Antimicrobial resistance monitoring in *Escherichia coli* from livestock

Evaluation & interpretation

Ayla Hesp

Antimicrobial resistance monitoring in *Escherichia coli* from livestock

Evaluation & interpretation

Ayla Hesp

2021

ISBN: 978-94-6423-504-3

Ayla Hesp, DVM
PhD thesis, Utrecht University, Utrecht, the Netherlands (2021)

Cover, photos and design: Jelle de Boer and Ayla Hesp
Lay-out: ProefschriftMaken || www.proefschriftmaken.nl

Antimicrobial resistance monitoring in *Escherichia coli* from livestock

Evaluation & interpretation

Monitoring van antimicrobiële resistentie in *Escherichia coli* uit landbouwhuisdieren

Evaluatie & interpretatie

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van de
rector magnificus, prof.dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op
donderdag 11 november 2021 des middags te 12.15 uur

door

Ayla Hesp

geboren op 7 december 1986
te Amsterdam

Promotoren:
Prof. dr. D.J. Mevius
Prof. dr. ir. G. van Schaik

Copromotor:
Dr. K.T. Veldman

Dit proefschrift werd mogelijk gemaakt met financiële steun van Wageningen Bioveterinary Research en Infection & Immunity Utrecht

Contents

Chapter 1

General introduction

General introduction

Effective antimicrobials are essential for adequate healthcare (WHO, 2015). Unfortunately, worldwide antimicrobial resistance (AMR) threatens this effectiveness (Walsh, 2003; Lai et al., 2014; Ventola, 2015), caused by the use of antimicrobials in humans, animals and in other applications (Walker et al., 2009; Chang et al., 2015; Hoelzer et al., 2017). The possibilities for development of antimicrobials are limited, and new antimicrobials will not become widely available (Blaskovich et al., 2017; Hutchings et al., 2019). This leaves prudent antimicrobial use (AMU) and other interventions to limit existing AMR as an important strategy (WHO, 2015). Therefore, AMR is monitored as a public health hazard, to enable the development of interventions by policy makers. Production animals are a relevant reservoir of AMR, because of AMU in livestock, and because AMR may be transmitted to humans directly, or indirectly via the food chain or the environment (Michael et al., 2014; Chang et al., 2015; Hoelzer et al., 2017). This thesis is about the monitoring of AMR in livestock as public health hazard in indicator organism *Escherichia coli*.

Definitions of monitoring and surveillance

Monitoring provides animal- or public health data from defined populations by systemic collection, analysis, interpretation and dissemination of results (Hoinville et al., 2013). *Surveillance* is defined as 'continuous or repeated measurement, providing descriptive information that is linked with action to mitigate risk' (Hoinville et al., 2013). The main difference with monitoring is that surveillance results are linked to interventions; there is a defined action plan in advance (Hoinville et al., 2013). Another relevant classification is *active* and *passive surveillance*. In 'active surveillance' investigators are actively collecting samples or data (Bisdorff et al., 2017), in 'passive surveillance' information comes to organisations by other means than active collection (Hoinville et al., 2013), for example the submissions of clinical samples from which data is analysed for surveillance purposes.

In the Netherlands, AMR monitoring results are reported yearly in Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN) (Figure 1) (MARAN, 2021) and by the European Food Safety Authority (EFSA) at European level (EFSA, 2018).

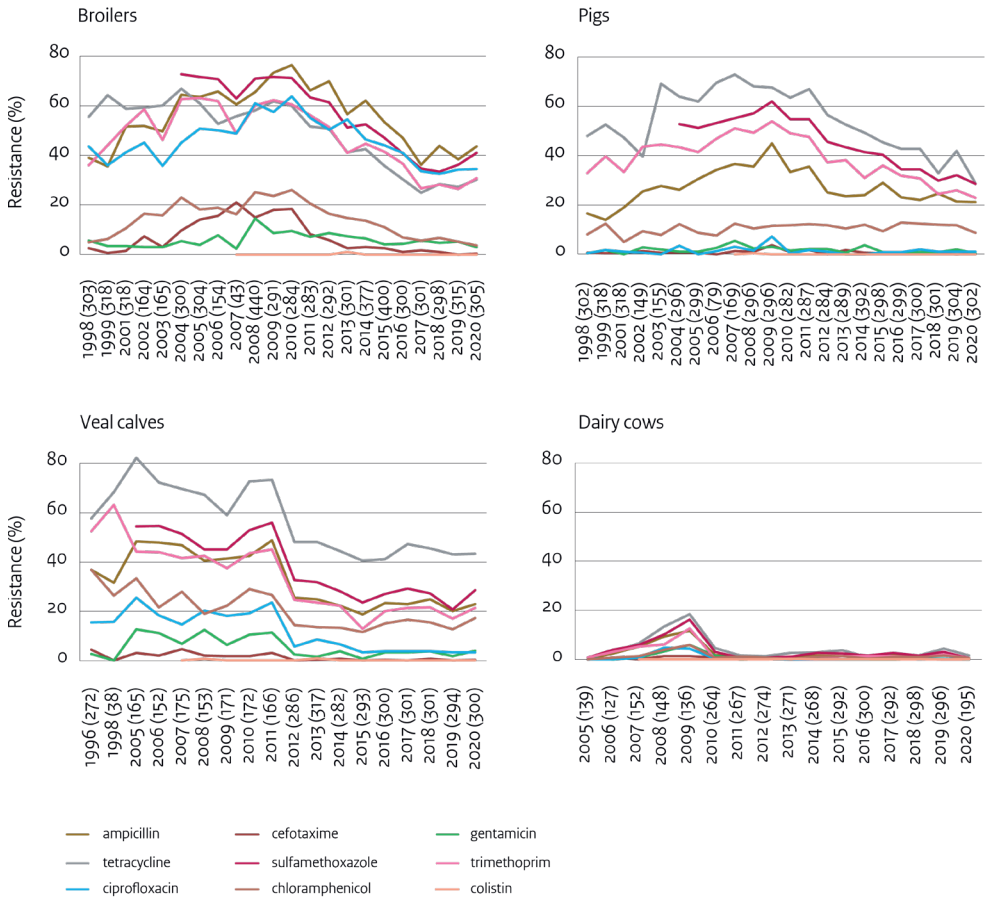


Figure 1. Trends in proportion of resistance (%) in *Escherichia coli* isolated from broilers, slaughter pigs, veal calves and dairy cattle in the Netherlands, 1998-2020

Adapted from MARAN, 2021. Veldman KT, Wit B, Franz E, Heederik D, 2021. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2020.

This monitoring program started in 1998, following recommendations of the Invitational European Union Conference 'The Microbial Threat', with the aim 'to monitor evolution and effects of interventions, through establishment of accurate surveillance systems on antimicrobial resistance in the human and veterinary sector' (Frimodt-Moller, 2004). *E. coli* was chosen as the indicator organism for gut microbiota in order to monitor the effects of antimicrobials with Gram-negative spectra. This program has long been 'monitoring': no intervening actions were defined based on the program. However, the rising

AMR trends found by this monitoring have initiated interventions (Mevius and Heederik, 2014), and the consequences of observing AMR in livestock have changed. Monitoring has (partly) shifted to surveillance. MARAN can be considered active surveillance, because samples are actively collected (Bisdorff et al., 2017). Some surveillance components of the current program can be defined as hazard-specific surveillance: specific resistance is actively screened for such as Extended-Spectrum Beta Lactamase (ESBL)- or AmpC- or carbapenemase-producing *Enterobacteriaceae*. Risk-based surveillance takes into account the probability of occurrence and magnitude of the biological and economic consequence of health hazards, to plan, design and interpret the results obtained from surveillance systems (Hoinville, 2013; Alban et al., 2016). For AMR, this is applicable when diseased animals instead of healthy animals are sampled to monitor resistance. In the rest of the thesis, we will use 'monitoring' as the most appropriate definition for the activities described, unless otherwise indicated.

Current AMR monitoring activities & gaps of knowledge

In the European Union, monitoring of AMR in animals as a public health hazard is performed under the Directive 2003/99/EC, and decisions 2013/652/EU and 2020/1729/EU. The directive obliges member states of the EU to monitor AMR in commensal *E. coli* and food-borne pathogens *Salmonella* and *Campylobacter* isolated from poultry, pigs and cattle. Guidelines on sample design, laboratory analytical methods for antimicrobial susceptibility testing (AST) and how to report data are provided in EU legislation 2013/652/EU and prescribed by the European Food Safety Authority (EFSA, 2019). The international legislation has helped to define the basic requirements for harmonisation and standardisation of AMR monitoring. Elements such as the sampling strategy and the microbiological methods are prescribed by legislation; they are 'input-based'. The trend is that the design of surveillance is left to experts, but output has to meet minimum requirements (Cameron, 2012). This 'output-based surveillance' creates room for improvement of prescribed ('input-based') surveillance (Cameron, 2012).

The evaluation and quantitative interpretation of AMR monitoring results is not prescribed by legislation but is challenging and will become more complex when more data is available. The updated EU legislation in 2020 has allowed whole-genome sequencing (WGS) as alternative method to culture-based AST in AMR monitoring (2020/1729/EU). So far, relevant statistical methods to validate the results of WGS-based AST versus culture-based AST were lacking. Ideally, the effects of interventions such as (reductions in) AMU are reflected in AMR monitoring data. The analyses so far have not allowed optimal evaluation

and interpretation of these effects. Also, evaluation of AMR monitoring itself can be optimized. Existing evaluation tools for monitoring of animal health could be applied to evaluate AMR monitoring, but to do so scientific expertise needs to be built. Active surveillance of AMR in healthy animals versus passive surveillance of AMR in diseased animals has not been evaluated. The relation between AMR in commensal and clinical bacterial isolates from the same animal population is mostly unknown.

Objective and outline of this thesis

The first aim of this thesis is to evaluate results of AMR monitoring commensal *E. coli,* with statistical methods and by assessing evaluation tools. Chapter 2 quantifies AMR trends in data from the Netherlands, 1998 to 2016, in broilers, slaughter pigs, and veal calves. Chapter 3 assesses different tools which can be used to evaluate AMR monitoring in different countries.

The second aim is to optimize the interpretation of AMR monitoring outcome. In Chapter 4, a multivariate cluster analysis was applied to AMR monitoring data from the Netherlands, 2007 to 2018, in broilers, slaughter pigs, veal calves, and dairy cows. This chapter aims to summarise AMR over multiple antimicrobial classes, as arguments for development of objective AMR monitoring outcome indicators. In Chapter 5, AMR is described in commensal *E. coli* from livestock in several European countries and the relationship with AMU and the EFSA outcome indicators was evaluated. Chapter 6 compares the active monitoring of non-wildtype susceptibility in commensal *E. coli* isolated from healthy animals with passive monitoring of clinical resistant *E. coli* from diseased broilers in the Netherlands, 2014 to 2019.

The third aim of this thesis to assess the value and test validity of WGS (Illumina high-throughput sequencing) to monitor AMR in livestock. In Chapter 7 Bayesian latent class analysis is used to evaluate the accuracy of WGS-based AST versus culture-based AST without a gold standard. In Chapter 8 we describe the benefits of WGS for monitoring purposes (apart from detection of resistance genes) in the same commensal *E. coli* isolates from livestock. Finally, the General discussion (Chapter 9) discusses the meaning of the findings in this thesis for future evaluation and interpretation of AMR monitoring in livestock.

References

Alban, L., Rugbjerg, H., Petersen, J.V., Nielsen, L.R., 2016. Comparison of risk-based versus random sampling in the monitoring of antimicrobial residues in Danish finishing pigs. Preventive veterinary medicine 128, 87-94.

Bisdorff, B., Schauer, B., Taylor, N., Rodríguez-Prieto, V., Comin, A., Brouwer, A., Dórea, F., Drewe, J., Hoinville, L., Lindberg, A., Martinez Avilés, M., Martínez-López, B., Peyre, M., Pinto Ferreira, J., Rushton, J., G, V.A.N.S., Stärk, K.D., Staubach, C., Vicente-Rubiano, M., Witteveen, G., Pfeiffer, D., Häsler, B., 2017. Active animal health surveillance in European Union Member States: gaps and opportunities. Epidemiol Infect 145, 802-817.

Blaskovich, M.A., Butler, M.S., Cooper, M.A., 2017. Polishing the tarnished silver bullet: the quest for new antibiotics. Essays Biochem 61, 103-114.

Cameron, A.R., 2012. The consequences of risk-based surveillance: Developing output-based standards for surveillance to demonstrate freedom from disease. Preventive veterinary medicine 105, 280-286.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., Hanage, W.P., 2015. Antibiotics in agriculture and the risk to human health: how worried should we be? Evol Appl 8, 240-247.

EFSA, 2018.European Food Safety Authority and European Centre for DiseasePrevention and Control. The European Union summary report on antimicrobial resistance inzoonotic and indicator bacteria from humans, animals and food in 2016. EFSA Journal 2018;16(2):5182, 270pp. https://doi.org/10.2903/j.efsa.2018.5182.

EFSA, 2019. Aerts, M., Battisti, A., Hendriksen, R., Kempf, I., Teale, C., Tenhagen, B.-A., Veldman, K., Wasyl, D., Guerra, B., Liébana, E., Thomas-López, D., Belœil, P.-A., 2019. Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. EFSA Journal 17, e05709.

Frimodt-Moller, N., 2004. Microbial Threat--The Copenhagen Recommendations initiative of the EU. J Vet Med B Infect Dis Vet Public Health 51, 400-402.

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., Coukell, A., 2017. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? BMC Vet Res 13, 211.

Hoinville, L.J., 2013. Animal Health Surveillance Terminology Final Report from Pre-ICAHS Workshop. http://www.fp7-risksur.eu/sites/default/files/partner_logos/icahs workshop-2011_surveillance_tewrminology_report_V1.2.pdf.

Hoinville, L.J., Alban, L., Drewe, J.A., Gibbens, J.C., Gustafson, L., Häsler, B., Saegerman, C., Salman, M., Stärk, K.D., 2013. Proposed terms and concepts for describing and evaluating animal-health surveillance systems. Preventive veterinary medicine 112, 1-12.

Hutchings, M.I., Truman, A.W., Wilkinson, B., 2019. Antibiotics: past, present and future. Current Opinion in Microbiology 51, 72-80.

Lai, C.C., Lee, K., Xiao, Y., Ahmad, N., Veeraraghavan, B., Thamlikitkul, V., Tambyah, P.A., Nelwan, R.H., Shibl, A.M., Wu, J.J., Seto, W.H., Hsueh, P.R., 2014. High burden of antimicrobial drug resistance in Asia. J Glob Antimicrob Resist 2, 141-147.

MARAN, 2021. Veldman KT, Wit B, Franz E, Heederik D, 2021. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2020.

Mevius, D., Heederik, D., 2014. Reduction of antibiotic use in animals "let's go Dutch". Journal für Verbraucherschutz und Lebensmittelsicherheit 9, 177-181.

Michael, C.A., Dominey-Howes, D., Labbate, M., 2014. The antimicrobial resistance crisis: causes, consequences, and management. Front Public Health 2, 145.

Ventola, C.L., 2015. The antibiotic resistance crisis: part 1: causes and threats. P t 40, 277-283.

Walker, B., Barrett, S., Polasky, S., Galaz, V., Folke, C., Engström, G., Ackerman, F., Arrow, K., Carpenter, S., Chopra, K., Daily, G., Ehrlich, P., Hughes, T., Kautsky, N., Levin, S., Mäler, K.-G., Shogren, J., Vincent, J., Xepapadeas, T., de Zeeuw, A., 2009. Looming Global-Scale Failures and Missing Institutions. Science 325, 1345-1346.

Walsh, C., 2003. Where will new antibiotics come from? Nat Rev Microbiol 1, 65-70.

WHO, 2015. Global action plan on antimicrobial resistance. Retrieved from https://www.who.int/antimicrobial-resistance/publications/global-action-plan/en/

I

Part I

Evaluation of antimicrobial resistance monitoring in livestock

Chapter 2

Monitoring antimicrobial resistance trends in commensal *Escherichia coli* from livestock, the Netherlands, 1998 to 2016

Hesp A, Veldman K, van der Goot J, Mevius D, van Schaik G. Monitoring antimicrobial resistance trends in commensal Escherichia coli from livestock, the Netherlands, 1998 to 2016. Euro Surveill. 2019 Jun;24(25):1800438. doi: 10.2807/1560-7917.ES.2019.24.25.1800438. PMID: 31241037; PMCID: PMC6593905.

https://doi.org/10.2807/1560-7917.ES.2019.24.25.1800438

Abstract

Background: Monitoring of antimicrobial resistance (AMR) in animals is essential for public health surveillance. To enhance interpretation of monitoring data, evaluation and optimisation of AMR trend analysis is needed.
Aims: To quantify and evaluate trends in AMR in commensal *Escherichia coli*, using data from the Dutch national AMR monitoring programme in livestock (1998–2016).
Methods: Faecal samples were collected at slaughter from broilers, pigs and veal calves. Minimum inhibitory concentration values were obtained by broth microdilution for *E. coli* for 15 antimicrobials of eight antimicrobial classes. A Poisson regression model was applied to resistant isolate counts, with explanatory variables representing time before and after 2009 (reference year); for veal calves, sampling changed from 2012 represented by an extra explanatory variable.
Results: Resistant counts increased significantly from 1998-2009 in broilers and pigs, except for tetracyclines and sulfamethoxazole in broilers and chloramphenicol and aminoglycosides in pigs. Since 2009, resistant counts decreased for all antimicrobials in broilers and for all but the phenicols in pigs. In veal calves, for most antimicrobials no significant decrease in resistant counts could be determined for 2009–16, except for sulfamethoxazole and nalidixic acid. Within animal species, antimicrobial-specific trends were similar.
Conclusions: Using Dutch monitoring data from 1998-2016, this study quantified AMR trends in broilers and slaughter pigs and showed significant trend changes in the reference year 2009. We showed that monitoring in commensal *E. coli is* useful to quantify trends and detect trend changes in AMR. This model is applicable to similar data from other European countries.

