurity was directly related to higher abundances of ARGs mainly encoding macrolide resistance. These effects of biosecurity were independent of AMU in mutually adjusted models.

Conclusions: Using resistome data in association studies is unprecedented and adds accuracy and new insights to previously observed AMU-AMR associations. Major components of the pig resistome are positively and independently associated with on-farm AMU and biosecurity conditions.

Introduction

In many European countries, antimicrobial resistance (AMR) rates in human and zoonotic bacteria are high and/or on the rise.[1,2] Unfortunately, antimicrobial drug discovery is not keeping pace with the rise in AMR.[3] In 2014, this lack of appropriate treatment options was estimated to result in ∼700000 attributable human deaths per annum worldwide.[4] While the need for research on new drugs is self-evident, aetiological research investigating determinants of AMR is equally warranted.

It is generally accepted that veterinary antimicrobial use (AMU) promotes AMR in animals, and AMR can be transmitted from animals to humans via the food chain or through direct animal

contact.[5] To date, the majority of association studies in food-producing animals have focused on the relationship between AMR and AMU or other farm practices within one particular resistant bacterial species and/or pathogen, e.g. studies on MRSA (pigs, veal)[6–9] or *Escherichia coli* (pigs, poultry, veal, dairy cows).[10–15] Few studies report on multi-country data, but mostly report on associations between AMU and AMR at the ecological level.[5,16] Since regional differences in AMR are observed between European countries,[1] insight into the role of on-farm practices that contribute to the observed differences is paramount to reduce AMR in farm animals.

In 2006 the term 'resistome' was proposed to designate the full collection of resistance genes in microorganisms and microbial populations.[17,18] In recent years, the decreasing costs of high-throughput metagenomic approaches[19] have enabled researchers to study the resistome in a multitude of different reservoirs, e.g. humans, animals, soil, toilet waste, sewage and permafrost.[20–22,24–28] Acquired resistance genes from the resistome can be transmitted between bacterial species through horizontal gene transfer. Rather than focusing on specific (pathogenic) resistant bacteria, studying this 'mobile resistome' can provide a more complete understanding of the epidemiology of AMR in the complex human–animal interface.[26] Currently, metagenomic resistome data are scarcely used in aetiological studies. In 2016, Xiao *et al.*[29] investigated the influence of overall AMU in pigs on the composition of the pig resistome. Despite the limited number of investigated farms (N=3), higher abundances of AMR genes (ARGs) were seen in pigs continuously fed low doses of antimicrobials.

More recently, our research group demonstrated positive associations between country-level AMU in pigs and the pig resistome (relative ARG abundances).[30] Within the present study we further build upon the latter study by including detailed farm-level data; in particular, we used a shotgun metagenomic approach to investigate associations between the pig resistome and farm-level risk factors (e.g. AMU classes, biosecurity, farm production figures). To the best of our knowledge, the inclusion of detailed farm-level data in epidemiological resistome models (a model including both farm-level AMU and the resistome) is unprecedented.

Materials and methods

Study design

Between 4 June 2014 and 9 December 2015, we conducted a cross-sectional study among a convenience sample of 181 pig farms in 9 European countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain). At each farm, 25 pig faecal samples were collected and pooled as described in the supporting materials (see Supplementary Methods, available at *JAC* Online). Figure 1 presents the distribution of sampled farms across Europe. Only conventional, non-mixed, farrow-to-finish farms (sporadic exceptions as mentioned before)[30] were selected and sampled across seasons. Farm recruiting occurred directly from a randomized database or through veterinarians and slaughterhouses. The selected herds had to have only one owner and no contact through trade. Additional selection criteria were required at the farm (location of the fatteners, minimum number of sows and fatteners present on the farm) and herd-level (all-in all-out batch production), yet not always met, as previously described in detail.[30]

Data collection

For an elaborate description of the pig sampling procedure, handling of samples and storage, sample pooling, DNA extraction, sequencing, metagenomic data clean-up and bioinformatics, the reader is referred to Munk *et al.*[30] The DNA sequences (reads) from the 181 metagenomic samples are deposited in the European Nucleotide Archive under project accession number PRJEB22062.

Brief summaries regarding sampling, pooling, laboratory work, metagenomics and questionnaire data are provided below and in the Supplementary data of the current paper. Owing to lack of complete data, six farms [farm identifiers (IDs): 1001, 1009, 9001–9003 and 9006] were excluded from the analysis resulting in 176 farms. Analyses using biosecurity data were performed in all countries except one, due to lack of contrast and being out of range of expected values (country I).

Questionnaire data

Standardized farm questionnaires (including AMU, farm technical and biosecurity questions) were completed by the farmers together with the visiting researcher. To avoid social desirability bias, certain biosecurity questions were verified during farm inspections. Questionnaire data were entered and checked for quality in EpiData 3.1. Further data quality checks were performed using ActivePerl 5.24.1[31] and SAS 9.4.[32] SAS was subsequently used for database management. To check data entry quality, 10% of all questionnaires were entered twice and compared. AMU data were re-evaluated in-depth after conversion to Microsoft Excel, while other farm data were analysed descriptively in 'R' v.3.3.1.[33] Inconsistencies were thoroughly re-evaluated by the researchers responsible for the data. Finally, a database was created that consists of all AMR data and meta-data (farm characteristics and AMU). All farms were anonymized to ensure that results could not be traced back to individual farms. Countries were anonymized as required by the farming organization in one participating country.

Calculation of antimicrobial treatment incidences

The calculation of antimicrobial treatment incidences (TIs) is summarized in the Supplementary data. AMU group TIs based on the Defined Daily Doses Animal (DDDvet) were computed at farm-level. A group treatment was defined as any treatment applied simultaneously to all animals present in, at least, the smallest housing unit, and includes any parental or other curative group treatment next to metaphylactic or prophylactic group treatments. TI expresses the percentage of pigs receiving a dose of antimicrobials each day, or equivalently, during the percentage of time a pig is treated with antimicrobials in a certain production phase or its entire life. TIs were determined for three age categories (TI sucklers, weaners, fatteners) and a standardized 200 day lifespan[34] across age categories (TI 200, which corrects for possible differences in slaughter ages between herds and countries), per farm and by AMU class.

Since multiple farms did not use any antimicrobials, we also performed a separate analysis while dichotomizing certain AMU variables. Country AMU variables, for which farms in at least seven countries expressed non-use, were dichotomized at the median value. If non-use for a specific AMU variable was observed in all farms, except for one country, we excluded this country from this variable.

Furthermore, TIs based on antimicrobial purchases were calculated, which includes AMU purchase data from a standardized time period before the farm visit.[59] Unlike AMU purchase data, AMU group treatments directly relate to the sampled batch. Therefore, we only used AMU purchase data within a sensitivity analysis.

Biosecurity indexes calculation

The algorithm behind Biocheck.ugent®, a risk-based scoring tool to evaluate the quality of biosecurity within a pig herd, was used to calculate a biosecurity score for each farm based on 108 biosecurity questions.[35,36] Each question was part of a biosecurity subcategory of external (measures to prevent disease introduction on farms) or internal (measures to prevent disease

Figure 1. Pig farm sampling across nine European countries. Yellow dots represent either single farms or a multitude of sampled pig farms for which GPS coordinates were available. Farm GPS coordinates used to prepare this map were anonymized and could therefore deviate up to 7 km from the true location. Sampling in France and Italy is respectively concentrated within Brittany and in northern Italy, as most pig farming is concentrated there [source: Eurostat: http://ec.europa.eu/eurostat/statistics-explained/index.php/File: Number_of_sows_by_region_(2013).png]. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

spread within farms) biosecurity. Within these two subcategories, weights were assigned to each subcategory (Table S1), and to each particular answer to a subquestion within each subcategory.[35] Total biosecurity was defined as the mean of external and internal biosecurity. One question from the original Biocheck.ugent® could not be included ('How frequently is the fluid of footbaths changed') and the algorithm was therefore slightly modified. Overall, three main and 12 subscores were calculated per farm (Table S1).