Introduction

Antimicrobial resistance (AMR) is recognised as one of the most urgent health issues worldwide [1-3]. Resistant bacteria emerge, evolve, persist and spread in livestock as animal reservoirs [4] selected by antimicrobial use (AMU) [5]. AMR can be transferred from animals to humans by direct contact or via the food chain and environment [4]. Therefore, monitoring of AMR in animals is an essential aspect of public health surveillance.

In 1998, a monitoring programme of AMR in livestock started in the Netherlands (NL). The programme was initiated following recommendations given at the Invitational European Union (EU) Conference 'The Microbial Threat' hosted by the Danish Government in Copenhagen in 1998 [6]. The recommendations were 'to monitor evolution and effects of interventions, through establishment of accurate surveillance systems on antimicrobial resistance in the human and veterinary sector' [7]; *Escherichia coli* was chosen as the indicator organism for gut microbiota in order to monitor the effects of antimicrobials with Gram-negative spectra. Since then, results have been reported annually in the report of the Monitoring programme of antimicrobial resistance and antibiotic usage in animals in the Netherlands (MARAN) [8]. From 1998 to 2009, increasing proportions of resistant isolates were observed for several antimicrobial classes, including third generation cephalosporins and fluoroquinolones, as well as high prevalence of multidrug-resistant isolates (resistant to three or more antimicrobial classes) in broilers, slaughter pigs and veal calves [9].

These findings together with high AMU in livestock compared with other European countries resulted in drastic policy changes [9]. In 2010, the Dutch government ordered the veterinary sector to reduce overall AMU sales with 50% within 4 years. A series of mandatory targets was set, starting with a 20% AMU reduction for livestock by 2011. By 2013, an additional reduction of 30% should be observed. In 2012, this target was renewed to 70% reduction by 2015 for total livestock production. The government set 2009 as reference year for this reduction target [10]. The first two targets were achieved in 2013 through a joint effort between livestock sectors, farmers and veterinarians but the 70% target has not been fully achieved in 2018. In 2016, total antimicrobial sales for veterinary use in NL had decreased by 64% compared with 2009, as reported by the Netherlands Veterinary Medicines Institute (SDa) [11]. During this period, trends in AMR and potential effects of AMU-interventions were monitored and reported in MARAN.

So far, no formal statistical methods have been applied for trend analysis of Dutch monitoring data from livestock. Trends were typically evaluated by

visual inspection of resistant proportions with confidence intervals (CIs). And to our knowledge, only a limited number of studies have been conducted to quantify trends in AMR monitoring data from livestock. For example, in 2015, a study by Hanon et al. reported resistance trends in commensal *E. coli* in the Belgium monitoring programme between 2011-14 [12]. In 2018, a descriptive trend analysis was performed by Boireau et al. to look at resistance in animal pathogens between 2002-15 [13].

Evaluation is needed of current statistical methods to optimise AMR monitoring in animals and enhance interpretation of monitoring data. The aim of this study, therefore, was to evaluate whether AMR trends could be quantified and changes detected in Dutch monitoring data from 1998 to 2016. We developed a model to quantify AMR trends over time relative to a chosen reference year in which a trend change may have occurred. Here, we describe the results of our evaluation and provide recommendations for quantitative trend analysis of AMR monitoring data.

Methods

Animal sampling and monitoring activities

In the Dutch monitoring programme, individual caecal samples are collected annually by the Netherlands Food and Consumer Product Safety Authority (NVWA) from broilers, pigs and veal calves in slaughterhouses. Broilers and pigs have been sampled since 1998, veal calves since 2005. Between 2005 and 2011, sampling in veal calves started with pooled faecal samples taken at farms, but from 2012 calves were sampled individually at slaughter. Since 2014, when AMR monitoring in commensal *E. coli* from livestock became mandatory by EU legislation, caecal samples have been taken from all prescribed animal species.

In the NL, ca 300 *E. coli* isolates are collected per animal species annually, which is more than the EU prescribed yearly sampling of 170 isolates per animal species. A two-stage random sampling procedure is followed to ensure that one animal per batch from one herd/flock is sampled and to minimise the risk of clustering as result of multiple samples from the same herd. First, all slaughter batches within a slaughterhouse are stratified (proportional to annual throughput of slaughtered animals) and one slaughter batch is randomly selected. Second, one animal is randomly selected from this slaughter batch for sampling.

Bacterial isolation and susceptibility testing

The terms ‘resistant’ and ‘resistance’ in this study refer to non-wild type

susceptibility, based on epidemiological cut-off (ECOFF) values as defined by The European Committee on Antimicrobial Susceptibility Testing [14]. No selective media were used to enhance detection of resistant isolates in this study. From each faecal sample, *E. coli* was isolated on MacConkey agar and one colony was randomly selected and identified as *E. coli* (biochemically by Indole test before 2012 and by matrix-assisted laser desorption/ionisation time-of-flight after 2012). Minimum inhibitory concentrations (MICs) were determined with broth microdilution, according to ISO 20776–1:2006, by commercially available microtitre plates (Sensititre EUVSEC by Thermo Scientific, East Grinstead, United Kingdom). Before antimicrobial panels were prescribed by European Food Safety Authority (EFSA) in 2008 [15] and EU-legislation in 2013, panels were periodically adjusted to improve efficiency; 10 different panels were used from 1998 to 2016. Some antimicrobials were replaced by others and MIC ranges were changed. Nevertheless, antimicrobials of relevant groups were continuously present. Amoxicillin and ampicillin were representatives of aminopenicillins; Cefotaxime and ceftazidime were representatives of cephalosporins. Gentamicin, neomycin and kanamycin were representatives of aminoglycosides. Tetracyclines were represented by doxycycline and tetracycline. Sulfamethoxazole and trimethoprim were representatives of folate pathway inhibitors. Amphenicols were represented by chloramphenicol and florfenicol. Ciprofloxacin represented the fluoroquinolones, nalidixic acid represented the quinolones.

An exception is colistin; before 2010, colistin was not in antimicrobial panels, or without sufficient MIC ranges to detect phenotypic colistin-resistance. Supplement S1, Table S1 gives an overview of panels and MIC ranges.

Between 1998 and 2016, the 12,491 isolates included in this study were collected and analysed at the Dutch National Reference Laboratory (NRL) for monitoring AMR in animals, at Wageningen Bioveterinary Research (WBVR, Lelystad, NL). Of which, 5,021 isolates were from broilers (1998–2016), 4,809 from slaughter pigs (1998–2016) and 2,651 from veal calves (2005–16). In the year 2000 no isolates were collected for any species.

Statistical analysis of trends in resistant counts

All statistical analyses in this study were performed in R version 3.3.3 (R Foundation, Vienna, Austria). Yearly resistant isolate counts (n) were aggregated separately for each antimicrobial per species (Supplement S1, Tables S2, S3 and S4), and exact 95% confidence intervals (CIs) for the counts were calculated, using yearly total numbers of isolates tested (N). Regression models were applied using the glm() function in R and models were selected by comparison of Akaike's Information Criterion (AIC).

The best fitting model for our purpose was a generalised linear model with Poisson distribution and a log link function (Poisson regression) for yearly resistance counts (n), with the log of the total number of strains per year (N) as offset. In our model, trends in AMR were modelled relative to a reference year for all animal species, to specifically test whether a trend change was observed. Two explanatory numerical variables were used: 'time in years 1998-2009 until start of AMU interventions' (x1) and 'time in years 2009-2016 since start of AMU interventions' (x2). The notation of the x-variables were:

x1 Time in years until reference year: -11,-10,-9,-8,-7,-6,-5,-4,-3,-2,-1, 0, 0, 0, 0, 0, 0, 0, 0

x2 Time in years since reference year: 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 1, 2, 3, 4, 5, 6, 7

The chosen reference year in the model was '0' in both explanatory variables, making this year the model intercept and the estimate for the mean resistant proportion in the year 2009. Estimates for x1 and x2 indicated whether a significant trend change occurred. The exponent of the estimates gave incidence rate ratios (IRRs), which quantified the mean increase or decrease per year, an IRR of 1 indicating the mean change of the resistant proportion per year is zero (no trend), an IRR > 1 indicating a mean increase over time and an IRR < 1 a mean decrease over time. This specific notation made the model flexible to analyse trend changes. By varying x1 and x2 and comparing model fit, we could also assess in which year a trend change had most likely taken place. Only results with 2009 as reference year are presented here, because this was set as index year by the government and to measure AMU reduction by the Netherlands Veterinary Medicines Institute [11] and was sufficient to illustrate our method. The 95% CIs for IRRs were calculated as were CIs for predicted values, using the inverse link-function.

To verify our method, we compared it with a generalised linear model with binomial distribution, using the same notation for x1 and x2. The Poisson model had lower AICs for most data. Goodness-of-fit was tested using the deviance chi-squared goodness-of-fit test, and assessing scaled deviances (with a dispersion parameter of 1; a scaled deviance of > 2 indicated overdispersion and a scaled deviance of < 0.5 indicated underdispersion). With overdispersion, the variance of the count is much larger than the mean, a common problem with count data. For a few antimicrobial-species combinations model fit was suboptimal i.e. Poisson's assumptions were not met. For cases with over/underdispersed data, a negative binomial or binomial distribution was applied, respectively, to improve model fit.

As the sampling of veal calves changed in 2011, an extra variable was added

to the model to detect possible effects of this sampling change. This variable x3 was '0' until 2011 and '1' from 2012.

Colistin resistance data was only available since 2010 (Supplement S1, Table S1), and was analysed with Poisson regression for 2010–16 for broilers and slaughter pigs, with 'x' representing time in years.

The following antimicrobials from the same class for which *E. coli* is considered to be cross-resistant: amoxicillin/ampicillin and doxycycline/tetracycline and neomycin/kanamycin [16] were modelled as if being equal.

Results

Broilers

Between 1998 and 2009 (x1), there were statistically significant increasing resistance trends for all antimicrobials (range IRR: 1.04–1.30), except for tetracyclines (IRR: 1.0; 95% CI: 0.99–1.02; p = 0.56) and sulfamethoxazole (IRR: 1.0; 95% CI: 0.98–1.03; p = 0.69). Between 2009 and 2016 (x2), significant decreasing resistance trends were observed for all antimicrobials in broilers (range IRR: 0.66–0.95) (Table 1 Figure 1).

Table 1. Estimates for antimicrobial resistance trends in yearly resistant counts of *Escherichia coli* from broilers, for time in years before (1998–2009)[a] and after (2009–2016)[b] antimicrobial use interventions, the Netherlands, 1998–2016

Antimicrobial	Variable	Estimate	P value[c]	IRR[d] (95% CI)	Scaled deviance[e] (0.5 < > 2)
Amoxicillin/ ampicillin	Intercept	- 0.27	0.00	0.76 (0.71–0.81)	1.21
	x1	0.06[a]	0.00	1.06 (1.05–1.07)	
	x2	- 0.06[b]	0.00	0.94 (0.93– 0.96)	
Cefotaxime	Intercept	- 1.53	0.00	0.22 (0.19–0.25)	2.68
	x1	0.21	0.00	1.24 (1.19–1.29)	
	x2	- 0.42	0.00	0.66 (0.61–0.71)	
Ceftazidime[f]	Intercept	- 1.59	0.00	0.20 (0.17–0.24)	1.55
	x1	0.24	0.00	1.27 (1.20–1.35)	
	x2	- 0.39	0.00	0.67 (0.63–0.72)	
Gentamicin	Intercept	- 2.20	0.00	0.11 (0.09–0.13)	1.93
	x1	0.12	0.00	1.13 (1.08–1.17)	
	x2	- 0.13	0.00	0.88 (0.83–0.92)	
Doxycycline/ tetracycline	Intercept	- 0.48	0.00	0.62 (0.57–0.66)	0.63
	x1	0.00	0.56	1.00 (0.99–1.02)	
	x2	- 0.09	0.00	0.91 (0.90–0.93)	

Sulfamethoxazole[f]	Intercept	- 0.31	0.00	0.73 (0.68–0.79)	0.39
	x1	0.01	0.69	1.01 (0.98–1.03)	
	x2	- 0.08	0.00	0.93 (0.91–0.95)	
Trimethoprim	Intercept	- 0.42	0.00	0.66 (0.61–0.70)	1.41
	x1	0.04	0.00	1.04 (1.02–1.05)	
	x2	- 0.08	0.00	0.92 (0.90–0.94)	
Chloramphenicol	Intercept	- 1.24	0.00	0.29 (0.26–0.32)	1.52
	x1	0.12	0.00	1.13 (1.10–1.16)	
	x2	- 0.17	0.00	0.84 (0.81–0.87)	
Florfenicol[f]	Intercept	- 3.02	0.00	0.05 (0.03–0.07)	1.49
	x1	0.27	0.00	1.30 (1.19–1.45)	
	x2	- 0.31	0.00	0.73 (0.60–0.89)	
Ciprofloxacin	Intercept	- 0.49	0.00	0.61(0.57–0.66)	0.69
	x1	0.05	0.00	1.05 (1.04–1.07)	
	x2	- 0.05	0.00	0.95 (0.93–0.97)	
Nalidixic acid[f]	Intercept	- 0.46	0.00	0.63 (0.58–0.69)	0.59
	x1	0.06	0.00	1.06 (1.03–1.10)	
	x2	- 0.07	0.00	0.94 (0.92–0.96)	
Neomycin/ kanamycin[f]	Intercept	- 1.85	0.00	0.16 (0.13–0.19)	1.25
	x1	0.05	0.01	1.05 (1.01–1.10)	
	x2	- 0.21	0.00	0.81 (0.73–0.89)	

E. coli: Escherichia coli; CI: confidence interval; IRR: incidence rate ratio.

[a] Estimate for antimicrobial resistance trends in years 1998–2009.

[b] Estimate for antimicrobial resistance trends in years 2009–16.

[c] P values < 0.05 indicate significant trends for variables x1 (1998-2009) and x2 (2009-16).

[d] IRR with 95% CI: for the intercept this number indicates the estimated resistant proportion for reference year 2009. For variables x1 and x2 this is the mean increase or decrease per year.

[e] Scaled deviance of > 2 indicates overdispersion of data, scaled deviance of < 0.5 indicates underdispersion of data.

[f] Data were not collected during whole length of testing period, see Supplement S1, Table S1.

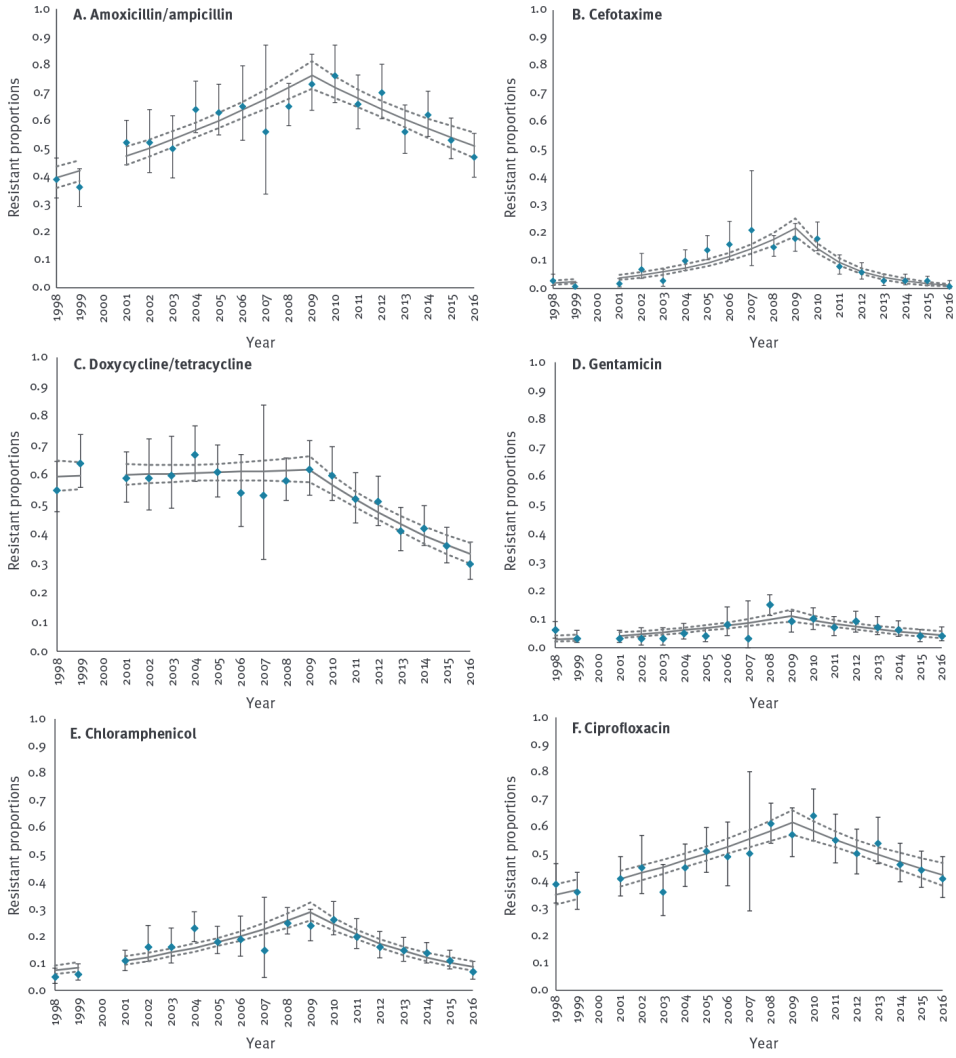


FIGURE 1. Resistant proportions in isolates from broilers, modelled as resistant counts with Poisson regression and time in years befo re[a] and after[b] 2009, the Netherlands, 1998–2016

[a] Time in years 1998–2009 (x1).
[b] Time in years 2009–16 (x2).
Vertical error bars indicate 95% confidence intervals (CIs) per yearly observation. Model predicted values are visualised in the grey line with their 95% CIs (grey dotted lines). A: Amoxicillin/ ampicillin; B: Cefotaxime; C: Doxycycline/tetracycline; D: Gentamicin; E: Chloramphenicol; F: Ciprofloxacin in broilers.

Additional analyses with the same modelling approach but using 2010 as reference year instead of 2009 showed that for most antimicrobials the decreasing trend started after 2010; the model with 2010 as reference year had a better fit (data not shown). However, overall, the model fit with 2009 as reference year was good reflected by the scaled deviances in Table 1 and CIs in Figure 1. The cefotaxime data was overdispersed (scaled deviance 2.68); a negative binomial distribution was applied, which better fit the data (scaled deviance 1.32) and gave similar estimates.

Slaughter pigs

The observed resistant counts were generally lower than in broilers, except for tetracyclines; the estimated resistant proportion for 2009 was 0.72 (0.67–0.77) (Table 2).

Between 1998 and 2009 (x1), there were statistically significant increasing resistance trends for all antimicrobials (range IRR: 1.03–1.43), except for chloramphenicol (IRR: 1.03; 95% CI: 1.00–1.06; p = 0.07). Between 2009 and 2016 (x2), significant decreasing resistance trends were observed for all antimicrobials in pigs (range IRR: 0.66–0.95), with exception of chloramphenicol- (IRR: 1.00; 95% CI: 0.96-1.05; p = 0.97) and florfenicol resistance (IRR: 1.09; 95% CI: 0.81–1.46; p = 0.56) (Table 2).

For quinolones and gentamicin, model fit was suboptimal, data were overdispersed. A model with a negative binomial distribution resulted in different estimates for quinolones and similar estimates for gentamicin (Supplement S1, Tables S5).

Table 2. Estimates for antimicrobial resistance trends in yearly resistant counts of *Escherichia coli* from slaughter pigs, for time in years before (1998–2009)[a] and after (2009–2016)[b] antimicrobial use interventions, the Netherlands, 1998–2016

Antimicrobial	Variable	Estimate	P value[c]	IRR[d] (95% CI)	Scaled deviance[e] (0.5 < > 2)
Amoxicillin/ ampicillin	Intercept	- 0.91	0.00	0.40 (0.37–0.44)	1.17
	x1	0.09[a]	0.00	1.09 (1.07–1.11)	
	x2	- 0.09[b]	0.00	0.91 (0.89–0.94)	
Cefotaxime	Intercept	-3.98	0.00	0.02 (0.01–0.03)	1.39
	x1	0.15	0.01	1.16 (1.05–1.30)	
	x2	- 0.23	0.01	0.79 (0.66–0.93)	
Ceftazidime[f]	Intercept	- 3.83	0.00	0.02 (0.01–0.03)	1.92
	x1	0.17	0.01	1.18 (1.05–1.39)	
	x2	- 0.23	0.00	0.80 (0.67–0.93)	

Gentamicin	Intercept	- 3.49	0.00	0.03 (0.02–0.04)	2.57
	x1	0.13	0.00	1.14 (1.06–1.24)	
	x2	- 0.16	0.01	0.85 (0.76–0.95)	
Doxycycline/ tetracycline	Intercept	- 0.33	0.00	0.72 (0.67–0.77)	0.72
	x1	0.03	0.00	1.04 (1.02–1.05)	
	x2	- 0.08	0.00	0.93 (0.91–0.95)	
Sulfamethoxazole[f]	Intercept	- 0.51	0.00	0.60 (0.55–0.66)	0.20
	x1	0.03	0.04	1.03 (1.00–1.07)	
	x2	- 0.08	0.00	0.93 (0.91–0.95)	
Trimethoprim	Intercept	- 0.65	0.00	0.52 (0.48–0.57)	0.71
	x1	0.04	0.00	1.04 (1.02–1.05)	
	x2	- 0.08	0.00	0.92 (0.90–0.95)	
Chloramphenicol	Intercept	-2.18	0.00	0.11 (0.10–0.13)	1.12
	x1	0.03	0.07	1.03 (1.00–1.06)	
	x2	0.00	0.97	1.00 (0.96–1.05)	
Florfenicol[f]	Intercept	- 4.61	0.00	0.01 (0.004–0.02)	1.15
	x1	0.22	0.02	1.25 (1.05–1.54)	
	x2	0.09	0.56	1.09 (0.81–1.46)	
Ciprofloxacin	Intercept	- 3.39	0.00	0.03 (0.02–0.05)	3.48
	x1	0.15	0.00	1.16 (1.07–1.27)	
	x2	- 0.43	0.00	0.65 (0.54–0.77)	
Nalidixic acid[f]	Intercept	- 3.25	0.00	0.04 (0.02–0.06)	3.29
	x1	0.36	0.00	1.43 (1.16–1.82)	
	x2	- 0.46	0.00	0.63 (0.52–0.76)	
Neomycin/ kanamycin[f]	Intercept	- 3.33	0.00	0.04 (0.02–0.05)	1.08
	x1	0.04	0.37	1.04 (0.95–1.14)	
	x2	- 0.47	0.00	0.62 (0.46–0.81)	

E. coli: Escherichia coli; CI: confidence interval; IRR: incidence rate ratio.
[a] Estimate for antimicrobial resistance trends in years 1998–2009.
[b] Estimate for antimicrobial resistance trends in years 2009–16.
[c] P values < 0.05 indicate significant trends for variables x1 (1998-2009) and x2 (2009-16).
[d] IRR with 95% CI: for the intercept this number indicates the estimated resistant proportion for reference year 2009. For variables x1 and x2 this is the mean increase or decrease per year.
[e] Scaled deviance of > 2 indicates overdispersion of data, scaled deviance of < 0.5 indicates underdispersion of data.
[f] Data were not collected during whole length of testing period, see Supplement S1, Table S1.

Veal calves

Results showed that trends between 2005 and 2009 (x1), and between 2009 and 2016 (x2), could not be analysed without taking into account variable x3, a binary variable representing the sampling change from 2012 (Table 3). When variable x3 was added, the fit increased significantly for all antimicrobials (Table 3). AICs improved and overdispersion was reduced for gentamicin, trimethoprim and quinolones (Table 3). Collinearity between x2 and x3 was not considered a problem; standard errors of explanatory variables were not greatly influenced by adding x3, and Variance Inflation Factors of x-variables

were acceptable.

When taking into account the sampling change from 2012, no significant decreasing trend could be estimated in the monitoring data from 2009 to 2016 in veal calves for all antimicrobials except sulfamethoxazole and naladixic acid (Table 3). In 2012, a sharp decrease in resistant counts was observed, due to the change in sampling strategy, explained by x3, illustrated for ciprofloxacin in Figure 2. For nalidixic acid, florfenicol and aminoglycosides, data were overdispersed (Table 3). A model with negative binomial distribution better fit these data, resulting in similar estimates as the Poisson model (data not shown). Modelling a subset of the veal calves data from 2012 to 2016, only resulted in significant decreases for sulfamethoxazole, trimethoprim, ciprofloxacin and nalidixic acid with IRRs of 0.90, 0.91, 0.85 and 0.76, respectively (data not shown).

TABLE 3. Estimates for antimicrobial resistance trends in yearly resistant counts of *Escherichia coli* from veal calves, for time in years before[a] and after[b] antimicrobial use interventions, with and without including a sampling change as extra explanatory variable[c], the Netherlands, 2005-16

Antimicrobial	Variable	Estimate	P value[d]	IRR[e] (95% CI)	AIC[f]	Scaled deviance[g] (0.5 < > 2)
Amoxicillin/ampicillin	Intercept	- 0.83	0.00	0.44 (0.38–0.50)	97.09	1.97
	x1	- 0.03[a]	0.41	0.97 (0.92–1.04)		
	x2	- 0.12[b]	0.00	0.89 (0.86–0.92)		
	Intercept	- 0.82	0.00	0.44 (0.38–0.50)	87.11	0.72
	x1	- 0.02[a]	0.44	0.98 (0.92–1.04)		
	x2	- 0.02[b]	0.62	0.98 (0.92–1.05)		
	x3	- 0.56[c]	0.00	0.57 (0.41–0.78)		
Cefotaxime	Intercept	- 3.65	0.00	0.03 (0.01–0.05)	47.49	0.99
	x1	- 0.03	0.79	0.97 (0.75–1.26)		
	x2	- 0.32	0.00	0.72 (0.58–0.88		
	Intercept	- 3.71	0.00	0.02 (0.01–0.05)	47.60	0.87
	x1	- 0.06	0.67	0.95 (0.86–1.64)		
	x2	- 0.07	0.73	0.93 (0.61–1.39)		
	x3	- 1.30	0.18	0.27 (0.04–1.72)		
Ceftazidime	Intercept	- 3.65	0.00	0.03 (0.01–0.05)	45.49	1.54
	x1	0.15	0.34	1.17 (0.86–1.64)		
	x2	- 0.44	0.00	0.64 (0.49–0.81)		
	Intercept	- 3.88	0.00	0.02 (0.01–0.04)	41.71	1.01
	x1	0.08	0.65	1.08 (0.79–1.52)		
	x2	0.10	0.69	1.11 (0.67–1.83)		
	x3	- 2.85	0.02	0.06 (0.00–0.60)		

Gentamicin	Intercept	- 2.37	0.00	0.09 (0.07–0.12)	88.38	3.51
	x1	- 0.05	0.44	0.95 (0.83–1.08)		
	x2	- 0.24	0.00	0.79 (0.72–0.86)		
	Intercept	- 2.46	0.00	0.09 (0.06–0.11)	73.54	1.85
	x1	- 0.08	0.23	0.92 (0.81–1.05)		
	x2	0.11	0.26	1.11 (0.92–1.34)		
	x3	- 1.83	0.00	0.16 (0.06–0.39)		
Tetracycline	Intercept	- 0.39	0.00	0.68 (0.61–0.75)	100.22	1.60
	x1	- 0.04	0.12	0.96 (0.92–1.01)		
	x2	- 0.08	0.00	0.93 (0.90–0.95)		
	Intercept	- 0.38	0.00	0.68 (0.61–0.75)	94.98	0.89
	x1	- 0.03	0.15	0.97 (0.92–1.01)		
	x2	- 0.02	0.42	0.98 (0.93–1.03)		
	x3	- 0.33	0.01	0.72 (0.57–0.91)		
Sulfamethoxazole	Intercept	- 0.65	0.00	0.52 (0.46–0.59)	96.57	1.71
	x1	- 0.01	0.83	0.99 (0.94–1.05)		
	x2	- 0.12	0.00	0.88 (0.86–0.91)		
	Intercept	- 0.65	0.00	0.52 (0.46–0.59)	93.56	1.30
	x1	0.00	0.87	1.00 (0.94–1.05)		
	x2	- 0.06	0.04	0.94 (0.88–1.00)		
	x3	- 0.33	0.03	0.72 (0.53–0.96)		
Trimethoprim	Intercept	- 0.82	0.00	0.44 (0.38–0.50)	98.43	2.23
	x1	0.00	0.98	1.00 (0.94–1.07)		
	x2	- 0.14	0.00	0.87 (0.84–0.90)		
	Intercept	- 0.82	0.00	0.44 (0.38–0.50)	92.63	1.53
	x1	0.00	0.96	1.00 (0.94–1.07)		
	x2	- 0.06	0.11	0.94 (0.88–1.01)		
	x3	- 0.47	0.01	0.63 (0.45–0.87)		

Chloramphenicol	Intercept	- 1.44	0.00	0.24 (0.20–0.28)	90.66	1.96
	x1	- 0.07	0.07	0.93 (0.86–1.01)		
	x2	- 0.10	0.00	0.91 (0.87–0.95)		
	Intercept	- 1.43	0.00	0.24 (0.20–0.28)	82.09	0.88
	x1	- 0.07	0.08	0.93 (0.87–1.01)		
	x2	0.03	0.56	1.03 (0.94–1.12)		
	x3	- 0.70	0.00	0.50 (0.32–0.76)		
Florfenicol[h]	Intercept	- 1.99	0.00	0.14 (0.10–0.18)	83.13	5.53
	x1	0.00	0.96	1.00 (0.90–1.13)		
	x2	- 0.14	0.02	0.87 (0.77–0.97)		
	Intercept	- 2.22	0.00	0.11 (0.08–0.15)	67.79	3.17
	x1	- 0.07	0.25	0.93 (0.82–1.05)		
	x2	0.29	0.01	1.33 (1.06–1.68)		
	x3	- 1.53	0.00	0.22 (0.11–0.45)		
Ciprofloxacin	Intercept	- 1.55	0.00	0.21 (0.17–0.26)	90.42	2.66
	x1	0.00	0.96	1.00 (0.91–1.09)		
	x2	- 0.25	0.00	0.78 (0.73–0.82)		
	Intercept	- 1.58	0.00	0.21 (0.17–0.25)	80.78	1.54
	x1	- 0.01	0.77	0.99 (0.90–1.08)		
	x2	- 0.06	0.34	0.94 (0.83–1.07)		
	x3	-1.00	0.00	0.37 (0.20–0.66)		
Nalidixic acid	Intercept	-1.54	0.00	0.21 (0.17–0.26)	90.80	2.83
	x1	0.00	0.98	1.00 (0.91–1.10)		
	x2	- 0.29	0.00	0.75 (0.70–0.80)		
	Intercept	- 1.57	0.00	0.21 (0.17–0.26)	86.34	2.37
	x1	- 0.01	0.82	0.99 (0.90–1.09)		
	x2	- 0.14	0.04	0.87 (0.76–1.00)		
	x3	- 0.78	0.01	0.46 (0.25–0.84)		

Neomycin/kanamycin[h]	Intercept	- 1.65	0.00	0.19 (0.15–0.24)	105.25	8.63
	x1	- 0.04	0.42	0.96 (0.87–1.06)		
	x2	- 0.20	0.00	0.82 (0.74–0.90)		
	Intercept	- 1.91	0.00	0.15 (0.11–0.19)	74.76	3.86
	x1	- 0.13	0.02	0.88 (0.79–0.98)		
	x2	0.30	0.00	1.35 (1.11–1.64)		
	x3	- 1.86	0.00	0.16 (0.08–0.29)		

AIC: Akaike's Information Criterion; *E. coli: Escherichia coli;* CI: confidence interval; IRR: incidence rate ratio.

[a] Estimate for antimicrobial resistance trends in years 2005–09.

[b] Estimate for antimicrobial resistance trends in years 2009–16.

[c] 0/1 variable for the change in sampling from 2012 onwards, from then individual animal samples were taken at slaughterhouses, from 2005-2011 floor samples were taken on veal farms.

[d] P values < 0.05 indicate significant trends for variables x1 (2005-09) and x2 (2009-16).

[e] IRR with 95% CIs, for the intercept this number indicates the estimated resistant proportion for reference year 2009. For variables x1 and x2 this is the mean increase or decrease per year.

[f] A measure for the fit of the model, lower values for the model including x3 indicate a better fit.

[g] Scaled deviance of > 2 indicates overdispersion of data, scaled deviance of < 0.5 indicates underdispersion of data.

[h] Data were not collected during whole length of testing period, see Supplement S1, Table S1.

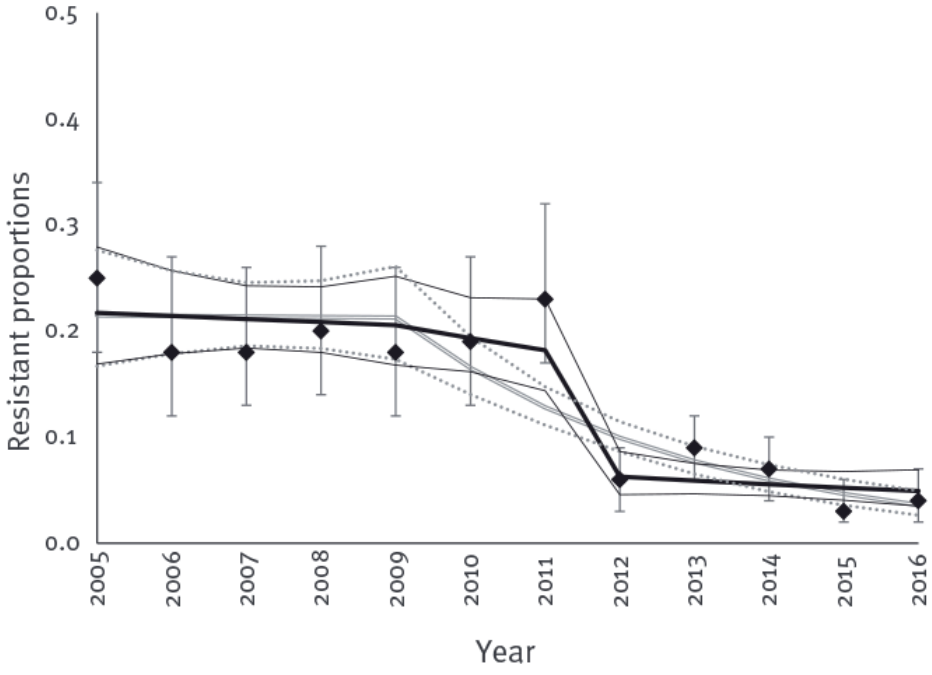


Figure 2. Resistant proportions per year for ciprofloxacin in isolates from veal calves, modelled as resistant isolate counts with Poisson regression and time in years before[a] and after 2009[b], with and without including the variable for the sampling change from 2012 onwards[c], the Netherlands, 2005–2016

[a] Time in years 1998–2009 (x1).
[b] Time in years 2009–16 (x2).
[c] 0/1 variable for the change in sampling from 2012 onwards (x3).
Vertical error bars indicate 95% confidence intervals (CIs) per yearly observation. The black bold line presents predicted values for the model including the variable for sampling change (x3), with the model's 95% CIs (black thin lines). The grey line in the background presents predicted values for the model without the variable for sampling change (x3), with the model's 95% CIs (grey dotted lines).