Table 1. Overview of sampled farms and batches of pigs

Characteristics	Country A	Country B	Country C	Country D	Country E	Country F	Country G	Country H	Country I	Overall
General farm information[a]										
no. of included farms	20	20	20	20	20	19	20	20	17	176 farms[b]
farms which held other animals at farm for non-commercial purposes, *n* (% of farms)**	14 (70.0)	4 (20.0)	16 (80.0)	5 (25.0)	11 (55.0)	17 (89.5)	3 (15.0)	9 (45.0)	5 (29.4)	84 (47.7)
people working at the farm in total, median (10th–90th %ile)**	2.0 (1.0–3.1)	3.0 (2.0–6.2)	3.5 (2.0–7.1)	5.0 (2.9–10.0)	3.0 (2.0–5.0)	3.0 (1.8–8.0)	4.0 (2.0–17.8)	5.0 (2.0–12.2)	5.0 (2.0–23.2)	3.0 (2.0–10.0)
farms with a certain type of batch management production system, *n* (%)***										
1 week (%)	3 (15.0)	7 (35.0)	11 (55.0)	10 (50.0)	3 (15.0)	10 (52.6)	11 (55.0)	11 (55.0)	6 (35.3)	72 (40.9)
2 week (%)	3 (15.0)	4 (20.0)	6 (30.0)	1 (5.0)	5 (25.0)	1 (5.3)	0	2 (10.0)	0	22 (12.5)
3 week (%)	4 (20.0)	5 (25.0)	1 (5.0)	8 (40.0)	9 (45.0)	2 (10.5)	7 (35.0)	4 (20.0)	0	40 (22.7)
4 week (%)	8 (40.0)	0	0	0	0	2 (10.5)	2 (10.0)	3 (15.0)	9 (52.9)	24 (13.6)
5 week (%)	2 (10.0)	3 (15.0)	0	1 (5.0)	3 (15.0)	3 (15.8)	0	0	2 (11.8)	14 (8.0)
other (%)	0	1 (5.0)	2 (10.0)	0	0	1 (5.3)	0	0	0	4 (2.27)
Characteristics of the sampled batch, median (10th–90th %iles)[a]										
fatteners sampled**	307 (109–909)	160 (93–2623)	394 (154–777)	342 (226–713)	319 (120–495)	200 (95–568)	240 (129–433)	1100 (641–3000)	600 (122–1753)	333 (106–1339)
age of the fatteners at sampling, days**	182 (168–214)	192 (170–210)	150[c] (123–172)	270 (232–287)	175 (161–189)	178 (165–191)	162 (149–172)	168 (150–182)	128 (110–164)	173 (135–246)
Farm technical data, median (10th–90th %iles)[a]										
fatteners set-up/year*	4442 (2920–11306)	4429 (2870–10039)	4600[c] (3292–7080)	13 700 (4602–23070)	6371 (4009–13692)	6473 (3016–17417)	5075 (3000–12818)	4225 (1980–7380)	2000 (720–54800)	5100 (2052–17248)
producing sows present at the farm/year**	266 (152–527)	341 (148–1353)	253 (149–629)	629 (276–1123)	276 (164–489)	290 (189–882)	250 (155–565)	550 (200–2056)	100 (44–750)	297 (149–1091)
producing boars present at the farm/year**	2.0 (1.0–5.1)	2.0 (1.0–5.8)	4.0 (2.0–8.0)	4.5 (1.9–13.1)	2.0 (1.0–5.1)	2.0 (1.0–5.0)	2.0 (1.0–6.2)	3.0 (2.0–8.1)	5.0 (2.6–10.0)	2.0 (1.0–7.7)
age of transfer of the weaners to growing-finishing department, days**	70 (63.9–84.6)	70 (67.8–85.5)	78[c] (69.0–84.0)	90 (81.8–111.0)	70 (62.9–81.3)	70 (58.4–81.6)	68.5 (56–77.7)	60 (60.0–75.0)	72 (68–75)	71 (60–90)
weaning age, days**	23.5 (20.0–25.2)	27.0 (21.0–28.0)	28.0 (25.0–32.1)	28.0 (24.6–28.0)	24.0 (21.0–28.0)	27.0 (22.6–28.0)	28.0 (24.6–28.0)	24.0 (21.0–28.0)	28.0 (28.0–35.0)	28.0 (21.0–29.0)
no. of weaned piglets/sow/year**	30.1 (23.5–32.1)	29.0 (22.3–31.0)	28.1 (26.3–31.1)	24.5 (22.4–27.1)	29.5 (27.9–31.6)	29.6 (27.7–31.3)	25.5 (22.9–30.1)	26.0 (22.9–27.6)	23.0 (18.0–26.0)	27.2 (22.5–30.9)

Percentages and percentiles are rounded to one or no decimal places. Letters A–I represent the nine countries.
[a]Significant difference between countries based on Kruskal–Wallis or continuity corrected χ^2 test: *, <0.01; **, <0.001; ***, not possible to test.
[b]In total, 33.0% (*N*=58) of all farms were visited in autumn, 16.5% (*N*=29) in spring, 38.6% (*N*=68) in summer and 11.9% (*N*=21) in winter.
[c]One observation was missing.