Table 4. Results of Poisson regression of antimicrobial resistance trends in yearly resistant counts of indicator *E. coli* resistant to colistin from broilers and slaughter pigs, the Netherlands, 2010–2016

Species	Year	n	Resistant Count (n)		Estimate	P value[a]	IRR[b] (95% CI)	AIC[c]	Scaled deviance[d] (0.5 < > 2)
Broilers	2010	284	3	Intercept	- 3.80	0.00	0.02 (0.01–0.06)	22.57	1.40
	2011	283	2	x	- 0.46	0.01	0.63 (0.42–0.88)		
	2012	292	3						
	2013	301	3						
	2014	377	0						
	2015	400	0						
	2016	300	0						
Slaughter pigs	2010	282	0	Intercept	- 4.92	0.00	0.01 (0.00–0.05)	14.31	1.14
	2011	287	2	x	- 0.53	0.16	0.59 (0.23–1.12)		
	2012	284	1						
	2013	289	0						
	2014	392	0						
	2015	298	0						
	2016	299	0						

AIC: Akaike's Information Criterion; *E. coli: Escherichia coli;* CI: confidence interval; IRR: incidence rate ratio.

[a] P values < 0.05 indicate significant trends for variables x.

[b] IRR with 95% CIs, for the intercept this number indicates the estimated resistant proportion for 2010. For variable x this is the mean decrease per year.

[c] A measure for the fit of the model, a lower value indicates a better fit.

[d] Scaled deviance of > 2 indicates overdispersion of data, scaled deviance of < 0.5 indicates underdispersion of data.

Colistin resistance

Colistin-resistant isolates were detected sporadically in Dutch monitoring programme data since 2010. Time trends for colistin can be seen in Table 4. No significant decrease was detected for slaughter pigs, however for broilers a significant decreasing trend was observed. For veal calves, a decreasing trend could not be distinguished reliably from the effect of the sampling change (data not shown).

Discussion

This study aimed to optimise interpretation of AMR monitoring data by modelling resistance trends in commensal *E. coli* from livestock and to evaluate if any trends (and trend changes) were observed from 1998 to 2016. We developed a model that optimised the quantification of resistance trends and the detection of trend changes as a likely effect of interventions in indicator commensal *E. coli* from livestock. We conclude that monitoring in indicator commensal *E.coli* is valuable to evaluate resistance trends in livestock on animal population level. For nearly all antimicrobials in broilers and slaughter pigs, significant and quantifiable changes were observed in NL monitoring from 1998 to 2016. Significant decreases since 2009 were mostly preceded by significant increases from 1998 to 2009 and there was high similarity in trends for all antimicrobials within animal species.

Broilers

An increasing veterinary therapeutic AMU was measured in NL between 1998 and 2009 [11], corresponding to the AMR trends we found in the broiler data over this time period. For most antimicrobials resistant proportions started to decrease from 2010, confirmed in an additional analysis by the better fit of broiler data in models with 2010 as reference year (data not shown). In 2010, the illegal prophylactic use of ceftiofur on day-old chicks in hatcheries ended following intensified control measures implemented by the Dutch Food Safety Authority. This may have resulted in the abrupt and significant decreases of cefotaxime- and ceftazidime resistant counts after 2010. Interestingly, however, the observed resistant proportions for ciprofloxacin in broilers remained high and although these proportions decreased significantly since 2009, it is at a slower rate than expected.

Fluoroquinolone-use has decreased considerably in broilers since 2009 [17]. As part of the intervention measures, fluoroquinolone-use in livestock was legally restricted as was the use of third generation cephalosporins.

Since January 2014, these antimicrobials are only allowed to be used after veterinarians have confirmed by antibiogram that no alternative antibiotics are available (with exception of ceftiofur, which was never licensed in poultry) [9]. The relative persistence of ciprofloxacin-resistant *E. coli* in broilers may be explained by chromosomal mutations, which have a low bacterial fitness cost [18]; ciprofloxacin-resistance is mostly not encoded on plasmids like cefotaxime-resistance is. It is speculated that ciprofloxacin-resistance may be transmitted between broiler flocks, or be introduced from parent stocks, from the farm environment or from hatcheries but it is currently unclear so further investigations are needed. Persistence of quinolone-resistance in livestock is very relevant since fluoroquinolones are marked as critically important antimicrobials by WHO [19].

Slaughter pigs

From 1998 to 2009, resistant counts increased in slaughter pigs, except for chloramphenicol and for the aminoglycosides. Resistant proportions of *E. coli* isolates decreased significantly since 2009 for all antimicrobials, except chloramphenicol and florfenicol; corresponding to data from the Netherlands Veterinary Medicines Institute who also observed an AMU decrease since 2009 [11]. In general, resistant proportions were lower in isolates from slaughter pigs than in broilers, with exception of tetracycline-resistance. Despite the fact that chloramphenicol has not been used in pigs since its ban in the early 1990s, resistance remained and has not decreased since 2009; the frequent use of florfenicol in pigs may be the cause of this as florfenicol selects for the presence of *floR* genes, which confers resistance to both chloramphenicol and florfenicol [16]. Furthermore, co-selection of *cat*-genes in Class 1 integrons by other substances (tetracyclines, aminoglycosides, sulfonamides or trimethoprim) as described by Wu et al. may explain this phenomenon [20]. Tetracycline use in pigs has decreased since 2009, but is still relatively high [11].

For gentamicin and quinolones, overdispersion of data in the Poisson model hampered trend analysis. For antimicrobials of which resistant proportions are nearly zero and when the data has many zero counts, determining aberrations in trends can be difficult. In general, with a negative binomial distribution these trends could still be assessed reliably in this study.

Veal calves

Changing from pooled samples from farms to individual animals at slaughter had a large impact on observed resistant counts in veal calves. Trends could not be assessed without including this sampling change in the model. We conclude that in spite of a substantial decrease in total AMU in veal calves from 2007

to 2015 (as reported by the SDa [11]) for most antimicrobials no significant decrease in resistant proportions of *E. coli* could be determined with the current monitoring system from 2009 to 2016, except for sulfamethoxazole and nalidixic acid. Looking specifically at the trend from 2012 to 2016, after the sampling change, a significant decreasing trend was observed for quinolones, sulfamethoxazole and trimethoprim, but not for other antimicrobials. Between 2005 and 2009, for most antimicrobials in veal calves no significant trend was observed.

Colistin as example of trend analysis in rare resistance

Quantifying trends in resistant isolates from livestock is needed to support treatment guidelines and AMR policy. When resistance is non-existent or rare, monitoring with a limited number of samples may not be able to detect emerging resistance, or resistance with a low prevalence. The statistical model used in this study was appropriate with a yearly sample of 300 isolates. Although we did not test it explicitly, this result for colistin may indicate that yearly sampling of 170 isolates, as currently prescribed by EFSA [15], may not be sensitive enough to detect changes in rare resistance traits especially when changes are small. The effect of different sampling strategies in the monitoring on both detecting emerging resistance and trend changes should be further investigated.

Commensal *Escherichia coli* as sentinel organism

Often, resistant proportions in sentinel organism *E. coli* are referred to as 'prevalence' of resistance. However, *E. coli* is only a minor fraction of gut microbiota and detected resistant proportions cannot be translated directly to AMR prevalence in livestock in general [21]. Nonetheless, commensal *E. coli* can be used as an indicator organism to study AMR-trends in Gram-negative bacteria in livestock, which are intrinsically susceptible to the antimicrobials used in the panel. Because *E. coli* is present in all faecal samples, randomisation of sampling is possible. Furthermore, the wildtype is susceptible to all of the tested antimicrobials and isolation methods for *E. coli* from animal faeces can be standardised. These are the characteristics which make *E. coli* a useful indicator. This study stresses that when standardised AMR monitoring in *E. coli* is performed continuously, time-trends can be analysed reliably. These trends indicate if AMU interventions are necessary and when measures are taken their effect on monitored resistant counts is reflected in the monitoring data.

Changes of antimicrobial panel

In the analysis, resistant proportions for amoxicillin/ampicillin, doxycycline/tetracycline and neomycin/kanamycin were modelled as if being one. This

enabled trend analysis for these antimicrobials and significant decreasing trends were shown in both broilers and slaughter pigs. For amoxicillin and ampicillin, resistance in *E. coli* is encoded by the same genes and the same resistance mechanisms are involved [16]. Doxycycline and tetracycline have different antibacterial potencies, but resistance genes and mechanisms are identical [16]. For these two pairs of antimicrobials, ECOFFs will identify identical non-wildtype susceptible populations. For the aminoglycosides neomycin and kanamycin, a variety of aminoglycoside-modifying enzymes can be involved [16]. In our experience, *E.coli* from animals are phenotypically mostly cross-resistant to these antimicrobials, but confirmation of absolute cross-resistance by typing of resistance genes is lacking.

Since 2008, the antimicrobial panel is decided by EFSA and included in EU legislation. In general, all antimicrobial classes of public health interest are represented in the panel. However, one of the disadvantages of using phenotypic methods is that the choice of specific antimicrobials is confined by the limited amount of wells in the Sensititre plates, to provide wide enough ranges for the tested substances. In the near future, phenotypic susceptibility testing for AMR surveillance in animals may be replaced by whole-genome sequencing, then any known resistance genes will be found.

Statistical analysis

We considered several modelling methods for time-series data. An autoregressive integrated moving average (ARIMA) model was explored, which best fit data with high density of observations in short time-periods. The generalised additive model (GAM) with spline-functions applied by Boireau et al. is useful to correct for recurring trends such as seasonality [13]. In our study, monitoring data came from a standardised random sampling procedure with a relatively small yearly sample. This standardisation is one of the qualities of the programme, resulting in very little noise in the data. We therefore decided to use generalised linear models that allow for different distributions and chose not to use splines. Although splines are useful to form hypotheses about when and how many trend changes occurred [13], splines are not helpful in quantification of trends.

A Poisson distribution was preferred over binomial distribution, giving priority to trend assessment in emerging and rare resistances. Poisson has a high accuracy for low counts. In general, the Poisson distribution fitted the data better than the binomial distribution; AICs were lower. In the Poisson distribution the mean is equal to the variance. As can be seen from over or underdispersion, this assumption is not met for all antimicrobials. In these cases, data can be remodelled with other distributions, such as binomial (for high counts) or negative binomial distributions (for very low counts). The use

of alternative distributions for over or underdispersion improved model fit for this data. However, estimates were similar, thus conclusions based on Poisson regression seem robust.

Poisson regression seems well suited for quantifying resistance time-trends over the past 20 years and to show trend changes as a result of interventions. However, when the aim is to compare recent monitoring data (a new year) with the previous years, this method may not be the most informative. Adding a new year of data to a time-series of multiple years will not affect estimates for time-trends of x1 and x2. Aberrations in new data can be detected by applying 'year' as a factor instead of a numerical in this Poisson regression model, showing separate estimates of each year relative to one reference year (data not shown).

In this study, we have investigated the best modelling approach to quantify trends over time and detect the effects of interventions within the current Dutch sampling frame. In the Technical specifications 2012 [22], EFSA has given recommendations (based on simulations) on how sampling strategy affects the power of detecting increases or decreases over time. Additional to the work in this study, it should be further investigated how different sample sizes or sampling intervals (every other year instead of a yearly sample) affect the ability of the monitoring programme to detect emerging resistances and trend changes.

Relating AMR trends to AMU

AMR trends were independently quantified and effects of AMU regulations were reflected by the choice of the reference year in the model (2009). The EU monitoring programme in commensal *E. coli* from livestock aims to monitor the effects of AMU-interventions. Relating AMR trends to AMU at a national level is challenging in the first place, because not all member states have detailed data of veterinary AMU. Although there is an ecological correlation between resistant proportions of *E. coli* from animals at slaughter and AMU in livestock, as shown earlier by Dorado Garcia et al. [10], this correlation does not refer to individual animals. Only with extensive sampling at farm level, AMR trends from isolates in faecal samples from livestock can be directly correlated with AMU.

Conclusion

This analysis of the standardised commensal *E. coli* dataset from the Dutch NRL for monitoring AMR in livestock, shows that monitoring in commensal *E. coli* is a useful tool to detect trends in phenotypic resistance in livestock relevant to public health (as defined by EFSA and EU legislation). We showed effective methods to quantify resistance trends in different antimicrobials and detect trend changes. The results of this study concern Dutch data, but this modelling

approach is applicable to similar data acquired in other EU countries. The method can be applied to a dataset of any size, although the method will perform better when there is more data available.

Acknowledgements

We thankfully acknowledge the technicians in the National Reference Laboratory at WBVR, Joop Testerink and Marga Japing, for determining the MICs. Also we thank Ben Wit and Petra Dop from Netherlands Food and Consumer Product Safety Authority, for coordinating the sampling with us. For statistical input, we would like to thank Bas Engel (Wageningen University) and Hans Vernooij (Utrecht University), and Ronald Petie (WBVR) for guidance in designing the R-scripts.

Funding statement: This research was supported by the Ministry of Agriculture, Nature and Food Quality (WOT-01-001-003) and Wageningen Bioveterinary Research.

Conflict of interest

None declared.

Authors' contributions

Ayla Hesp, Gerdien van Schaik, and Jeanet van der Goot designed the analysis. Dik Mevius and Kees Veldman started the Dutch national monitoring program in 1998, acquired all MIC data during AMR monitoring activities at WBVR from 1998 to 2016, and performed the microbiological interpretation. Ayla Hesp conducted the analysis and wrote the main manuscript. Ayla Hesp, Kees Veldman, Jeanet van der Goot, Dik Mevius, and Gerdien van Schaik interpreted results and wrote and reviewed the manuscript.

References

1. Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P&T. 2015;40(4):277-83. PMID:25859123
2. Walker B, Barrett S, Polasky S, Galaz V, Folke C, Engström G, et al. Environment. Looming global-scale failures and missing institutions. Science. 2009;325(5946):1345-6. http://dx.doi.org/10.1126/science.1175325 PMID:19745137
3. World Health Organization (WHO). Antimicrobial resistance - global report on surveillance. World Health Organization, 2014.Geneva: WHO; 2014. Available from: https://www.who.int/drugresistance/documents/surveillancereport/en/
4. Chang Q, Wang W, Regev-Yochay G, Lipsitch M, Hanage WP. Antibiotics in agriculture and the risk to human health: how worried should we be? Evol Appl. 2015;8(3):240-7. http://dx.doi.org/10.1111/eva.12185 PMID:25861382
5. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. Front Public Health. 2014;2:145. http://dx.doi.org/10.3389/fpubh.2014.00145 PMID:25279369
6. Frimodt-Møller N. Microbial Threat--The Copenhagen Recommendations initiative of the EU. J Vet Med B Infect Dis Vet Public Health. 2004;51(8-9):400-2. http://dx.doi.org/10.1111/j.1439-0450.2004.00786.x PMID:15525373
7. Commission of the European Communities. Brussels, 20.06.2001 COM(2001) 333 final. Proposal for a council recommendation on the prudent use of antimicrobial agents in human medicine. Brussels: Commission of the European Communities. Available from: http://ec.europa.eu/transparency/regdoc/rep/1/2001/EN/1-2001-333-EN-F1-2.Pdf
8. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN) in. 2017. Lelystad: MARAN; 2018. Available from: https://www.wur.nl/upload_mm/7/b/0/5e568649-c674-420e-a2ca-acc8ca56f016_Maran%202018.pdf
9. Mevius D, Heederik D. Reduction of antibiotic use in animals "let's go Dutch". J Verbraucherschutz Lebensmsicherh. 2014;9(2):177-81. http://dx.doi.org/10.1007/s00003-014-0874-z
10. Dorado-García A, Mevius DJ, Jacobs JJ, Van Geijlswijk IM, Mouton JW, Wagenaar JA, 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(12):3607-19. http://dx.doi.org/10.1093/jac/dkw308 PMID:27585970
11. Veterinary Medicines Institute (SDa). Usage of Antibiotics in Agricultural Livestock in the Netherlands in 2016. Utrecht: SDa; 2016. Available from: https://cdn.i-pulse.nl/autoriteitdiergeneesmiddelen/userfiles/Publications/engels-def-rapportage-2016-deel-1-en-2-22-09-2017.pdf

12. Hanon JB, Jaspers S, Butaye P, Wattiau P, Méroc E, Aerts M, et al. A trend analysis of antimicrobial resistance in commensal Escherichia coli from several livestock species in Belgium (2011-2014). Prev Vet Med. 2015;122(4):443-52. http://dx.doi.org/10.1016/j.prevetmed.2015.09.001 PMID:26423778
13. Boireau C, Morignat É, Cazeau G, Jarrige N, Jouy É, Haenni M, et al. Antimicrobial resistance trends in Escherichia coli isolated from diseased food-producing animals in France: A 14-year period time-series study. Zoonoses Public Health. 2018;65(1):e86-94. http://dx.doi.org/10.1111/zph.12412 PMID:29110404
14. European Committee on Antimicrobial Susceptibility testing (EUCAST). Antimicrobial wild type distributions of microorganisms 2017. Växjö: EUCAST; 2017. Available from: http://www.eucast.org/mic_distributions_and_ecoffs/
15. European Food Safety Authority (EFSA). Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal Escherichia coli and Enterococcus spp. from food animals. EFSA J. 2008;141:1-14.
16. van Hoek AH, Mevius D, Guerra B, Mullany P, Roberts AP, Aarts HJ. Acquired antibiotic resistance genes: an overview. Front Microbiol. 2011;2:203. http://dx.doi.org/10.3389/fmicb.2011.00203 PMID:22046172
17. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN) in. 2016. Lelystad: MARAN; 2017. Available from: https://www.wur.nl/upload_mm/c/8/4/50343a1f-a2ad-4389-8208-d0e595b9a946_Maran%20report%202017.pdf
18. Machuca J, Briales A, Labrador G, Díaz-de-Alba P, López-Rojas R, Docobo-Pérez F, et al. Interplay between plasmid-mediated and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in Escherichia coli. J Antimicrob Chemother. 2014;69(12):3203-15. http://dx.doi.org/10.1093/jac/dku308 PMID:25139837
19. World Health Organization (WHO). Critically important antimicrobials for human medicine, 5th revision. Geneva: WHO; 2017. Available from: https://www.who.int/foodsafety/publications/antimicrobials-fifth/en/
20. Wu RB, Alexander TW, Li JQ, Munns K, Sharma R, McAllister TA. Prevalence and diversity of class 1 integrons and resistance genes in antimicrobial-resistant Escherichia coli originating from beef cattle administered subtherapeutic antimicrobials. J Appl Microbiol. 2011;111(2):511-23. http://dx.doi.org/10.1111/j.1365-2672.2011.05066.x PMID:21645183
21. Munk P, Andersen VD, de Knegt L, Jensen MS, Knudsen BE, Lukjancenko O, et al. A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. J Antimicrob Chemother. 2017;72(2):385-92. http://dx.doi.org/10.1093/jac/dkw415 PMID:28115502

22. European Food Safety Authority (EFSA). Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in Salmonella, Campylobacter and indicator Escherichia coli and Enterococcus spp. bacteria transmitted through food. EFSA J. 2012;10(6):2742. Available from: https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2012.2742

Chapter 3

Assessment of evaluation tools for integrated surveillance of antimicrobial use and resistance through selected case studies

Sandberg M, Hesp A, Aenishaenslin C, Bordier M, Bennani H, Bergwerff U, Chantziaras I, De Meneghi D, Ellis-Iversen J, Filippizi ME, Mintiens K, Nielsen LR, Norström M, Tomassone L, van Schaik G, Alban L. Assessment of Evaluation Tools for Integrated Surveillance of Antimicrobial Use and Resistance Based on Selected Case Studies. Front Vet Sci. 2021 Jul 8;8:620998. doi: 10.3389/fvets.2021.620998. PMID: 34307513; PMCID: PMC8298032.

https://doi.org/10.3389/fvets.2021.620998

Abstract

Regular evaluation of integrated surveillance for antimicrobial use (AMU) and resistance (AMR) in animals, humans and the environment is needed to ensure system effectiveness, but the question is how. In this study, six different evaluation tools were assessed after being applied to AMU and AMR surveillance in eight countries: 1) ATLASS: the Assessment Tool for Laboratories and AMR Surveillance Systems developed by the Food and Agriculture Organization (FAO) of United Nations, 2) ECoSur: Evaluation of Collaboration for Surveillance tool, 3) ISSEP: Integrated surveillance system evaluation project 4) NEOH: developed by the EU COST Action 'Network for Evaluation of One Health' 5) PMP-AMR: The Progressive Management Pathway tool on AMR developed FAO, 5) and 6) SURVTOOLS: developed in the FP7-EU project 'RISKSUR'. Each tool was scored using i) 11 pre-defined functional aspects (e.g., workability concerning the need for data, time and people), ii) a SWOT-like approach of user experiences (e.g., things that I liked, or that the tool covered well), and iii) eight predefined content themes related to scope (e.g., development purpose, collaboration). PMP-AMR, ATLASS, ECoSur and NEOH are evaluation tools that provide a scoring system to obtain semi-quantitative results, whereas ISSEP and SURVTOOLS will result in a plan for how to conduct evaluation(s). ISSEP, ECoSur, NEOH and SURVTOOLS allow for in-depth analyses and therefore require more complex data, information and specific training of evaluator(s). PMP-AMR, ATLASS and ISSEP were developed specifically for AMR-related activities – only ISSEP included production of a direct measure for "integration" and "impact on decision-making". NEOH and ISSEP were perceived as the best tools for evaluation of OH aspects, and ECoSur as best for evaluation of the quality of collaboration. PMP-AMR and ATLASS seemed to be the most user-friendly tools, particularly designed for risk managers. ATLASS was the only tool focusing specifically on laboratory activities. Our experience is that adequate resources are needed to perform evaluation(s). In most cases, evaluation would require involvement of several assessors and/or stakeholders, taking from weeks to months to complete. This study can help direct future evaluators of integrated AMU and AMR surveillance towards the most adequate tool for their specific evaluation purpose.

Introduction

The importance of combatting antimicrobial resistance (AMR) was highlighted in the Global Action Plan (GAP) released by the World Health Organisation (WHO) in 2015 (1). It was further adopted by the Tripartite Collaboration consisting of the members of the WHO, Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) and endorsed by political leaders and the United Nations (UN) General Assembly (2). The Tripartite Collaboration acknowledges that the AMR challenge needs to be addressed using a One Health (OH) approach to reflect that the development and spread of AMR does not respect boundaries between sectors and, therefore, requires cross-sectoral collaboration and prevention activities. One of the main objectives of the GAP is to initiate and maintain cost-effective integrated surveillance of antimicrobial use (AMU) and AMR at the global and national levels (1).

Ideally, combatting AMR requires engagement from actors within all sectors of animal health, food safety, environmental protection, plant health and human health (3). All sectors need to be involved in surveillance to identify emerging resistance, understand the AMR epidemiology and develop effective policies for AMU and AMR reduction. In short, the integration of sector activities and robust collaboration are essential for successful surveillance and control of AMU and AMR. According to Stärk et al., (4), OH surveillance describes the systematic collection, validation, analysis, interpretation of data and dissemination of information collected in humans, animals and the environment to inform decisions for more effective, evidence-based interventions. AMR genes are present in bacteria and spread amongst humans, animals and the environment. A programme of integrated surveillance of AMR in foodborne bacteria includes coordinated sampling and testing of antimicrobial susceptibility of bacteria from food-producing animals, food and humans using epidemiological (including sampling) and microbiological methods that enable comparisons of results. The use of comparable methods is necessary to allow comparison of antimicrobial susceptibility results between different areas, countries and regions (5, 6). Currently, integrated, OH AMU and AMR surveillance and monitoring systems exist or are under development in many countries (4). However, the surveillance programmes do not always address all necessary sectors and they are rarely fully integrated (7). An integrated approach provides a better understanding of the epidemiology of AMR, an easier identification of the best intervention points and enhance the timeliness of surveillance by providing early warning of emergence of new resistant strains from one sector to another. Furthermore,

cross-sectoral collaboration may lead to knowledge/resource sharing, expertise exchange and capacity building (8), which may result in cost savings and create more efficient and effective systems (9). Full integration might not be necessary to achieve the wanted outputs and integration and collaboration in itself can be costly without always improving outputs (7, 10). A surveillance approach implies planning, data collection, analysis, interpretation and dissemination of a given activity. It is useful to apply collaboration across different surveillance activities and integration in all or some of the activities. Identification of the optimal levels of integration to obtain the information needed for decision-making is an important task in OH surveillance systems (7, 10).

Aenishaenslin et al. (7) suggested that the value of OH surveillance for AMR can be conceptualised and measured across a selection of different outcomes that can be classified in three dimensions (i) immediate; (ii) intermediate; or (iii) ultimate. Immediate outcomes include increased understanding of the AMR epidemiology at the human, animal and environment health interface and the value would lie in the intellectual or social capital generated. Intermediate outcomes include changes in policy or behaviours, and the expected value is the reduction in AMU and AMR that results from these changes. Ultimate outcomes include tangible benefits such as improved animal, human and environmental health and associated socioeconomic benefits.

Apart from appropriate planning and designing, surveillance programmes also need regular evaluation to remain operational, efficient and cost-effective. Moreover, evaluation is needed to ensure that the goal is underpinned by the on-going activities and shared with the essential stakeholders (11). Evaluation is complex and requires agreement on an evaluation objective, a process usually led by food safety/health authorities in consultation with other stakeholders. Secondly, an appropriate evaluation tool should be selected, which requires expertise and knowledge of surveillance evaluation.

Existing tools for evaluation of surveillance (e.g. 12, 13) are not necessarily appropriate for integrated surveillance as they might not address aspects such as collaboration across sectors (12, 14). Characteristics of OH surveillance programmes have been described, and recently, tools to evaluate integrated surveillance systems have emerged, targeting different aspects of the OH or other integrated surveillance activities (7, 11, 15, 16, 17, 18, 19). A tool may have been made for evaluation of a particular type of surveillance system, such as animal health surveillance. Still, it might also be used to assess other types of surveillance systems such as AMR surveillance, covering aspects such

as sampling-strategies and sample-sizes of surveillance protocols. The latter may not be covered in details by the tools developed specifically for AMR surveillance evaluation. The different tools vary in their approaches, layouts and user-friendliness, comprehensiveness, terminology, aspects covered, capacity, training and resources required to use them, as well as their specific usefulness for the evaluation of AMU and AMR surveillance. Hence, a characterisation and meta-evaluation of the existing evaluation tools is called for to provide guidance on how to identify the best match between the evaluation objective, the resources available, and the selected evaluation tool.

During 2019-2020, an international network of scientists in the project "Co-Eval-AMR - Convergence in evaluation frameworks for integrated surveillance of AMR" (20) developed guidance for choosing an assessment approach from an inventory of tools suitable for evaluating integrated AMU and AMR surveillance systems, according to the needs of the users. The results presented here originate from the Co-Eval-AMR network aiming to guide assessors in their future selection of evaluation tools. A pilot version of the present study, using one surveillance-system-case and the first version of the assessment criteria, was published by Nielsen et al. in 2020 (21). The objective of the present study was to describe and assess the characteristics, functionalities and suitability of tools that might be used for evaluation of integrated AMU and AMR surveillance.

Materials and methods

Overview of the evaluation tools

In the following section, the six tools used are presented in brief.

2.1.1 ATLASS (Assessment Tool for Laboratories and AMR Surveillance Systems)

The Assessment Tool for Laboratories and AMR Surveillance Systems (ATLASS) is a tool designed by FAO for assessing and defining targets to improve national AMR surveillance systems in the food and agriculture sectors (18). It is composed of two modules: a surveillance module and a laboratory module. Each module includes two standardised questionnaires, which are to be completed by the assessors. The assessments generate a baseline and classify a "stage" for AMR laboratory capacity detection, AMR surveillance, and dissemination of information.

2.1.2 ECoSur (Evaluation of Collaboration for Surveillance)

The Evaluation of Collaboration for Surveillance tool (ECoSur) aims at evaluating

the organisation, functioning and functionalities of collaboration taking place in a multi-sectoral surveillance system (11). The final purpose is to assess whether collaboration as planned and implemented is relevant and functional to produce the expected collaborative outputs. The tool relies on the scoring of 22 attributes and three indexes characterising the organisation of collaboration at the governance and operation level and nine attributes referring to core functions of collaboration to ensure the sustainable operation of an effective multi-sectoral surveillance system. Three automatically generated outputs display the evaluation results for attributes and indexes and support the identification of strengths and weaknesses of collaboration and the formulation of recommendations for its amelioration.

2.1.3 ISSEP (Integrated surveillance system evaluation project)

The AMR Integrated Surveillance Systems tool (ISSEP) is a conceptual tool developed in Canada with the aim to structure an evaluation of the added value of integrated surveillance systems for AMR (7). It comprises five evaluation levels that target the evaluation of OH integration in the surveillance system, its capacity to produce integrated information and expertise, to generate actionable knowledge, to influence decision-making, and health and economic impacts. For each level, a set of evaluation questions are defined, and links are made with existing evaluation tools. A semi-quantitative scale is applied to show the level of integration of the surveillance system (19).

2.1.4 NEOH (Network for Evaluation of One Health)

The Network for Evaluation of One Health (NEOH) tool is part of a framework resulting from the EU COST Action "Network for Evaluation of One Health" to provide science-based guidance for the evaluation of One Health and other integrated approaches to health (16, 20, 21). There are four elements namely "System definition and description of OH initiative within the system", "Theory of Change" (ToC), "Assessment of OH-ness" and "Outcome evaluation". Qualitative assessment as well as semi-quantitative scorings are used for the evaluation of the degree and of the "OH-ness" (OH-index and OH-ratio) and metrics for different outcomes. Illustrative web-diagrams of the distribution of scores for gap-identification are presented in the Excel-tool for assessment of OH-ness (20).

2.1.5 PMP-AMR (Progressive Management Pathway tool for AMR)

The Progressive Management Pathway tool for AMR tool (PMP-AMR) is a self-assessment tool designed by FAO to provide guidance to countries for implementation of their National Action Plans (NAP) for AMU and AMR (17, 21). It includes four focus areas for evaluation: Awareness, Evidence, Governance

and Practices. For each focus area, specific activities, achievements and key performance indicators (KPI) are listed. The tool provides a dashboard, showing the progress made for each focus area towards an optimal and sustainable use of antimicrobials.

2.1.6 SURVTOOLS

SURVTOOLS was developed as a part of the EU FP7 funded project RISKSUR: Risk-based Animal health Surveillance Systems. The evaluation tool (EVA-TOOL) is a support tool for the evaluation of animal health surveillance systems, developed to provide guidance for evaluation of animal health surveillance including economic evaluation (12, 21). When planning an evaluation, the user is guided through three main steps: defining the evaluation context; defining the evaluation question; selecting the evaluation attributes and the economic criteria. Furthermore, the tool provides additional information and guidance on how to use the evaluation plan to perform the evaluation, and how to report on the evaluation outputs. An online web version of the EVA tool is available (12).

The case study approach

A total of eight country-based case studies of AMU and AMR surveillance systems were included in the study (Table 1). Each country-based case study was undertaken by individuals or a group of individuals with expertise on the respective national cases (hereafter called the assessors), making a total of 20 assessors. The choice of case was the National Action Plan on AMR or parts of it in the respective assessor's country. To collect the information needed to carry out the assessment, the assessors reached out to additional experts and other sources.

The assessors, met regularly and initially there was developed an assessment methodology in collaboration with selected members of the Co-Eval-AMR network group. The methodology included two standardised scoring schemes, a SWOT-like analysis scheme and templates for reporting and instructions. The evaluation tools were applied on the country-based case studies using one or more tools on each case. Overall, the outcome was the users' experience regarding applicability of the tool. A total of six tools were assessed, and each tool was assessed between one and four times.

Methodology used to assess the tools

The details of the scoring scheme for functional aspects, the SWOT-like approach and the scoring scheme for the themes describing the scope of the tools are presented below.

2.3.1 Scoring functional aspects

A scoring scheme aiming at assessing 11 functional aspects was developed and answers were scored numerically; where 1 = not covered, 2 = not well covered, 3 = more or less covered, 4 = well covered. With each score, a comment was requested explaining the score. The 11 aspects were: 1) User friendliness, 2) Compliance with evaluation objectives 3) Efficiency (number of people, time taken vs what the evaluation should be used for), 4) Use of a step-wise approach to the evaluation, 5) Overall appearance, 6) Generation of actionable evaluation outputs, 7) Evaluation of OH aspects, 8) Workability in terms of required data, 9) Workability in terms of required people to include, 10) Workability in terms of analysis to be done, 11) Time taken for application of the tool.

The combined scores for each tool were presented in a heat map. In the case one assessor/assessor group scored over a range of numbers, averaging was used followed by rounding up if necessary to obtain a whole number for the total score. A crude summary score for each tool was calculated and presented in heat maps. The scores should only be interpreted relatively within this study material. The justification for each score, provided by the individual assessors, was condensed by the first author and checked for correctness by the other authors, and the "condensed results" were then presented.

2.3.2 A SWOT-like approach

A SWOT-like scheme was developed asking the assessors to answer four questions: 1) Things that I liked, or that the tool covered well; 2) Things that I struggled with when using this tool; 3) Things people should be aware of when using this tool; and 4) Things that this tool covers insufficiently. A qualitative synthesis of the result was done in two steps. First, all individual phrases were captured. In a second step, phrases with the similar meaning were reduced into one, implying that a phrase was simplified or made into one word, if possible. It also implied that no phrase or word was repeated for each of the SWOT analyses and tools. The first synthesis was carried out by the assessors for the tools they had applied. The second synthesis was condensed by two of the authors and the condensed results were checked for correctness by the other authors and subsequently presented.

2.3.3 Scoring themes for the scopes

A second scoring scheme consisted of eight themes to describe the scope of the tool: Developed specifically for AMU and AMR, Collaboration, Resources, Output and use of information, Integration, Governance, Adaptivity and Technical operations. Seven of the themes included in the scheme were developed in the Co-Eval-AMR project (22). Additionally in this study, the theme Governance

was added. The objective was to score how well each theme was covered by the specific evaluation tool. A more detailed description of the individual themes scope is given in Table 2. The same scoring scale as in Section 2.3.1 was used. The combined scores for each tool were presented in a heat map, based on a similar way for estimation as described in 2.3.1. A crude summary score for each tool was calculated, but this should only be interpreted relatively within this study material. Again, the free text justifications behind the scores provided by the assessors were synthesized by the first author, checked for correctness by the other authors and subsequently presented.

Results

All detailed answers and justifications from the scoring of the functional aspects and the themes and from using the SWOT-like approach are published on the Co-Eval-AMR project webpage (https://coevalamr.fp7-risksur.eu/) and in Nielsen et al., 2019 (21).

Scoring of the functional aspects of the tool

The results from the scoring of the case studies according to the 11 functional aspects of AMU and AMR surveillance systems are shown in Table 3. A summary of the justifications behind the scores is shown in Table 4. A crude summary of the scores showed that ISSEP and NEOH had the lowest scores, 25 and 30 respectively, of the total 44 that could have been achieved. ATLASS and PMP-AMR had the highest, 39 of the 44 possible

For OH aspects, ATLASS and NEOH scored the highest. PMP-AMR, ATLASS, EcoSur and NEOH provide semi-quantitative scores for the aspects evaluated, whereas ISSEP and SURVTOOLS will result in a plan for how to conduct evaluation(s). ISSEP, ECoSur, NEOH and SURVTOOLS allow for in-depth analyses and, therefore, require more complex data, information and specific training of the evaluator(s). PMP-AMR and ATLASS seemed to be the most user-friendly tools, particularly designed for food safety authorities managing the surveillance system.