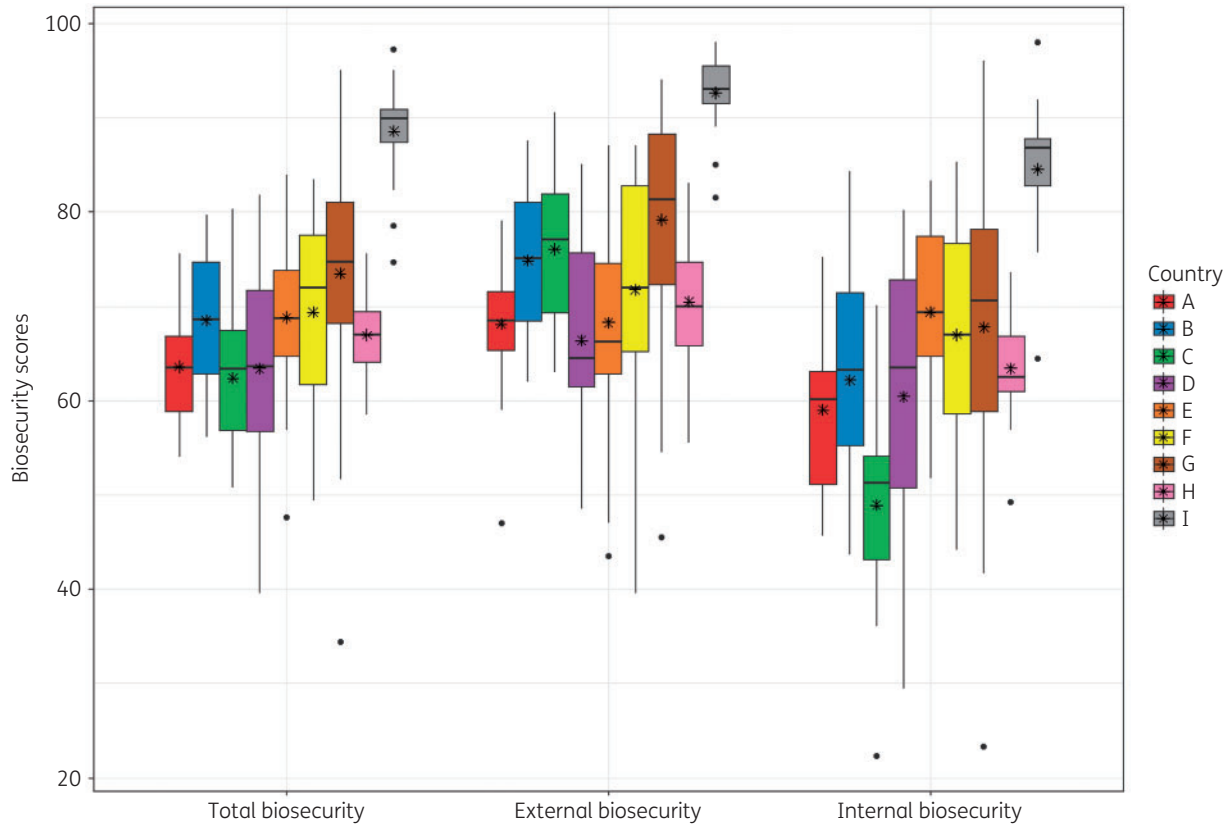

Figure 2. Boxplots: an overview of the three main biosecurity scores (total, external and internal) computed per farm (176 farms) presented by country. Scores can vary between 0% (low) and 100% (high). Box represents the IQR and centre line depicts the median. Dots represent outliers (values smaller than Q1–1.5×IQR or larger than Q3+1.5×IQR). Asterisk shows the mean by country. Letters A–I represent the nine countries. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Statistical analysis

'R' software (version 3.3.2)[33] was used for statistical analysis and creating figures. Farm technical variables were selected and checked for correlation. In case of high correlation (Spearman's $\rho \geq 0.7$), we chose the most appropriate variable based on the risk factor literature. To increase power and to evaluate known cross-resistances, all AMR variables were clustered by AMU class, and all AMU class variables were used as input for the models, together with several combinations of these AMR and AMU class variables (Table S1). Since AMR was previously linked to local temperature, we also included sampling season as a potential determinant.[37]

As described in the supporting materials, relative ARG abundances [fragments per kilobase reference per million bacterial fragments (FPKM)] were generated and aggregated (cluster levels: 90% identity level and AMR class). Based on previous analyses[30] a large country effect was expected and therefore a random-effects meta-analysis (DerSimonian-Laird estimator, linear regression, by country) was chosen to relate, univariately, each ARG cluster to AMU, biosecurity and technical farm data ('R' packages *rmeta*, *metafor*). ARG abundances and AMU data were strongly skewed to the right requiring $\log_{10}$ transformation. Owing to excess zeros, a pseudocount of 1 was added before the $\log_{10}$ transformation.

Choosing meta-analysis as a statistical technique inevitably results in country exclusion when no AMU/AMR is observed. To avoid spurious associations and overestimation of results, summary estimates were only preserved when estimates from at least four countries were incorporated.

The meta-analysis returned two *P* values: one regarding the overall effect size [Benjamini–Hochberg false discovery rate (FDR) adjusted, $q \leq 0.1$ threshold], and the other was between-country heterogeneity ($p_h > 0.05$, absence of heterogeneity). Within a random-effects meta-analysis weights are assigned by country which takes the between and within-country variation into account. To prevent certain countries from influencing these weights excessively, ARG clusters were standardized (mean=0, SD=1) by country. Models were first adjusted for potential confounders from the main list of univariate significant associations ($P < 0.05$, $q > 0.1$). To exclude confounding by AMU and biosecurity, we separately re-analysed the data adding both internal biosecurity and AMU to a model with its main associated AMR type. Internal biosecurity was chosen over other biosecurity variables (total biosecurity, internal biosecurity subcategories) based on high correlations (Spearman's $\rho \geq 0.7$) between biosecurity variables. For the most important associations defined in the univariate meta-analysis, the lower aggregation ARG clusters (90% identity level) were regressed on AMU and biosecurity.

Overall, the meta-analyses provide estimates of the association between the determinants and the relative ARG clusters (FPKM). These estimates should be considered as associations without direct straightforward and practical implementation. First, FPKM values are interpreted as the number of fragments of a certain clustered resistance gene within the full bacterial population present within a faecal sample, and do not allow direct absolute gene count comparison between samples. Since we added a pseudocount prior to $\log_{10}$ transformation (AMU and ARGs) 'calculating back' to easily interpretable estimates becomes challenging. Lastly, since we scaled the ARG abundances, the change in the determinants shall be interpreted as a change in standardized units of the ARGs.

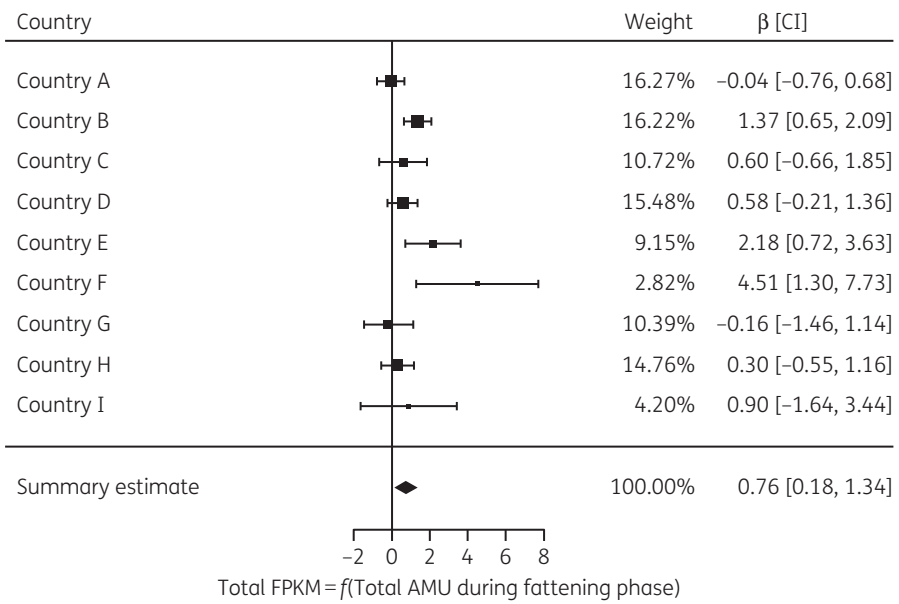

Figure 3. Univariate meta-analysis by country: Total AMU during fattening phase versus total relative ARG abundance (total FPKM), $P<0.01$. Heterogeneity test (p_h) is significant only when including country F (very low AMU). Without country F: summary estimate β=0.64, $P=0.014$ and $p_h>0.05$. β [CI], estimate of the linear regression and [upper and lower CI]. p_h, *P* value heterogeneity test. Weight, the assigned weight per country.

Results

Farms

Questionnaires and resistome data were available from 176 farms. At least one farm per season was visited within each country, except for countries F and H (no winter), and country I (no spring and winter). Table 1 provides summary statistics of the sampled farms and batches. Certain variables indicate large within- and between-country differences, e.g. the number of fatteners sampled, fatteners set-up per year and producing sows.

Pig resistome and AMU group treatment data

Earlier reported resistome[30] and AMU data.[59] for the 176 farms included in this study are summarized in the Supplementary data (Figures S1-S5).