The SWOT-like approach

The results of the SWOT-like approach applied to assess the tools is shown in Table 5. The variation in answers to the four SWOT-like questions was low among the assessors of each tool, indicating consistency regarding the general impression of the tools. The PMP-AMR and ATLASS were liked for the semi-quantitative scorings which could be made directly and that the tools were

particularly made for evaluation of AMR surveillance systems. What is not covered in these two tools, is the environmental-, plant- and human part of surveillance.

The ECoSur was liked because it allowed evaluation of collaboration in detail; however, the level of abstraction in the language in the existing version of the tool was a struggle. ISSEP was liked because it described the relationship between the integrated surveillance activities for AMU and AMR, OH outputs produced and the different expected outcomes very well.

NEOH was liked for being comprehensive, multi-facetted and fit for a transversal analysis of OH initiatives. The main struggle related to NEOH was that it was cumbersome and time-consuming to use. Similarly SURVTOOLS was liked because information for evaluation of all aspects of a surveillance system including the epidemiological part is provided as scientific references. Further an epidemiological calculator is provided. However, SURVTOOLS is one of the tools that only provide an evaluation plan.

Scoring of the themes describing the scope of the tool

The results from the scoring of each tool for the eight themes describing the scope of the tool in relation to surveillance are shown in Table 6. A summary of the justifications behind the scores are shown in Table 7. A crude summary of the scores for the tools, regarding which themes they covered, showed limited variation. ATLASS had the highest crude summary scores of 28 followed by ISSEP and ECoSur both with 25.

PMP-AMR, ATLASS and ISSEP have been developed specifically for AMR-related activities. NEOH and ISSEP were perceived as the best tools for evaluation of all OH aspects, and ECoSur and ISSEP for evaluation of the quality of collaboration. ATLASS is the only tool evaluating laboratory activities specifically. Only ISSEP produced a direct measure of the "integration" and "impact on decision-making". SURVTOOLS has an epi-sample size calculator and is, hence, the only tool providing a quantitatively assessment of the technical operations in surveillance.

Discussion

4.1 Tools developed specifically for evaluating AMU and AMR surveillance

Only PMP-AMR, ATLASS and ISSEP have been developed especially for evaluating AMU and AMR surveillance. Generally speaking, ISSEP was the only tool assessed that addressed AMU and integration aspects. The strengths of PMP-

AMR and ATLASS are governance, hence, strategic implementation of NAPs. PMP-AMR neither addresses evaluation of design of surveillance nor integration or collaboration. ATLASS is structured in such a way that detailed information about the sectors involved and the laboratories in the surveillance system can be captured. Hereby it addresses the gaps in a laboratory's capacity to implement surveillance testing. A quantitative evaluation of the epidemiological designs is impossible in ATLASS. Moreover, ATLASS does not provide an output of the level of integration – but all data collated could provide the evaluator with an impression of the level of integration in the system evaluated.

However, the other evaluation tools were also considered suitable for evaluation of AMU and AMR surveillance programmes. In fact, several of the tools showed a high degree of flexibility and were applicable to different surveillance evaluation objectives. Still, the most accurate evaluations originated from the tools that match the specific evaluation questions. Generally speaking, evaluation of integrated AMU and AMR surveillance systems will benefit from using tools developed specifically for evaluating AMR surveillance and OH aspects since specific characteristics are encountered.

4.2 User friendliness and potential value

The PMP-AMR and ATLASS tools are to a high extent self-instructive and the questions were, therefore, easy to answer. The structure of PMP-AMR was very easy to understand, whereas ATLASS was more complicated to fill in, since it comprises of many questions at all levels of organisation. The handbook/guidance/surveillance evaluation wiki to SURVTOOLS was, perceived by some of the assessors, as very clear and easy to read. It also provides advice on how to cover many of the required aspects of evaluation. The online evaluation tool itself looks very aesthetic but covers less information than the handbook and is not fully self-instructive for all evaluation objectives. NEOH requires knowledge of both the relevant context (in the NEOH framework denoted 'the underlying system and its system boundaries') and the integrated surveillance activities ('the initiative under evaluation') in question, because the assessor must define all components that form part of the underlying system (the context) included in or affected by the surveillance. NEOH allows the assessor to identify and assess expected outcomes based on the ToC of the initiative. ToC is a specific type of methodology for planning, participation and evaluation that is used in companies, philanthropy, not for-profit and government sectors to promote social change. Further, it defines long-term goals and then maps backward in time to identify the necessary preconditions and actions to be taken. The ToC focus will lead to learning and perhaps a better understanding of the surveillance

and its potential societal impacts. It is easy to get lost in the extensive handbook published to assist in using NEOH, and a quick guide is currently missing. The many detailed questions about integration such as OH implementation including systemic organisation and level of sharing (infrastructure aspects) and learning (operational aspects) allows for nuances in the answers, and hereby, a better quality of the results. However, the evaluator should be aware that applying this tool requires time investment and training, including specific training in "systems thinking".

ISSEP, ECoSur, and SURVTOOLS also allow for an in-depth analysis requiring collection of more complex data and information. For SURVTOOLS, specific training in design of epidemiological studies and a wide spectrum of analytical methods is needed before a full exploitation of the tool can be expected. Many of the tools could also be used to guide the design of AMU and AMR surveillance systems in addition to evaluation of existing systems.

Many of the tools, especially ATLASS, produce intermediate outputs of how well the different parts of the programme are integrated and how well the partners collaborate. In contrast, the interpretation of evaluation results of ECoSur supports the identification of strengths and weaknesses of collaboration and the formulation of recommendations. Among the six tools investigated, this tool allows for addressing collaboration in most detail and in different dimensions.

It became clear during this study that adequate resources are needed to perform a full evaluation, sometimes requiring involvement of many assessors and/or stakeholders, and it might take weeks to months to finalise. For all tools, training and instructions would be required to understand the tools sufficiently well to work effectively. Further, the assessor should preferably have a moderate level of understanding of surveillance processes. Moreover, it is important to balance the degree of complexity of the evaluation tool with the available resources in terms of number of people, data, and time.

4.3 Output and use of information (impact)

The ISSEP and partly SURVTOOLs approaches provide a conceptual basis for structuring the evaluation of different surveillance outcomes, from the level of integration to the evaluation of the decisions as well as economic efficiency. The outputs of an evaluation may consist of first-level outputs, such as epidemiological performance measures, as well as intermediate output, such as how well the system is integrated. For successful AMU and AMR surveillance, the final impact would be that there are antibiotics available to treat future

generations of humans and animals against infections. PMP-AMR and ATLASS only produces intermediate outputs through the theme collaboration. It remains unknown whether this and similar themes really reflect what is necessary to implement to reach the final desired impact in the AMU and AMR surveillance. ATLASS and PMP-AMR is contributing to this final impact by providing evaluation of the governance, strategic support and budgets for surveillance. Evaluation of impact of surveillance will be further addressed in a Phase 2 of the Co-Eval-AMR, just initiated, as a follow-up project funded by Joint Programming Initiative for AMR (JPIAMR) https://www.jpiamr.eu/project/coeval-amr-phase-2/.

4.4 The limitations of the study

We have presented the experiences of eight country-based case study groups in using six evaluation tools. Due to resource constraints, some tools were only assessed in a limited number of case studies. Some of the tools were only scored by two assessors, by two assessor groups, or by the creator(s) of the tool. For NEOH, ECoSur, ISSEP, PMP-AMR co-developers of the tools were involved in the assessment, but the tools were also assessed by other case study groups. The assessments were done by different persons and the scores were perceived as crude and subjective. The assessors had varying levels of understanding of the evaluation tools; some were involved in the development of one of the tools, whereas others were trained in using a specific tool. The first group of assessors may have had greater insights into the tool(s) that they assessed and may have been biased in some aspects of the assessment e.g. user friendliness. During the assessment process, there was some convergence in the scoring done by the assessors due to the development of a common understanding of the words and sentences used in the tools. Therefore, the results of the scoring of the functional criteria had a higher variation than the results of the scoring of the attributes that was done later in the process. The qualitative assessments are probably more informative for the pros and cons of each tool than the actual scores. SURVTOOLS and ATLASS were assessed by "non-developers".

Monitoring and stewardship of AMU as part of AMR surveillance was not addressed in the assessment. In the second phase of the Co-Eval-AMR, additional assessments using other tools are planned. Moreover, focus will be on how to assess the impact of integrated surveillance systems for AMU and AMR as well as on how to evaluate governance. The online assessment system made by the Co-Eval-AMR project group can also be used by other scientist for doing similar comparisons and hence more experiences will be collected (https://coevalamr.fp7-risksur.eu/). Most of the participants in the case study groups were veterinarians or professionals working within veterinary public health. Persons

in human health only participated indirectly when being interviewed, and there was no focus on the environment. In Phase 2 of the project, collaboration among others, with social scientists will broaden the scope and the way of looking at surveillance and evaluations.

4.5 Development of assessment methodology and reporting the results to capture the variation in the underlying reasoning

In the Co-Eval-AMR project, the methodology was developed to capture the usability of the tools for evaluation of AMU and AMR surveillance activities in a systematic way, allowing for comparisons between assessors. The assessment methodologies covered aspects known as contributing to controlling AMR e.g., evaluation of OH aspects, mentioned by for instance Holmes et al. (3). The 11 functional aspects included elements such as user-friendliness and whether the tool meet evaluation needs/produces actionable outputs and the resource needed related to data, manpower, and time. In the second phase of the Co-Eval-AMR-project improvements in assessment criteria will be considered.

As opposed to the other tools, ISSEP and SURVTOOLS generated only a plan for how to conduct the actual evaluation based on the chosen evaluation questions. Hence, scoring these for some of the 11 functional aspects and the eight themes was difficult. The PMP-AMR, ATLASS, ECoSur and NEOH tools provide semi-quantitative evaluation outputs. PMP-AMR and ATLASS measure the progress over time and can be used repeatedly. Moreover, PMP-AMR and ATLASS seemed suitable for non-scientists too, since they do not require specific knowledge of epidemiology and surveillance for their application. The tools are not interchangeable – they do not have common scopes and objectives; therefore, one cannot choose a tool only based on the appreciation as assessed only by these case studies. Some lack of consistency exists between the work done in the different working groups of the Co-Eval-AMR project, because some of the development of methodologies was undertaken simultaneously in all working groups, e.g., governance was therefore only assessed by "country case study groups" with a few exceptions. The latter reflected in the missing data given as a footnote in Table 3.

4.6 Establishing a data capture-system for generation of assessment experiences

The developed reporting template enables other assessors to report their experiences using the tools in a comparable way. The template consists of four sections; 1. General information, 2. Scoring of ten functional aspects, 3. SWOT-like approach, 4. Scoring of eight themes describing the scope of the tool. The

idea was to develop a kind of user experience scoring overview similar to many internet applications such as TripAdvisor and Google reviews providing the readers with quick, yet detailed, insights of the tools. The template is placed in an online platform on the homepage of Co-Eval-AMR (https://coevalamr.fp7-risksur.eu/). We encourage users of the tools to provide their inputs and expect that over time a growing collection of experiences will help users in choosing more easily among the existing tools.

4.7 Conclusion

Evaluation of integrated surveillance is needed at regular intervals using robust tools. It is important to choose a tool that adequately addresses the specific evaluation objectives. We provided a portfolio of the experiences of 20 users representing eight country-based case studies in which six different tools were applied, to highlight their attributes, pros and cons and requirements.

Only PMP-AMR, ATLASS and ISSEP have been developed especially for evaluating AMU and AMR surveillance – with ISSEP being the only tool providing a semi-quantitative score of AMU and AMR integration. All six tools demonstrate a high degree of complementarity. Depending on the evaluation questions selected, assessors may choose among the different tools to conduct the evaluation as such, namely ECoSur for addressing collaboration, NEOH for the OH-ness and the relationship between ToC and expected outcomes of the surveillance, ATLASS for the laboratory capacities, and SURVTOOL for epidemiological and economic performance.

An online platform for reporting of users' experiences will help users interested in conducting an evaluation of AMU and AMR surveillance in choosing the most adequate tools for their specific evaluation needs: https://guidance.fp7-risksur.eu/. Furthermore, this platform could help to further extend general user experience of AMU and AMR surveillance evaluation tools.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. Marion Bordier was involved in the development of ECoSur and Liza Rosenbaum Nielsen was involved in the development of NEOH.

Author Contributions

Marianne Sandberg, Ayla Hesp, Marion Bordier and Lis Alban were substantially involved, and took the lead, in all steps of the study from the conception to the

design of the work. All authors contributed the testing or and/or interpretation of the tool testing results. Most of the authors initially drafted parts of the paper. Ayla Hesp and Ursula Bergwerff designed Table 3.

All authors approved the final version of the paper. They also agreed to be accountable for all aspects of the work, in ensuring that questions related to the accuracy or integrity of any part of the work were appropriately investigated and resolved.

Funding

Co-Eval-AMR was funded by JPIAMR (www.jpiamr.eu) and led by Dr. Barbara Häsler from grant holder Royal Veterinary College in the UK, receiving funding from the Medical Research Council, grant number MR/S037721/1.

Acknowledgements

We thank all participants of Co-Eval-AMR network project for their contribution as well as the stakeholders in the different case studies, who provided us with information to perform the assessments.

References

World Health Organization. Global action plan on antimicrobial resistance. *WHO Press* (2015) 1–28. Accessed October 2020: http://www.who.int/drugresistance/global_action_plan/en/

WHO/FAO/OIE report. Monitoring and evaluation of the global action plan on antimicrobial resistance: framework and recommended indicators. (2019). Accessed October 2020: https://www.oie.int/fileadmin/Home/eng/Media_Center/docs/pdf/PortailAMR/EN_MandE_GAP_AMR.pdf

Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, Guerin PJ, Piddock LJ. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet (2016)387:176–87 doi: 10.1016/S0140-6736(15)00473-0.

Stärk KDC, Arroyo Kuribreña M, Dauphin G, Vokaty S, Ward MP, Wieland B. One Health surveillance - More than a buzz word? *Prev Vet Med* (2015) 120(1):124–30. doi: 10.1016/j.prevetmed.2015.01.019

World Health Organization report 2017. Integrated Surveillance of Antimicrobial Resistance in Foodborne Bacteria: Application of a One Health Approach, ISBN 978-92-4-151241-1: Accessed March 2020: https://apps.who.int/iris/bitstream/handle/10665/255747/9789241512411-eng.pdf?sequence=1

Schnall, J, Rajkhowa, A, Ikuta, K. Surveillance and monitoring of antimicrobial resistance: limitations and lessons from the GRAM project. *BMC Med* (2019) 17:176. https://doi.org/10.1186/s12916-019-1412-8

Aenishaenslin, C, Häsler, B, Ravel, A, Parmley, J Stärk, K, Buckeridge, D. Evidence needed for antimicrobial resistance surveillance systems. *Bull World Health Organ* (2019) 97(4): 283 89. http://dx.doi.org/10.2471/BLT.18.218917

Houe H, Nielsen SS, Nielsen LR, Ethelberg S, Mølbak K. Opportunities for Improved Disease Surveillance and Control by Use of Integrated Data on Animal and Human Health. *Front Vet Sci* (2019) 6:301. doi: 10.3389/fvets.2019.00301

Falzon, LC, Alumasa, L, Amanya, F, Kang'ethe, E, Kariuki, S, Momanyi, K, Muinde, P, Murungi, MK, Njoroge, SM, Ogendo, A, Ogola, J, Rushton, J, Woolhouse MJE, Fèvre, EM. One Health in Action: Operational Aspects of an Integrated Surveillance System for Zoonoses in Western Kenya. *Front Vet Sci* (2019) 6:252. https://doi.org/10.3389/fvets.2019.00252

Falzon, L, Lechner, I, Chantziaras, I, Collineau, L, Courcoul, A, Filippitzi, M, Laukkanen-Ninios, R, Peroz, C, Pinto Ferreira, J, Postma, M, Prestmo, PG, Phythian, C, Sarno, E, Vanantwerpen, G, Vergne, T, Grindlay, DJC, Brennan, M. Quantitative Outcomes of a One Health approach to Study Global Health Challenges. *EcoHealth* (2018) 15:209–227. https://doi.org/10.1007/s10393-017-1310-5

Bordier, M, Delavenne, C, Thuy Thi Nguyen, D, Goutard, FL, Hendrikx, P. One Health Surveillance: A Matrix to Evaluate Multisectoral Collaboration. *Front Vet Sci* (2019) 6:109. https://doi.org/10.3389/fvets.2019.00109

Peyre, M, Hoinville, M, Njorogec, J, Cameron, A, Traon, D, Goutard, F, Calba, C, Grosbois, V, Delabouglise, A, Varant, V, Drewe, J, Pfeiffer, D, Häsler, B. The RISKSUR EVA tool (Survtool): A tool for the integrated evaluation of animal health surveillance systems. *Prev Vet Med* (2019) 173:104777. https://doi.org/10.1016/j.prevetmed.2019.104777

Calba, C, Goutard, FL, Hoinville, L, Hendrikx, P, Lindberg, A, Saegerman, C, Peyre M. Surveillance systems evaluation: a systematic review of the existing approaches, BMC Public Health (2015) 15:448 doi: 10.1186/s12889-015-1791-5.

EMPRESTOOLS, Surveillance Evaluation Tool (SET). Accessed October 2020: http://www.fao.org/ag/againfo/programmes/en/empres/tools_SET.html

Bordier M, Uea-Anuwong T, Binot A, Hendrikx P, Goutard FL. Characteristics of One Health surveillance systems: A systematic literature review. *Prev Vet Med* (2018) pii: S0167-5877(18)30365-9. doi: 10.1016/j.prevetmed.2018.10.005

NEOH. EU Cost Action Network for Evaluation of One Health 'NEOH'(TD1404) action website. Accessed October 2020: http://neoh.onehealthglobal.net/

FAOa. FAO Progressive Management Pathway for Antimicrobial Resistance (PMP-AMR): Accessed October 2020: http://www.fao.org/antimicrobial-resistance/resources/tools/fao-pmp-amr/en/

FAOb. FAO Assessment Tool for Laboratories and AMR Surveillance Systems (ATLASS). Accessed October 2020: http://www.fao.org/antimicrobial-resistance/resources/tools/fao-atlass/en/

Aenishaenslin C, Häsler B, Ravel A, Parmley EJ, Mediouni S, Bennani H, Stärk KDC and Buckeridge DL. Evaluating the Integration of One Health in Surveillance Systems for Antimicrobial Use and Resistance: A Conceptual Framework. *Front Vet Sci* (2021) 8:611931. doi: 10.3389/fvets.2021.611931

Rüegg SR, Nielsen LR, Buttigieg SC, Santa M, Aragrande M, Canali M, Ehlinger T, Chantziaras I, Boriani E, Radeski M, Bruce M, Queenan K and Häsler B. A Systems Approach to Evaluate One Health Initiatives. *Front Vet Sci* (2018) 5:23. doi: 10.3389/fvets.2018.00023

Nielsen LR, Alban, L, Ellis-Iversen, J, Mintiens, K, Sandberg, M. Evaluating integrated surveillance of antimicrobial resistance: experiences from use of three evaluation tools. *Clin Microbiol Infect* (2019). https://doi.org/10.1016/j.cmi.2020.03.015

JPIAMR. Accessed October 2020: https://www.jpiamr.eu/supportedprojects/7th-call-results/

Table 1 | Overview of eight country-based case studies involving six different tools for evaluation of surveillance of antimicrobial use and resistance, 2019.

Country	Tools	Name of surveillance programme	Component(s) covered
Belgium	PMP-AMR, NEOH	Belgian AMR Surveillance Programme (as suggested in the Belgian National Action Plan)	Swine, veal calves, poultry (broilers/laying hens), humans
Denmark	PMP-AMR, ATLASS, ECoSur, NEOH, SURVTOOLS	Danish Integrated AMR Surveillance Programme (DANMAP) – selected parts	Pigs
Canada	ISSEP	Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS)	Humans, livestock, food chain
Italy	NEOH, PMP-AMR, SURVTOOLS	Italian ClassyFarm Surveillance Programme (Data from the Piedmont region)	Pigs
Norway	PMP-AMR, NEOH	NORM-VET Monitoring Program for antimicrobial resistance in the veterinary and food production sectors (NORM-vet)	Broilers
The Netherlands	SURVTOOLS, NEOH	Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands (MARAN)	Broilers, slaughter pigs, veal calves, dairy cows
United Kingdom	ISSEP	Surveillance of AMU and AMR in the United Kingdom	Humans, livestock, food chain
Vietnam	ECoSur	Surveillance of AMR in Vietnam	Humans, Food products, Animals

Table 2 | Description of the themes describing the scope of the tool in relation to surveillance identified in the Co-Eval-AMR project and used for the additional assessment of the evaluation tools for surveillance programs/activities.

Theme	Description of the themes
AMU and AMR	Questions that are specifically addressing the case of AMR (occurrence, prevention, or response) or AMU (recording and management)
Collaboration	Questions on the framework of collaboration (organisation of roles and responsibilities) and the object of collaboration (exchange of data, information and knowledge, sharing of capacities). This category also covers questions about the inclusive participation of stakeholders (e.g. considering gender)
Resources	Questions quantitatively addressing human, physical and financial resources. Questions on the training level of human resources are also considered in this category
Output and use of information	Questions on surveillance outputs that are provided to inform public and private stakeholders, their use to inform decision-making and the benefits from this use (expected, perceived or measured)
Integration	Questions considering three levels of integration: • integration of data systems (within organizations and at national, regional, or international level, data systems interoperation, adherence to international testing and data standards) • integration between sectors and disciplines (knowledge integration, shared decision-making and planning, formulation of common goals) • integration in the national and international context motivating the need for surveillance (link to decision-making, shared decision-making and planning between countries)
Governance*	Questions related to the legislative framework as well as the steering and coordinating mechanisms for the surveillance system: Legislation, Steering, Criteria (limits and goals for reduction)

Adaptivity	Questions on any structural elements allowing for the surveillance system to adapt and evolve. This may include tools, plans and agreements to evolve (e.g. continuous learning programs, external evaluation), but also the features of management and governance allowing for regular evaluation and adaptation of operations (e.g. frequency of meeting, regularity of progress reports)
Technical operations	Questions on technical features of surveillance operations (surveillance design, laboratory capacities, management of specimens, tests applied, data management and analysis), their quality management (SOP, traceability), and the assessment of their performance (sensitivity, specificity)

*Governance was included as a separate theme in this study, but is not a separate theme on the https://guidance.fp7-risksur.eu/welcome/decision-support/.

Table 3 | Result of the scoring of all six tools with respect to the 11 functional aspects, shown as a heat map (number of times the tool was assessed, is given in the bracket). The scoring scale used was where 1 (red) = not covered, 2 = not well covered (orange), 3 = more or less covered (yellow), 4 = well covered (green).

	ISSEP (2)	**ECoSur** (2)	**ATLASS** (1)	**PMP –AMR** (4)	**NEOH** (5)	**SURVTOOLS** (2)
User friendliness	2	3	4	4	2	4
Meets evaluation needs/requirements	3	4	2	3	4	3
Efficiency	2	4	4	4	3	3
Use of a step-wise approach to the evaluation*	3	2	4	4	3	2
Overall appearance**	2	3	4	4	2	4
Generation of actionable evaluation outputs	2	4	4	4	3	2
Allows evaluation of One Health aspects	3	3	4	2	4	2
Workability in terms of required data: (1: very complex, 4: simple)	2	3	1	4	2	3
Workability in terms of people to include: (1: many, 4: few)	2	3	4	3	2	4
Workability in terms of analysis to be done: (1: difficult, 4: simple)	2	4	4	4	3	3
Time taken for application of tool: Time (1: > 2 month, 2: 1-2 months, 3: 1 week - 1 month, 4: < 1 week).	2	3	4	3	2	3
Crude summary score	25	36	39	39	30	33

*Only scored by 11 of the 20 of the assessors.
**Only scored by one of the two assessors of ISSEP.

Table 4 | Results of synthesis of the underlying reasoning's for the scoring according to the 11 functional aspects.

	ISSEP (CA, UK)	ECoSur (VN, DK)	ATLASS (DK)	PMP-AMR (BE, DK, IT, NO)	NEOH (DK, BE, IT, NO, NL)	SURVTOOLS (DK, NL)
User friendliness	Conceptual framework easy to follow Evaluation(s) more complicated	Relatively easy to understand, could be improved with a web interface	Can be used without much preparation	Easy to understand and fill in without training	Complex without training, long/exhausting. Scoring OH attributes is relatively simple	Tool itself is easy to fill in, but more complex to conduct evaluations
Meets evaluation needs/ requirements	Relationships of integrated surveillance activities/outputs described. No guidance on evaluation	Measurement of the level of collaboration, but not the overall added-value of collaborating for surveillance activities	Predefined network is comprehensive, but measurement of smaller progressions not possible	Qualitative scoring system could be improved. Partially meeting needs for AMU and AMR evaluation(s)	Comprehensive, less intuitive to use for specific technical details/ laboratory part	Epidemiological performance easiest to perform, other parts more difficult
Efficiency	Requires a lot of time to conduct evaluation(s)	Evaluation matrix easy to understand/apply. Validation meeting with stakeholder required	Questionable whether all data are really needed?	Easy to fill in. Immediate generation of results. Suitable for administrators	Takes a long time to fill in tool. "Theory of Change" (ToC) could be better integrated. Not a management tool	Take some time to fill in the tool, and longer time for evaluations
Use of a step-wise approach to the evaluation	The tool has five evaluation levels	Only possible to follow progress of collaboration if evaluation repeatedly done	Follow step-wise approach with areas containing sub-categories reflecting level of implementation and geography	Follows (inherent) a step-wise approach with 4 levels with logic progression. Level 1 : planning of activity/ locally, level 2, 3 and 4: undertaking activities / regionally/nationally	Stepwise approach to evaluation with the following steps: context description, initiative-within-context description, OH-ness and ToC (outcome and impact). If evaluation of progress: repeated evaluations over time needed	Do not follow step-wise approach. Order would be given by choice of evaluation question(s) and not by the toll itself
Overall appearance	The conceptual framework is well presented.	Well structured, web-platform needed	Useful for evaluation of AMU and AMR and residue-surveillance at laboratory level	The general assessment part excellent, the sector specific less so. Nice layout, some parts could be improved	Extensive handbook, Excel tool is mostly understandable but too compressed in layout	Generate evaluation plan. Take time to evaluate integrated surveillance. Objective results

Actionable evaluation outputs	No clearly defined actionable outputs	Generation of 3 graphical outputs of results: one for organizational attributes, one for organizational indexes, one for functional attributes	Monitors progress and suggests next level	Actions can be agreed upon during assessment. Graphics could be improved. Gaps in sector evaluation	A web-diagram makes it easy to identify gaps. Scoring is subjective: may lead to biased results	Not generated by tool. Evaluation could generate first level actionable outputs (e.g., effect of designs) other outputs on e.g., awareness more difficult to obtain
Evaluation of OH aspects	Comprehensive	Existence of specific attributes measuring OH aspects, e.g., shared leadership	All sectors covered, measure integration	Not addressed in particular	Major strength of the system's approach and the tool	Can be used for all aspects. Layout does not support all components
Workability regarding required data (1: very complex, 4: simple)	Large amounts of data required	Dependent on the complexity of the surveillance system evaluated	Large amounts of data required	Apparently simple. Data are easily accessible.	Requires effort/time to gather data. Some data complex to get (e.g., learning/system organization)	Relatively simple to get the data for filling in tool, but for some evaluation questions/ objectives it is complex to acquire the data
Workability regarding required people (1: many, 4: few)	Stakeholders from all sectors required	Meant to be applied by an evaluation team	Need expertise from several areas	All stakeholders invited to evaluation-meetings (2 days). One person can do evaluation, but then data capture needed (e.g., through interviews)	Interview of essential actors and stakeholders, but only one evaluator needed	Few people needed
Workability regarding analysis to be done (1: difficult, 4: simple)	No guidance on analysis provided	Easy identification of the criteria influencing the evaluation results to support formulation of recommendations	Automated analysis	Generated by the tool. Mostly yes/no answers to questions	Once tool filled in it provides support for analyses. Comparing ToC and scoring difficult	Dependent on number and complexity of evaluation question(s)
Time (1: > 2 month, 2: 1-2 months, 3: 1 week - 1 month, 4: < 1 week).	Long time required for evaluation(s)	Dependent on the complexity of the surveillance system evaluated	If assessor experienced in surveillance or detailed NAP report available: take relatively short time	Take relatively short time	Filling in the Excel tool is relatively fast once you have the information ready, Defining the ToC and gathering data is time consuming	Short time to fill in tool. Long time for some of the evaluation objectives/questions

Table 5 | Synthesis of phrases provided in the SWOT analysis of six different evaluation tools used in eight country-based case studies.

	ISSEP (CA-UK)	ECoSur (VN, DK)	ATLASS (DK)	PMP-AMR (BE, DK, IT, NO)	NEOH (DK, BE, IT NO, NL)	SURVTOOLS (DK, NL)
Like	Provision of a conceptual model for integrated surveillance of AMU and AMR surveillance	Comprehensive evaluation of collaboration Participatory evaluation Provision of a clear guidance	Automated analyses Progress monitoring Easy to communicate results	Easy Progress monitoring Participatory evaluation Evaluation of the implementation levels	Comprehensive and multi-facetted OH assessment Evaluation of implementation quality	Objectivity Comprehensive framework for different evaluation aspects
Difficulty	No provision of guidance to collect and analyse of data	Evaluation of collaboration only	Why need for such detailed data?	Subjectivity Crude scoring method	Cumbersome	Requirement of training for conducting evaluation Time consuming for evaluation of complex aspects
Be aware of	Necessary combination with other tools depending on the evaluation question	Characterization and evaluation of integration regarding collaborative objectives and context	Not possible to measure minor progress of epidemiological performance	Complexity in terms of people to include Self-assessment tool Results not comparable across countries	Requirement of training for application Resource-demanding	Provision of an evaluation plan only, not AMU and AMR specific
Not covering	Guidance for conducting evaluation	Surveillance performance	Environment and plant sector specifically	One Health assessment Distinction between ongoing and incomplete activities Evaluation of quality of activities	Progress monitoring Surveillance performance	Laboratory aspects One Health assessment

Table 6 | Results of scoring of six tools for AMR surveillance evaluation according to eight themes describing the scope of the evaluation tool (the number of times the tool was assessed, is given in the bracket). The scoring scale used was 1= not covered (red), 2= not well covered (orange) 3= more or less covered (yellow), 4= well covered (green).

	ISSEP (2)	**ECoSur** (2)	**ATLASS** (1)	**PMP-AMR** (4)	**NEOH** (5)	**SURVTOOLS** (2)
AMU and AMR specific	4	2	4	4	3	2
Collaboration	4	4	4	2	4	2
Resources	2	4	3	3	3	3
Output and use of information	4	3	3	3	3	2
Integration	4	4	3	2	4	2
Governance*	3	2	4	4	1	2
Adaptivity	2	4	4	4	3	2
Technical operations	2	2	3	2	2	2
Crude summary score	25	25	28	24	23	17

*Governance was included in this study by 9 of the 20 of the assessors (however, not a separate theme on the https://guidance.fp7-risksur.eu/welcome/decision-support/.

Table 7 | Synthesis of the underlying reasoning's for the scoring according to the eight themes describing the scope of six AMR surveillance evaluation tools.

Themes	ISSEP (CA-UK)	ECoSur (DK, VN)	ATLASS (DK)	PMP (BE, DK, IT, NO)	NEOH (DK, BE, IT, NO)	SURVTOOLS (DK, NL)
AMU and AMR	Framework developed specifically for AMU and AMR	Not specific for AMU and AMR but can be easily applied for AMU and AMR	Designed for AMU and AMR and residues	Designed for AMU and AMR. Misses components besides farm animals	Not designed for this purpose but can be adapted (e.g., under 'objectives of the initiatives') Most, if not all of these questions are expected to be included as part of elements 2 and 3	Not developed for AMU and AMR
Collaboration	Allows evaluation of collaboration between the different organizations involved	Collaboration at the heart of the tool, e.g., cross sectors/ professions/disciplines/ public/private organizations/ geographical/governance/ implementation	Between sectors, all actors and all levels	Reporting, not data exchange. Participation stakeholders/actors considered for institutions. Gender not considered. Promotes knowledge share	Collaboration included in all aspects (in element 1).	No particular guidance; difficult to understand how to evaluate amount of collaboration
Resources	Questions not included, but data can be collected if economic analysis is part of evaluation	Financial aspects addressed in detail at different levels: planning, allocation, use	Ask for un-limited or limited budget	Only present in 'governance'	Only covered in 'planning' and 'sharing' aspects of OH-ness evaluation. Focus on allocation: resources to achieve objectives of the initiative (human/ physical/ financial resources, training). In NEOH handbook: chapter about economic evaluation of OH	Generate a framework for economical evaluation. Epi-calculator available
Output and use of information	Allows to evaluate the outputs of integration and the impacts of integration on decision making and on health and economic outcomes	Allows conclusion about appropriateness of collaborative activities for the expected collaborative outputs (eg improving the epidemiological performance). No quantification of impacts on the surveillance value and of costs.	Intermediate level outputs best addressed	Outputs evaluated (better than impacts), e.g. production of guidelines on prudent use of AM, data reporting to organizations. Not covered in 'awareness'	Reveals gaps in OH and where impact of the initiative being evaluated might be improved. Outcomes/impacts depend on type of OH initiative and boundaries of the contextual "system" and resulting ToC. Hence, the evaluator must take into account the appropriate parameters (data, disciplinary paradigms)	If full evaluation, most of aspects would be covered and impact/output might be possible to measure. Unclear how to measure for inter-mediate outputs/ impacts

Integration	Allows evaluating impacts of integration on decision making/ health /economic outcomes	Assessment of the organization and functions of collaboration to achieve the desired level of integration, in coherence with the context	Addressed for many areas, not in depth	Questions on data reporting, adherence to international testing/data standards/level of knowledge/shared decision making. Not across sectors	Integration measures on many levels e.g. data integration in organizations, national, regional, or international level, and systems interoperation between different sectors. International testing/data standards not included, unless it is included in "initiative" being evaluated	Not included or advanced to evaluate
Governance	Partly considered when looking at the overall organization / management	Inclusion of many aspects: rationale and objective of collaboration, responsibilities of stakeholders, functionality of governance mechanisms, etc.	Addressed for many areas	Well covered, one main focus of the tool	Partially in the thinking and systemic organization of the OH-ness evaluation. The tool includes consideration of legislation and National Action Plan, if Nation is identified as dimensions in the "system"	Not included, but some aspects might be covered if conducting process-evaluation
Adaptivity	The tool does not cover this aspect.	No monitoring of the progress of collaboration. Monitoring and evaluation of collaboration. performance	Measure progress	Designed for measuring improvement	Can be assessed through repeated evaluations. If a dedicated process evaluation done, the progress can be studied. Evaluator and framework-design are "key"	Obtainable if evaluation is done twice (over time) to identify improvement
Technical operations	Includes questions on technical aspects e.g., sampling/ methodology	No evaluation of surveillance performance, even if taken into account evaluation certain collaboration attributes	Quality of epi-designs not covered	Includes questions on the targets of surveillance (e.g. pathogens). Low without ATLASS	Not among evaluation objectives. Include few questions probing for capacities/data handling. Could be part of operations assessment of OH-ness, but extent lies upon evaluator and framework followed	Cover technical efficiency/ performance, other laboratory aspects not guided/covered

II

Part II

Interpretation of antimicrobial resistance monitoring data

Chapter 4

Antimicrobial resistance clusters in commensal *Escherichia coli* from livestock

Hesp A, Ter Braak C, van der Goot J, Veldman K, van Schaik G, Mevius D. Antimicrobial resistance clusters in commensal Escherichia coli from livestock. Zoonoses Public Health. 2021 May;68(3):194-202. doi: 10.1111/zph.12805. Epub 2021 Jan 17. PMID: 33455079; PMCID: PMC8048968.

https://doi.org/10.1111/zph.12805

Abstract

To combat antimicrobial resistance (AMR), policymakers need an overview of evolution and trends of AMR in relevant animal reservoirs, and livestock is monitored by susceptibility testing of sentinel organisms such as commensal *E. coli*. Such monitoring data is often vast and complex and generates a need for outcome indicators that summarize AMR for multiple antimicrobial classes. Model-based clustering is a data driven approach that can help to objectively summarize AMR in animal reservoirs. In this study, a model-based cluster analysis was carried out on a dataset of minimum inhibitory concentrations (MIC), recoded to binary variables, for 10 antimicrobials of commensal *E. coli* isolates (N=12 986) derived from four animal species (broilers, pigs, veal calves, and dairy cows) in Dutch AMR monitoring, 2007-2018. This analysis revealed four clusters in commensal *E. coli* in livestock containing 201 unique resistance combinations. The prevalence of these combinations and clusters differs between animal species. Our results indicate that to monitor different animal populations, more than one indicator for multidrug resistance seems necessary. We show how these clusters summarize multidrug resistance, and have potential as monitoring outcome indicators to benchmark and prioritise AMR problems in livestock.