Biosecurity scores

Figure 2 displays the main calculated biosecurity scores from the 176 farms, showing large within- and between-country variation. The higher the score, the higher the respective type of farm biosecurity. Overall, farm external biosecurity (median=73.9) was higher than internal biosecurity (median=65.0). Furthermore, within these two biosecurity categories, the medians differed significantly between countries (Kruskal–Wallis, $P<0.001$).

Random-effects meta-analysis

In total, we regressed 16 main ARG classes (clustering of ARGs based on AMR class) on 14 farm meta-variables (including season), 15 main AMU classes and 15 biosecurity scores in a random-effects meta-analysis by country (excluding AMU/AMR combinations; Table S1). The univariate meta-analysis showed a positive association between total AMU during the fattening phase and total ARG (AMR class clustering) in pigs (β=0.76, $P<0.01$; Figure 3), but not for AMU during other life phases. In total, 122 significant associations between ARG classes and determinants ($P<0.05$) were identified including potential confounders. After excluding confounders and correcting for multiple testing, 18 associations remained ($q<0.1$, $p_h>0.05$; Table 2).

Among the main determinants for AMR, we found positive corresponding AMU-ARG associations for macrolides and tetracyclines. Interestingly, no similar associations existed between the most commonly used antimicrobials colistin and aminopenicillins and their corresponding ARG classes. When aminopenicillin use was high in sucklers or weaners, and low in fatteners, no association was found with the corresponding ARG class (β-lactam resistance was relatively low). Similarly, colistin use was high in sucklers, lower in fatteners (only 9% of the farms use colistin in fatteners, compared with 18% that use macrolides) and low colistin resistance was observed.

We also found positive associations between non-corresponding AMU-ARG classes such as β-lactam use (penicillins, aminopenicillins and cephalosporins) and amphenicol resistance. Next to AMU, we identified higher internal biosecurity as a determinant for (mainly) macrolide resistance. These latter associations remained after adjustment for AMU (Table 2).

Many farms did not use critically important antimicrobials such as (fluoro)quinolones or aminoglycosides, and dichotomous AMU was deemed more appropriate in these cases. This partly dichotomized analysis confirmed the results from the main analysis (except the association between penicillin/aminopenicillin use and amphenicol resistance), although after correcting for the FDR, these associations were no longer statistically significant. Furthermore, in comparison with the main analysis, amphenicol use (TI 200) was positively associated with amphenicol resistance

Table 2. Results random-effects meta-analysis: AMR class clusters ($q<0.1$, $p_h>0.05$)

AMU group treatments and biosecurity scores	AMR class	β	β*	β**	[LCI, UCI]	[LCI, UCI]*	[LCI, UCI]**	*q*	*P**	*P***	Countries included***
Lincosamides & macrolides use (fatteners)•	macrolide, oxazolidinone+	1.076	1.047	1.018	[0.602, 1.550]	[0.530, 1.563]	[0.567, 1.469]	0.006	0.0001	<0.0001	8
Lincosamides & macrolides use (fatteners)•	macrolide	1.076	1.046	1.018	[0.602, 1.550]	[0.530, 1.563]	[0.567, 1.469]	0.006	0.0001	<0.0001	8
Internal biosecurity	macrolide	0.027	0.022	0.029	[0.014, 0.039]	[0.007, 0.038]	[0.015, 0.042]	0.013	0.0049	<0.0001	8
Internal biosecurity	macrolide, oxazolidinone+	0.027	0.022	0.029	[0.014, 0.039]	[0.007, 0.038]	[0.015, 0.042]	0.013	0.0049	<0.0001	8
Macrolide use (fatteners)	macrolide	1.061	1.118	1.258	[0.537, 1.585]	[0.506, 1.730]	[0.795, 1.721]	0.015	0.0003	<0.0001	7
Macrolide use (fatteners)	macrolide, oxazolidinone+	1.061	1.118	1.258	[0.537, 1.585]	[0.506, 1.730]	[0.795, 1.721]	0.015	0.0003	<0.0001	7
Lincosamides & macrolides use (200 days)•	macrolide, oxazolidinone+	0.825	0.925	0.750	[0.410, 1.239]	[0.458, 1.393]	[0.191, 1.310]	0.016	0.0001	0.0085	8
Lincosamides & macrolides use (200 days)•	macrolide	0.825	0.926	0.750	[0.410, 1.240]	[0.459, 1.393]	[0.191, 1.309]	0.016	0.0001	0.0085	8
IB: Cleaning & disinfection	macrolide	0.015	0.011		[0.007, 0.023]	[0.002, 0.020]		0.024	0.0126		8
IB: Cleaning & disinfection	macrolide, oxazolidinone+	0.015	0.011		[0.007, 0.023]	[0.002, 0.020]		0.024	0.0126		8
IB: Measures compart. & equipment use	vancomycin	0.016	0.016		[0.008, 0.025]	[0.006, 0.025]		0.025	0.0017		8
β-lactam use (fatteners)#	phenicol (amphenicol)	1.212	1.332		[0.569, 1.855]	[0.619, 2.044]		0.025	0.0002		7
Tetracycline use (200 days)••	tetracycline	0.754	0.558		[0.346, 1.163]	[0.089, 1.027]		0.031	0.0197		9
IB: Measures compart. & equipment use	macrolide	0.016	0.016		[0.007, 0.024]	[0.007, 0.025]		0.032	0.0006		8
IB: Measures compart. & equipment use	macrolide, oxazolidinone+	0.016	0.016		[0.007, 0.024]	[0.007, 0.025]		0.032	0.0006		8
Total biosecurity	macrolide	0.032	$p_h<0.05$		[0.014, 0.051]	$p_h<0.05$		0.042	$p_h<0.05$		8
Total biosecurity	macrolide, oxazolidinone+	0.032	$p_h<0.05$		[0.014, 0.051]	$p_h<0.05$		0.042	$p_h<0.05$		8
β-lactam use (fatteners)##	phenicol (amphenicol)	1.163	1.275		[0.500, 1.825]	[0.561, 1.989]		0.044	0.0005		7

All figures are rounded. Presented *q* value is only applicable to β. [LCI, UCI], lower and upper CI. β* [LCI, UCI] and associated *P* value corrected for three confounders: 'number of fatteners set-up/year', 'number of producing sows present/year' and 'sampling age'. β** [LCI, UCI] and associated *P* value corrected for internal biosecurity (AMU) and corrected for AMU (internal biosecurity). A correction was not performed for the internal biosecurity subcategories and total biosecurity due to high overall correlation (Spearman's $\rho>0.7$) with internal biosecurity. Internal biosecurity is corrected for 'TI fatteners lincosamide and macrolide'. When internal biosecurity is corrected for 'TI 200 lincosamide and macrolide' or 'TI fatteners macrolide', the internal biosecurity estimates and CIs become respectively 0.029 [0.014, 0.045] and 0.035 [0.015, 0.054]. ***Countries included in each association: countries were excluded if no AMU was recorded on all farms from the respective country. All biosecurity analyses included eight countries.

Variable explanations: lincosamide and macrolide use (fatteners)•, macrolide+lincosamide+lincomycin/spectinomycin AMU in fatteners. Lincosamide and macrolide use (200 days)•, macrolide+lincosamide+lincomycin/spectinomycin AMU corrected for a lifespan of 200 days. β-lactam use (fatteners)#, β-lactam AMU (penicillin+aminopenicillin+cephalosporin) in fatteners. β-lactam use (fatteners)##, β-lactam AMU (penicillin+aminopenicillin) in fatteners. Tetracycline use (200 days)••, tetracycline use corrected for a lifespan of 200 days. Macrolide, oxazolidinone+, macrolide+macrolide, oxazolidinone and phenicol AMR. IB: Cleaning & disinfection, internal biosecurity subcategory 'Cleaning & disinfection'. IB: Measures comp. & equipment use, internal biosecurity subcategory 'Measures between compartments & use of equipment'.

($q<0.085$, $P<0.001$). Another sensitivity analysis using AMU purchase data instead of AMU group treatments only confirmed the positive associations involving macrolide resistance (results not shown).

Figure 4 displays an overview of the top 10 ARG clusters (90% identity level) within the main ARG classes identified in the meta-analysis. Large between-country differences (20%–40%) were observed when examining the contribution of the most abundant gene cluster within each antimicrobial class, e.g. for macrolide, *mefA_3_AF227521*; tetracycline, *tetQ*; and amphenicol, *cat2*. The univariate meta-analysis was repeated by including the ARG clusters, for which the most important associations ($q<0.1$, $p_h>0.05$) are presented in Table 3.

Discussion

The present study evaluates associations between farm characteristics and faecal (largely) mobile resistomes of conventionally held fattening pigs across nine European countries. The amount of macrolides and tetracyclines used was associated with the abundance of macrolide and tetracycline resistance genes. Furthermore, internal biosecurity was independently associated with (mainly) increased macrolide resistance.

Our results show robust evidence of direct ARG selection by these two widely used antimicrobial classes (macrolides, tetracyclines). These effects were observed when evaluating both levels of ARG clustering (ARG class and 90% identity level). Macrolide and tetracycline use in pigs have previously been linked to, respectively, macrolide and tetracycline resistance in specific bacterial species.[10,38–41] We also showed evidence for cross-ARG selection (combination of macrolide, lincosamide and lincomycin/spectinomycin use versus macrolide resistance) and co-selection of ARGs (a combination of penicillin, aminopenicillin and cephalosporin use versus amphenicol resistance). The observed cross-resistance to macrolide and lincosamide use is expected based on the mechanism behind macrolide resistance.[42,43] However, to the best of our knowledge, the association between β-lactam use and amphenicol ARGs has not been described before in pigs. Nevertheless, co-selection in pigs has been suggested in metagenomic studies. It was, for example, previously observed that in-feed ASP250 (chlortetracycline, sulfamethazine and penicillin) treatment in experimentally held pigs, increased ARG abundance beyond the administered ASP250, including amphenicol resistance.[44]

Sensitivity analyses using antimicrobial purchase and dichotomized AMU data did not fundamentally change our main findings involving macrolide AMU-AMR associations. None the less, dichotomization of AMU only confirmed our results prior to FDR correction. We consider this a negligible discrepancy since FDR correction could be considered conservative[45] and dichotomizing continuous variables likely results in loss of information and statistical power.[46]

In our study, the observed AMU-ARG associations might be characterized in different ways. Most antimicrobial group treatments were administered during early production stages (suckling and weaning), but we mainly found AMU-ARG associations corresponding to treatments administered during the fattening period (with the least AMU recorded).[59] Interestingly, when some antimicrobial classes were highly and specifically used during early life (e.g. aminopenicillins and colistin), no AMU-ARG relationships and only few colistin ARGs were found. Considering the assessment of the resistome in the final fattening phase, these findings suggest short-term selective pressure posed by early age AMU. Declining or lower AMR in the (final) fattening phase compared with AMR during earlier ages has been described before in longitudinal studies regarding ESBLs[47] and MRSA[48] in pigs. Although read-mapping was chosen over assembly- based strategies for greater sensitivity, this lack of association might also be assigned to low(er) ARG cluster levels (e.g. *mcr* or $bla_{CTX\text{-}M}$) detected within our metagenomic survey [e.g. in comparison to phenotypic assays ($bla_{CTX\text{-}M}$)[49] and since we only mapped against ResFinder[50]]. Another way to characterize these findings is by hypothesizing that the lack of AMU-ARG associations related to high colistin and β-lactam use potentially reflects natural history. It could be imagined that sustained past high use of certain antimicrobials, together with potentially differential bacterial fitness costs related to resistance,[51,52] developed and established the currently circulating pool of resistance genes.

Biocheck.ugent® scores for internal and total biosecurity were shown to be positively associated with macrolide resistance. The separate country estimates are consistently positive in most countries. Since the estimates hardly changed when adding both internal biosecurity and AMU to the model for macrolide resistance, we conclude that internal biosecurity is an independent determinant of macrolide resistance. Within internal biosecurity, the subcategories 'cleaning and disinfection' (eight countries with positive slope) and 'measurements between compartments and use of equipment' (M&E) (seven countries with positive slope) are shown to be positively associated with macrolide and vancomycin resistance (only M&E). In recent decades, evidence has built pointing to cross-resistance to antimicrobials and biocides (e.g. quaternary ammonium compounds) mediated by the hyperexpression or acquisition of bacterial efflux pumps.[53] Moreover, co-resistance has been suggested by linkage/co-occurrence of ARGs and biocide and metal resistance genes in bacteria and on plasmids.[23,53–55] Since both internal biosecurity 'cleaning and disinfection' (largely) and M&E (restrictively) are based on questions related to farm disinfection, a positive association between internal biosecurity and resistance might potentially be explained by high use of specific types of disinfectants.

Munk *et al.*[30] showed a strong correlation between the pig resistome and genus-level bacteriome by means of a Procrustes analysis. This analysis suggested that much of the between-country variation was explained by bacteriome differences. To the best of our knowledge, the present study is a first attempt at finding associations between the pig faecal resistome and farm-level data. To avoid spurious associations and to support the data complexity (~20 farms in nine countries), we took the strong between-country variation in resistomes and risk factors into account using a random-effects meta-analysis. Future larger studies might focus on implementing zero-inflated binomial or Hurdle models[56] to support further the excess zeros when analysing more thoroughly the ARGs at lower aggregation levels.

Adjustments for confounding were made during analysis, but other bias potentially remains. Our study design made an extensive effort to randomize farm selection, but did not always succeed. The farmers' willingness to participate might correlate with an interest in AMU/AMR reduction and consequently low AMU/AMR

Table 3. Results random-effects meta-analysis: lower aggregation resistance gene clusters (90% identity, $q<0.1$, $p_h>0.05$)

AMU and biosecurity	AMR class clustering	90% identity level clustering	β	[LCI, UCI]	q	Countries included*
Lincosamide & macrolide use (fatteners)•	macrolide	erm(T)_4_AJ488494	1.274	[0.823, 1.726]	<0.001	8
		erm(B)_clust	1.225	[0.769, 1.680]	<0.001	8
		erm(G)_clust	0.991	[0.305, 1.678]	0.047	8
		erm(F)_clust	0.702	[0.188, 1.217]	0.068	8
Macrolide use (fatteners)	macrolide	erm(B)_clust	1.392	[0.918, 1.867]	<0.001	7
		erm(T)_4_AJ488494	1.323	[0.834, 1.812]	<0.001	7
		mef(A)_3_AF227521	0.980	[0.453, 1.507]	0.011	7
		erm(A)_3_EU348758	0.757	[0.250, 1.265]	0.038	7
		erm(Q)	0.745	[0.175, 1.316]	0.088	7
Lincosamide & macrolide use (200 days)•	macrolide	erm(T)_clust	0.864	[0.365, 1.362]	0.017	8
		erm(F)_clust	0.724	[0.300, 1.148]	0.018	8
		mef(A)_3_AF227521	0.684	[0.262, 1.106]	0.023	8
		mef(A)_10_AF376746	0.626	[0.206, 1.047]	0.038	8
		msr(D)	0.687	[0.210, 1.165]	0.047	8
		erm(B)_clust	0.700	[0.162, 1.238]	0.089	8
Internal biosecurity	macrolide	erm(F)_clust	0.027	[0.014, 0.040]	0.002	8
		erm(B)_clust	0.023	[0.010, 0.036]	0.013	8
		erm(G)_clust	0.022	[0.009, 0.035]	0.020	8
		mef(A)_3_AF227521	0.019	[0.007, 0.032]	0.036	8
		erm(T)_4_AJ488494	0.018	[0.005, 0.031]	0.070	8
		mph(B)	0.017	[0.004, 0.030]	0.087	8
Tetracycline use (200 days)⁺	tetracycline	tet(M)	0.947	[0.437, 1.457]	0.011	9
		tet(W)	0.735	[0.318, 1.152]	0.014	9
		tet(40)	0.605	[0.190, 1.020]	0.046	9
		tetB(P)	0.615	[0.190, 1.040]	0.047	9
		tet(L)_clust1	0.673	[0.146, 1.199]	0.094	9
β-Lactam use (fatteners)#	phenicol (amphenicol)	cat_2	1.184	[0.268, 2.099]	0.089	7
β-Lactam use (fatteners)##	phenicol (amphenicol)	cat_2	1.174	[0.268, 2.080]	0.089	7

All figures are rounded. [LCI, UCI], lower and upper CIs. *Countries included in each association: countries were excluded if no AMU was recorded on all farms from the respective country. All biosecurity analyses included eight countries. Variable explanations: Lincosamide & macrolide use (fatteners)•, macrolide+lincosamide+lincomycin/spectinomycin AMU in fatteners. Lincosamide & macrolide use (200 days)•, macrolide+lincosamide+lincomycin/spectinomycin AMU corrected for a lifespan of 200 days. β-lactam use (fatteners)#, β-lactam AMU (penicillin+aminopenicillin+cephalosporin) in fatteners. β-Lactam use (fatteners)##, β-lactam AMU (penicillin+aminopenicillin) in fatteners. Tetracycline use (200 days)⁺, tetracycline use corrected for a lifespan of 200 days.

levels. No AMU/AMR also leads to a decreased sample size and potentially spurious associations, as the influence of non-use/no AMR cannot be estimated in a country-wise linear regression meta-analysis. We consequently excluded associations including less than four countries. Furthermore, while good farm AMU administration records were required, social desirability and recall bias could nevertheless have resulted in AMU and biosecurity misreporting or AMU underreporting and hence misclassification of exposure. Likewise, although the majority of treatments in pig production consist of group treatments,[57,58] the exclusion of individual treatments (due to an even higher risk of recall bias), might have led to misclassification of exposure. Despite strict protocols, bias could have resulted from countries employing their own field and lab workers.

Using resistome data provides a great level of detail and multiple outcome variables. This makes classical risk factor analysis less straightforward in comparison with studies involving specific AMR bacteria. First, ARG data are expressed as relative abundances (FPKM) and consequently less intuitive to interpret. Second, these ARGs could be localized in clinically irrelevant or dysfunctional/dead bacterial species, and no phenotypic resistance patterns (the combined expression of multiple ARGs) are studied. Third, less prevalent but still important resistance types (e.g. β-lactam resistance: $bla_{CTX\text{-}M}$) are more difficult to detect when using metagenomic sequencing data despite the high sequencing depth in our study. Finally, our study was limited by the ARGs present within the ResFinder database (largely acquired ARGs).

Macrolide	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
mef(A)_3_AF227521	32.13	31.90	29.07	34.11	24.70	33.52	48.25	51.61	22.24	33.67
lnu(C)	27.88	38.48	25.77	36.72	26.02	29.30	31.30	15.04	27.73	23.11
erm(F)_clust	18.74	20.36	9.78	3.94	28.73	23.27	8.12	13.12	22.28	13.29
erm(B)_clust	10.96	5.93	17.98	5.54	10.14	8.49	5.48	10.61	16.42	14.83
erm(G)_clust	3.58	0.98	4.34	10.83	2.38	2.72	2.69	3.97	4.17	3.67
lnu(A)	1.77	0.22	1.80	0.22	3.87	0.21	0.04	0.13	2.81	4.42
mef(A)_10_AF376746	1.11	0.83	1.89	1.48	1.11	1.07	1.25	1.33	0.82	0.86
msr(D)	1.01	0.23	2.31	5.89	0.38	0.31	1.68	0.91	0.26	0.86
lnu(B)	0.88	0.51	3.62	0.69	0.57	0.22	0.37	0.60	0.92	1.39
erm(Q)	0.52	0.24	1.75	0.30	0.51	0.34	0.37	0.38	0.54	0.62
Tetracycline	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
tet(Q)	38.57	42.05	27.54	44.25	39.34	35.28	46.63	51.54	27.48	41.93
tet(W)	30.78	33.51	34.42	33.22	29.01	31.88	28.81	24.76	33.16	26.63
tet(40)	11.55	7.51	8.71	4.46	15.38	15.69	9.50	5.94	18.79	10.97
tet(O)	7.36	7.22	9.07	9.05	6.35	8.13	7.54	5.74	6.91	6.83
tet(44)	5.14	5.23	8.51	6.05	3.96	4.23	4.78	4.24	4.96	4.98
tet(X)_clust	1.89	2.10	0.85	1.03	2.98	2.62	0.75	1.81	2.32	1.23
tet(L)_clust1	1.79	0.80	5.73	0.25	0.60	0.33	0.36	1.44	3.69	2.17
tetA(P)	0.73	0.45	2.15	0.71	0.52	0.46	0.57	0.55	0.58	0.70
tet(32)	0.73	0.74	1.50	0.55	0.38	0.75	0.59	0.44	0.89	0.62
tet(M)	0.59	0.16	0.47	0.13	0.90	0.35	0.13	1.24	0.60	1.24
Amphenicol	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
cat_2	60.05	48.95	25.59	27.41	63.40	30.32	42.28	16.14	41.06	54.47
catP	14.36	8.93	7.43	7.16	16.77	2.67	7.17	7.19	4.37	1.77
cat(pC194)	10.49	10.48	12.33	1.72	10.40	45.50	15.74	2.45	6.02	10.22
fexA	4.50	1.69	0.40	0.00	4.06	0.20	2.10	7.90	10.97	6.61
floR_clust	3.32	1.85	19.29	5.02	1.21	0.92	3.63	51.60	4.89	9.70
catQ	3.08	3.75	3.08	7.78	2.90	0.00	4.37	3.33	2.69	4.65
cmx	1.23	4.62	12.10	2.79	0.27	6.29	2.99	2.96	18.52	2.09
catS	0.69	6.32	5.53	23.30	0.43	3.17	13.75	2.44	1.67	0.23
cml_clust	0.48	2.02	3.45	0.00	0.05	0.30	0.00	0.75	5.25	2.30
cat_clust	0.48	0.00	5.19	0.89	0.07	0.92	0.00	0.56	0.86	3.20
Vancomycin	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
VanT-G	16.77	19.64	15.44	19.30	9.42	17.75	18.45	18.81	15.71	14.92
VanXY-G	16.15	16.47	12.68	18.95	8.77	18.99	15.77	17.38	17.15	20.17
VanW-G	13.32	13.55	11.36	14.31	9.08	15.01	13.73	13.73	13.62	17.13
VanG	11.50	10.55	8.96	12.71	7.77	11.35	14.49	13.35	12.17	12.34
VanX-B	5.70	7.41	6.21	5.20	10.82	2.58	5.39	4.86	4.53	4.13
VanH-B	4.96	4.20	6.70	3.42	9.24	4.86	4.96	4.23	4.41	1.51
VanA-B_1_AF192329	4.80	5.22	5.56	4.91	8.72	3.59	4.49	3.80	3.70	1.96
VanR-G	4.78	4.45	4.26	3.36	3.88	7.12	4.64	5.54	6.69	2.07
VanS-B	4.33	3.71	6.51	4.54	7.05	2.60	3.27	4.42	3.09	2.76
VanY-B	4.04	3.96	6.27	3.02	7.63	2.50	3.82	3.02	3.57	1.52
Oxazolidinone & Macrolides & Amphenicols	Overall %	A %	B %	C %	D %	E %	F %	G %	H %	I %
cfr_clust	100.00	0.00	100.00	0.00	100.00	100.00	0.00	100.00	100.00	100.00

Figure 4. Prevalence of the lower aggregation resistance gene cluster (90% identity) within a certain AMR gene class cluster identified in the meta-analysis. Only the top 10 (highest abundance) lower aggregation clusters within the AMR gene class cluster are presented. Country percentages: contribution (%) of a lower aggregation cluster within the AMR class cluster detected in a particular country. Blue scale indicates high (dark blue) to lower (light blue) contributions. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

In conclusion, our study is (as far as we are aware) unique in combining a large resistome dataset with risk factors. We established associations between ARGs and farming practices, which provides more insight into the biological pathways of resistance. Our approach enabled the evaluation of a large set of antimicrobial classes in parallel while checking for cross- and co-resistances (~2000 associations within nine countries). We therefore believe that our study provides an important first step in inferring the main drivers for AMR in pigs at the metagenomic level.

Acknowledgements

The authors would like to thank all participating farmers. Furthermore, the authors would like to express their sincere gratitude and appreciation for all laboratory assistance at the Danish Technical University (DTU) and the Institute for Risk Assessment Sciences, Utrecht University (IRAS, UU) and all others participating within the sampling and lab campaigns: Belgium: Marjolijn Schlepers. Bulgaria: Teodora Ivanova, Nikolay Cholakov, Eva Gurova-Mehmedova, Krasen Penchev. Germany: Franziska

Nienhaus. Denmark: Cecilie Liv Nielsen, Pia Ryt-Hansen, Bonnie Hølstad, Bettina Rasmussen, Kirstine Nielsen. France: Jenna Coton, Virginie Dorenlor, Florent Eono, Eric Eveno, Sophie Le Bouquin, Denis Leon, Rodolphe Thomas. Italy: Mario Gherpelli, Marco Pegoraro, Virginia Carfora. The Netherlands: Daisy de Vries. Poland: Beata Gawlik and Dorota Krasucka.

The abstract of this paper was presented at The 5th International One Health Congress, 23 June 2018, Saskatoon, Canada (Abstract number: 5 AMR 03).

Members of the EFFORT consortium

Haitske Graveland (UUVM), Alieda van Essen (WBVR), Bruno Gonzalez-Zorn (UCM), Gabriel Moyano (UCM), Pascal Sanders (ANSES), Claire Chauvin (ANSES), Julie David (ANSES), Antonio Battisti (IZSLT), Andrea Caprioli (IZSLT), Thomas Blaha (TIHO), Katharina Wadepohl (TIHO), Maximiliane Brandt (TIHO), Tine Hald (DTU), Ana Sofia Ribeiro Duarte (DTU), Dariusz Wasyl (NVRI), Magdalena Skarżyńska (NVRI), Magdalena Zając (NVRI), Andrzej Hoszowski (deceased) (NVRI), Hristo Daskalov (NDRVI), Helmut W. Saatkamp (BEC) and Katharina D. C. Stärk (SAFOSO).

Funding

This work was part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, co-funded by the European Commission, 7th Framework Programme for Research and Innovation (FP7-KBBE-2013–7, grant agreement: 613754).

Transparency declarations

None to declare.

Supplementary data

Supplementary Methods, Table S1 and Figures S1–S5 are available as Supplementary data at *JAC* Online.

References

1 European Food Safety Authority, European Centre for Disease Prevention and Control. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014. *EFSA J* 2016; **14**: 4380.

2 European Centre for Disease Prevention and Control. *Antimicrobial Resistance Surveillance in Europe-2015 Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net)*. Stockholm, 2017. https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/antimicrobial-resistance-europe-2015.pdf%0Ahttp://www.ecdc.europa.eu/en/publications/Publications/antimicrobial-resistance-surveillance-europe-2013.pdf.

3 Brown ED, Wright GD. Antibacterial drug discovery in the resistance era. *Nature* 2016; **529**: 336–43.

4 O'Neill J. *Review on Antimicrobial Resistance: Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*. London, UK, 2014. http://www.jpiamr.eu/wp-content/uploads/2014/12/AMR-Review-Paper-Tackling-a-crisis-for-the-health-and-wealth-of-nations_1-2.pdf.

5 European Centre for Disease Prevention and Control, Authority EFS, Agency EM. ECDC/EFSA/EMA second joint report on the integrated analysis of the consumption of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals. *EFSA J* 2017; **15**: 4006.

6 Graveland H, Wagenaar JA, Heesterbeek H *et al*. Methicillin resistant *Staphylococcus aureus* ST398 in veal calf farming: human MRSA carriage related with animal antimicrobial usage and farm hygiene. *PLoS One* 2010; **5**: e10990.

7 Bos MEH, Graveland H, Portengen L *et al*. Livestock-associated MRSA prevalence in veal calf production is associated with farm hygiene, use of antimicrobials, and age of the calves. *Prev Vet Med* 2012; **105**: 155–9.

8 Dorado-García A, Dohmen W, Bos MEH *et al*. Dose-Response Relationship between Antimicrobial Drugs and Livestock-Associated MRSA in Pig Farming. *Emerg Infect Dis* 2015; **21**: 950–9.

9 Dorado-García A, Graveland H, Bos MEH *et al*. Effects of reducing antimicrobial use and applying a cleaning and disinfection program in Veal Calf Farming: experiences from an Intervention Study to Control Livestock-Associated MRSA. *PLoS One* 2015; **10**: e0135826.

10 Dewulf J, Catry B, Timmerman T *et al*. Tetracycline-resistance in lactose-positive enteric coliforms originating from Belgian fattening pigs: degree of resistance, multiple resistance and risk factors. *Prev Vet Med* 2007; **78**: 339–51.

11 Persoons D, Haesebrouck F, Smet A *et al*. Risk factors for ceftiofur resistance in *Escherichia coli* from Belgian broilers. *Epidemiol Infect* 2011; **139**: 765–71.

12 Akwar HT, Poppe C, Wilson J *et al*. Associations of antimicrobial uses with antimicrobial resistance of fecal *Escherichia coli* from pigs on 47 farrow-to-finish farms in Ontario and British Columbia. *Can J Vet Res* 2008; **72**: 202–10.

13 Dunlop R, McEwen S, Meek A *et al*. Associations among antimicrobial drug treatments and antimicrobial resistance of fecal *Escherichia coli* of swine on 34 farrow-to-finish farms in Ontario, Canada. *Prev Vet Med* 1998; **34**: 283–305.

14 Dorado-García A, Mevius DJ, Jacobs JJH *et al*. Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. *J Antimicrob Chemother* 2016; **71**: 3607–19.

15 Dohmen W, Dorado-García A, Bonten MJM *et al*. Risk factors for ESBL-producing *Escherichia coli* on pig farms: a longitudinal study in the context of reduced use of antimicrobials. *PLoS One* 2017; **12**: e0174094.

16 Chantziaras I, Boyen F, Callens B *et al*. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J Antimicrob Chemother* 2014; **69**: 827–34.

17 D'Costa VM, McGrann KM, Hughes DW *et al*. Sampling the antibiotic resistome. *Science* 2006; **311**: 374–7.

18 Wright GD. The antibiotic resistome: the nexus of chemical and genetic diversity. *Nat Rev Microbiol* 2007; **5**: 175–86.

19 Thomas T, Gilbert J, Meyer F. Metagenomics—a guide from sampling to data analysis. *Microb Inform Exp* 2012; **2**: 3.

20 D'Costa VM, King CE, Kalan L *et al*. Antibiotic resistance is ancient. *Nature* 2011; **477**: 457–61.

21 Pal C, Bengtsson-Palme J, Kristiansson E *et al*. The structure and diversity of human, animal and environmental resistomes. *Microbiome* 2016; **4**: 54.

22 Forsberg KJ, Patel S, Gibson MK *et al*. Bacterial phylogeny structures soil resistomes across habitats. *Nature* 2014; **509**: 612–6.

23 Pal C, Bengtsson-Palme J, Kristiansson E *et al*. Co-occurrence of resistance genes to antibiotics, biocides and metals reveals novel insights into their co-selection potential. *BMC Genomics* 2015; **16**: 964.

24 Nordahl Petersen T, Rasmussen S, Hasman H *et al*. Meta-genomic analysis of toilet waste from long distance flights; a step towards global surveillance of infectious diseases and antimicrobial resistance. *Sci Rep* 2015; **5**: 11444.

25 Bengtsson-Palme J, Hammarén R, Pal C *et al*. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. *Sci Total Environ* 2016; **572**: 697–712.

26 Hu Y, Yang X, Li J *et al*. The bacterial mobile resistome transfer network connecting the animal and human microbiomes. *Appl Environ Microbiol* 2016; **82**: 6672–81.

27 Noyes NR, Yang X, Linke LM *et al*. Resistome diversity in cattle and the environment decreases during beef production. *Elife* 2016; **5**: e13195.

28 Munk P, Andersen VD, de Knegt L *et al*. A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. *J Antimicrob Chemother* 2017; **72**: 385–92.

29 Xiao L, Estellé J, Kiilerich P *et al*. A reference gene catalogue of the pig gut microbiome. *Nat Microbiol* 2016; **1**: 16161.

30 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.

31 ActiveState. *ActivePerl*. 2017. https://www.activestate.com/activeperl.

32 SAS Institute Inc. *SAS Software 9.4*. 2017.

33 R Core Team. *R: A Language and Environment for Statistical Computing*. 2017. https://www.r-project.org/.

34 Sjölund M, Postma M, Collineau L *et al*. Quantitative and qualitative antimicrobial usage patterns in farrow-to-finish pig herds in Belgium, France, Germany and Sweden. *Prev Vet Med* 2016; **130**: 41–50.

35 Laanen M, Persoons D, Ribbens S *et al*. Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds. *Vet J* 2013; **198**: 508–12.

36 Ghent University, Faculty of Veterinary Medicine, Department of Reproduction Obstetrics and Herd Health, Veterinary Epidemiology Unit MB. Biocheck.uGent® 2018. https://biocheck.ugent.be.

37 MacFadden DR, McGough SF, Fisman D *et al*. Antibiotic resistance increases with local temperature. *Nat Clim Chang* 2018; **8**: 510–4.

38 Jackson CR, Fedorka-Cray PJ, Barrett JB *et al*. Effects of tylosin use on erythromycin resistance in enterococci isolated from swine. *Appl Environ Microbiol* 2004; **70**: 4205–10.

39 Aarestrup FM, Carstensen B. Effect of tylosin used as a growth promoter on the occurrence of macrolide-resistant enterococci and staphylococci in pigs. *Microb Drug Resist* 1998; **4**: 307–12.

40 Aarestrup FM, Kruse H, Tast E *et al*. Associations between the use of antimicrobial agents for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and Norway. *Microb Drug Resist* 2000; **6**: 63–70.

41 Delsol AA, Anjum M, Woodward MJ *et al*. The effect of chlortetracycline treatment and its subsequent withdrawal on multi-resistant *Salmonella enterica* serovar Typhimurium DT104 and commensal *Escherichia coli* in the pig. *J Appl Microbiol* 2003; **95**: 1226–34.

42 Roberts MC, Sutcliffe J, Courvalin P *et al*. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. *Antimicrob Agents Chemother* 1999; **43**: 2823–30.

43 Roberts MC. Update on macrolide-lincosamide-streptogramin, ketolide, and oxazolidinone resistance genes. *FEMS Microbiol Lett* 2008; **282**: 147–59.

44 Looft T, Johnson TA, Allen HK *et al*. In-feed antibiotic effects on the swine intestinal microbiome. *Proc Natl Acad Sci USA* 2012; **109**: 1691–6.

45 Storey JD, Taylor JE, Siegmund D. Strong control, conservative point estimation and simultaneous conservative consistency of false discovery rates: a unified approach. *J R Stat Soc Series B Stat Methodol* 2004; **66**: 187–205.

46 Altman DG, Royston P. The cost of dichotomising continuous variables. *BMJ* 2006; **332**: 1080.

47 Hansen KH, Damborg P, Andreasen M *et al*. Carriage and fecal counts of cefotaxime M-producing *Escherichia coli* in pigs: a longitudinal study. *Appl Environ Microbiol* 2013; **79**: 794–8.

48 Weese JS, Zwambag A, Rosendal T *et al*. Longitudinal investigation of methicillin-resistant *Staphylococcus aureus* in piglets. *Zoonoses Public Health* 2011; **58**: 238–43.

49 Hammerum AM, Larsen J, Andersen VD *et al*. Characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. *J Antimicrob Chemother* 2014; **69**: 2650–7.

50 Zankari E, Hasman H, Cosentino S *et al*. Identification of acquired antimicrobial resistance genes. *J Antimicrob Chemother* 2012; **67**: 2640–4.

51 Rhouma M, Beaudry F, Thériault W *et al*. Colistin in pig production: chemistry, mechanism of antibacterial action, microbial resistance emergence, and one health perspectives. *Front Microbiol* 2016; **7**: 1–22.

52 Melnyk AH, Wong A, Kassen R. The fitness costs of antibiotic resistance mutations. *Evol Appl* 2015; **8**: 273–83.

53 Hegstad K, Langsrud S, Lunestad BT *et al*. Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health? *Microb Drug Resist* 2010; **16**: 91–104.

54 Fernández-Fuentes MA, Abriouel H, Ortega Morente E *et al*. Genetic determinants of antimicrobial resistance in Gram positive bacteria from organic foods. *Int J Food Microbiol* 2014; **172**: 49–56.

55 Hartmann EM, Hickey R, Hsu T *et al*. Antimicrobial chemicals are associated with elevated antibiotic resistance genes in the indoor dust microbiome. *Environ Sci Technol* 2016; **50**: 9807–15.

56 Xu L, Paterson AD, Turpin W *et al*. Assessment and selection of competing models for zero-inflated microbiome data. *PLoS One* 2015; **10**: e0129606.

57 Callens B, Persoons D, Maes D *et al*. Prophylactic and metaphylactic antimicrobial use in Belgian fattening pig herds. *Prev Vet Med* 2012; **106**: 53–62.

58 Regula G, Torriani K, Gassner B *et al*. Prescription patterns of antimicrobials in veterinary practices in Switzerland. *J Antimicrob Chemother* 2009; **63**: 805–11.

59 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 9 European countries based on single batch and purchase data. *J Antimicrob Chemother* 2019; **74**: 807–16.