Introduction

Antimicrobial resistance (AMR) threatens the core of modern medicine, since effective antimicrobials are prerequisites not only for curing infectious diseases, but are also part of routine surgery procedures to prevent bacterial infections (World Health Organization, 2015). Action plans have been developed by international healthcare organisations and governments to restrict AMR in both human- and veterinary medicine, and avoid possible transmission of AMR from animal- or environmental reservoirs to healthcare settings (World Health Organization, 2015; O'Neill, 2016). One crucial aspect of these action plans is monitoring of AMR in relevant reservoirs (O'Neill, 2016).

In food animals in the EU, AMR monitoring is performed following EU legislation (European Commission, 2013), by annually performing standardised antimicrobial susceptibility testing of a fixed number of indicator organisms like commensal *Escherichia coli*, and food borne pathogens such as *Salmonella* and *Campylobacter* species from animals and food thereof. Such monitoring programs result in complex data, which are only interpretable by experts. However, policymakers need a clear overview of development of AMR in reservoirs to further develop, adjust, and validate implemented policies timely. Policy informing organs like European Food Safety Authority (EFSA) have expressed the need for outcome indicators of AMR in tested microorganisms such as commensal *E. coli* (European Food Safety Authority, 2017). These outcome indicators should summarize AMR for multiple antimicrobial classes in a bacterial population, to allow an overall assessment of AMR in samples from relevant reservoirs, such as food animals.

Candidates for such AMR indicators have been tested on datasets of commensal indicator *E. coli* from food animals by EFSA, European Centre for Disease Prevention and Control (ECDC) and European Medicines Agency (EMA) (European Food Safety Authority, 2017). Suggestions were for example the pan-susceptible proportion, and multi-drug resistance proportion (resistant to three or more antimicrobial classes) (European Food Safety Authority, 2017). In this joint scientific opinion it was concluded that the pan-susceptible proportion is the most suitable summary indicator, because a high negative correlation was found with overall AMU. Resistance to three or more antimicrobials was suggested as secondary outcome indicator when very few isolates in an animal population were fully susceptible, and the proportion of fluoroquinolone resistant isolates and the prevalence of ESBL-producing *E. coli* were suggested as other secondary indicators. However, as mentioned

in that joint scientific opinion, the pan-susceptible proportion or resistance to three or more antimicrobials may not be specific enough to adjust AMU policy, especially for sector-specific measures (European Food Safety Authority, 2017). Other suggestions in literature have been to weigh antimicrobial classes, ranked by their relevance for public health as performed by a panel of experts in Havelaar et al.(2017), or to weigh observed AMR for antimicrobial classes by antimicrobial use (AMU) of that same class in an animal population; examples are Dorado Garcia et al.(2016), Laxminarayan et al.(2011). The downside of applying weights to such calculations is that subjective choices have to be made, which may bias the results.

Currently, objective arguments for suitable AMR monitoring outcome indicators are lacking. Few studies have succeeded in reducing complexity of AMR monitoring data over antimicrobial classes in understandable output parameters. To develop specific and applicable outcome indicators of AMR monitoring, we performed a model-based cluster analysis on a dataset of minimum inhibitory concentrations (MIC) for 10 antimicrobials of commensal *E. coli* isolates derived from four animal species (broilers, pigs, veal calves, and dairy cows) in Dutch AMR monitoring, 2007-2018. Here, we show how model-based clustering can be used as a data driven method to summarize resistance patterns, resulting in four clusters that have potential as monitoring outcome indicators to follow AMR trends and effects of AMU (-interventions) in livestock.

Methods

The data used for this analysis were MIC of 12 986 bacterial isolates, all being randomly isolated commensal indicator *E. coli* isolates from faecal or caecal samples of livestock as prescribed by EU-legislation(3): 3 602 from broiler chickens, 2 958 from dairy cows, 3 491 from slaughter pigs, and 2 935 from veal calves. All isolates were collected in the Dutch national monitoring program for AMR in livestock, from 2007 to 2018. Details of data collection and antimicrobial susceptibility testing in this monitoring program were described extensively by Hesp et al.(2019).

We used as definition for antimicrobial resistance: non-wildtype susceptibility, based on epidemiological cut-off (ECOFF) values as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (European Committee on Antimicrobial Susceptibility Testing, 2019). The MIC of all 12 986 isolates were recoded to binary variables (0 for susceptible, 1 for resistant)

using the EUCAST ECOFF values, for ten different antibiotics: gentamicin (GEN), ceftazidime (TAZ), cefotaxime (FOT), chloramphenicol (CHL), trimethoprim (TMP), sulfamethoxazole (SMX), ampicillin (AMP), tetracycline (TET), nalidixic acid (NAL) and ciprofloxacin (CIP). These antimicrobials were included in the analysis because they were continuously tested in the susceptibility panel from 2007 to 2018. They represent the following antimicrobial classes: aminoglycosides (GEN); 3rd generation cephalosporins (FOT/TAZ); amphenicols (CHL); folate pathway inhibitors (TMP/SUL); aminopenicillins (AMP); tetracyclines (TET) and (fluoro)quinolones (NAL/CIP).

After recoding the MIC to binary variables, the data were explored with multivariate analyses, using the 12 986 isolates as (statistical) units with their resistance for the 10 antimicrobials as binary outcome variables (accordingly, 10 variables). Dimension reduction techniques were explored to describe the multidrug resistance patterns. Principal component analysis and multiple correspondence analysis were considered but rejected, because these methods summarize pairwise correlations and associations (i.e. joint resistance patterns) only, thus largely neglecting multiple resistance of higher order, and the interpretation of their output is complicated. Instead, we chose for model-based cluster analysis (Vermunt et al., 2002) also known as latent class analysis, which derives clusters from data based on a statistical model, without the need to choose heuristically a similarity coefficient as in hierarchical clustering. For binary variables, the model has four key assumptions: (i) each unit belongs to one of K clusters (although the posterior membership is a probability, i.e. fuzzy), (ii) the resistance probability of each outcome variable (probability that the outcome is 1) depends on the cluster, (iii) for each cluster, the joint probability of the outcome variables is the product of the individual resistance probabilities (i.e. the outcome variables within a cluster are independent) and (iv) the overall joint distribution is a mixture of the joint probabilities of the clusters with mixing proportions equal to the relative cluster sizes. The Flexmix package (Leisch et al., 2004) in R (R Core Team, 2017) was used for fit this model for K = 10, with 1000 random restarts in the stepFlexmix function. The most likely number of clusters was chosen by the model based on an information criterion, specifically the integrated completed likelihood criterion (ICL), because ICL gives more parsimonious solutions than Akaike's information criterion (AIC) and Bayesian information criterion (BIC) (Biernacki et al., 2000). Finally, isolates are assigned to the cluster on the basis of maximum posterior membership probability according to the model described in Appendix S1. Other resistant isolates than in this dataset can be predicted similarly (Supplementary Appendix S1). Note that if the outcome variables would be independent in the full data set, the method

would not subdivide the isolates in clusters (the result would be one cluster). In other words, model-based cluster analysis uses the associations between the outcome variables to subdivide the isolates in clusters so that these variables lack association within clusters.

The composition of the clusters was further investigated by analyzing the occurrence of combinations of resistance phenotypes within the clusters and how they differed between animal species. The clusters from this analysis were compared with the outcome indicators suggested by ECDC, EFSA and EMA to show how the clusters relate to those indicators, and to investigate the potential of model-based clustering to quantitatively summarize monitoring outcomes.

Results

Model-based clustering showed that four clusters best described the data (illustration in figure S1). The composition of the four clusters, i.e. the mean probability of resistance per antimicrobial per cluster, is presented in Table 1 and a graphical representation can be found in Figure 1. Out of the 1024 possible combinations of resistance within individual isolates, 201 unique combinations were found. An overview of the overall frequency from high to low of the 201 resistance combinations is presented in supplementary material (Table S1) with the cluster they were assigned to and their posterior membership probabilities, and Tables S2-S5 present resistance combinations per cluster, per species. A graphical representation of the yearly proportions of isolates in the clusters over time for the different animal species can be found in Figure 2.

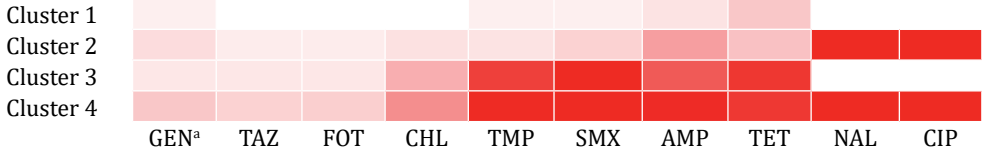


Figure 1. Heatmap showing the resistance probability for the ten tested antimicrobials[a] per cluster in the four clusters from model-based clustering, in commensal *E. coli* isolates (N=12 986) from broilers, dairy cows, slaughter pigs and veal calves from the Netherlands, 2007-2018

[a]GEN = gentamicin, TAZ = ceftazidime, FOT = cefotaxime, CHL = chloramphenicol, TMP = trimethoprim, SMX = sulfamethoxazole, TET, AMP = ampicillin, NAL = nalidixic acid, CIP = ciprofloxacin

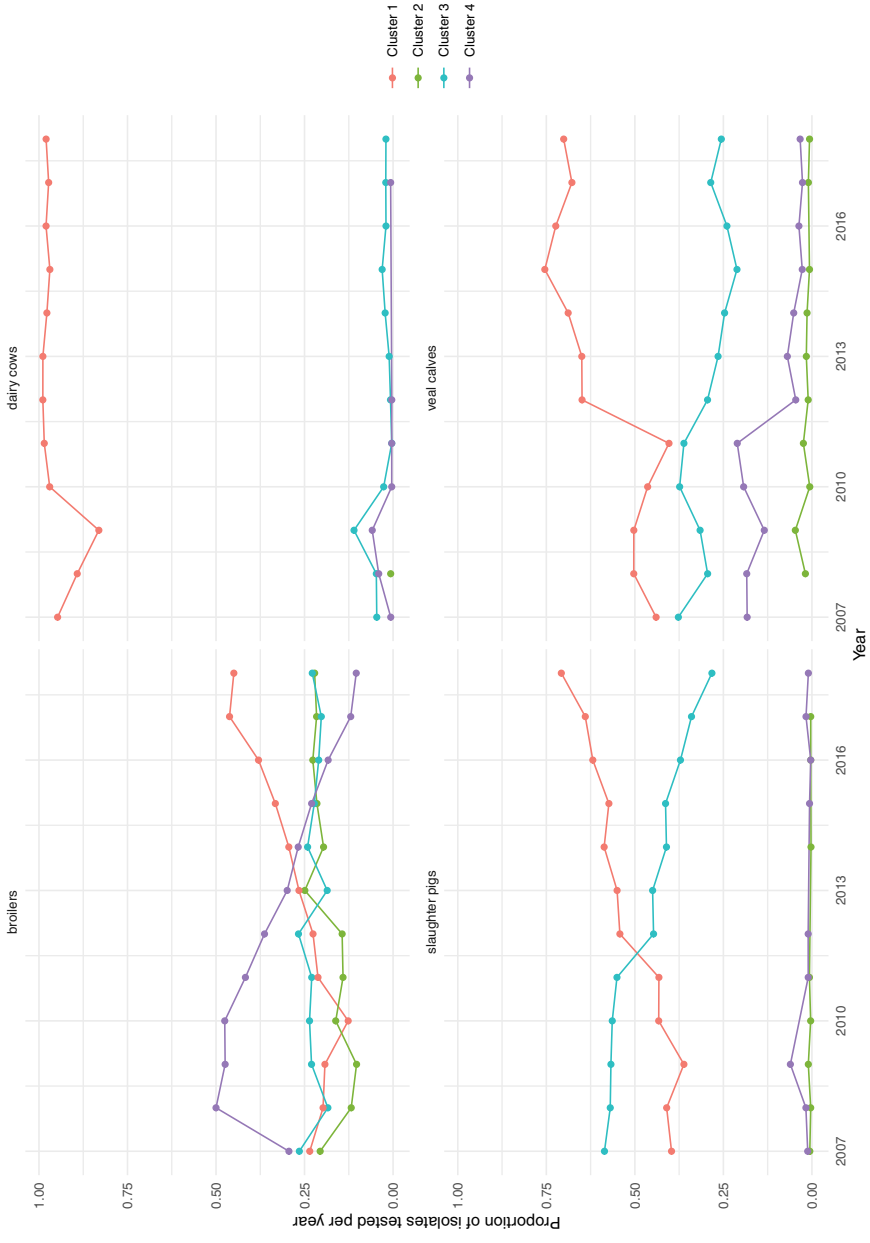


Figure 2. Proportion of isolates in the four clusters (1-4) derived from model-based clustering of multidrug-resistance in commensal *E. coli* isolates (N=12 986) of broilers, dairy cows, slaughter pigs and veal calves from the Netherlands, 2007-2018

Cluster 1 (n=7 566 isolates) is the cluster which is mostly pan-susceptible, besides (mostly) single resistance against TET, AMP, AMP TET, SMX, TMP, GEN, GEN TET and CHL with low probability (<0.16) (Table 1, Table S2). The single resistance phenotype with only TET resistance is almost exclusively present in pigs (Table S2). Cluster 2 (n=698) is mostly susceptible, but carrying only CIP and NAL resistance, sometimes in combination with other resistance traits, of which AMP and TET have the highest resistance probabilities (0.33 and 0.18, respectively). Cluster 2 is almost exclusively present in broilers and not in other animal species (Table 2, S3). Cluster 3 (n=3 289) mostly consists of multidrug-resistant isolates, with high probability of resistance against SMX, TET, TMP and AMP (proportions of 0.94, 0,81, 0.77, and 0.66 of the isolates respectively, Table 1), and against CHL with moderate probability (0.26) but with low probability (<0.05) against 3rd generation cephalosporins FOT and TAZ (Table 1, Table S4). Cluster 4 (n=1 433) contains the most multidrug resistant isolates: almost all (0.99) are resistant against CIP NAL (Table 1, S5), and to SMX, TET, TMP, AMP and CHL with even higher probability than in cluster 3, and with some probability (0.12 and 0.13 respectively) to 3rd generation cephalosporins (TAZ, FOT). To summarize the clusters:

Cluster 1: Isolates that are mostly susceptible against all tested antimicrobials

Cluster 2: Isolates that are mostly susceptible against all tested antimicrobials, except the (fluoro)quinolones

Cluster 3: Multidrug-resistant isolates that are (fluoro)quinolone susceptible

Cluster 4: Multidrug-resistant isolates that are also (fluoro)quinolone resistant

Note that the clusters are numbered in such a way that the resistance increases in probability with cluster number for each antimicrobial, except for (fluoro) quinolones (CIP and NAL). Clusters 1 and 3 are (fluoro)quinolone susceptible, whereas clusters 2 and 4 are highly (fluoro)quinolone resistant.

Table 2 shows how the isolates from the different animal species are divided over the four clusters and Table 3 presents the comparison between the four clusters and the outcome indicators defined by ECDC, EFSA and EMA. All isolates with pan-susceptibility (the primary outcome indicator defined by ECDC, EFSA and EMA) from the total of isolates tested (n=12 986) belong to cluster 1 (bottom of Table 3, Table S2). Isolates belonging to the secondary outcome indicator (as defined by ECDC, EFSA and EMA) multidrug-resistant (>=3) are divided over three clusters: 2, 3 and 4. The highest proportion of multidrug resistance is found in cluster 3 (0.21), followed by cluster 4 (0.11), and lastly 0.05 of the multidrug resistant isolates belongs to cluster 2 (Table 3).

Table 1. Resistance probability per cluster (rows) of a commensal *E. coli* isolate against an antimicrobial (columns). Isolates (N=12 986) are from broilers, dairy cows, slaughter pigs and veal calves in the Netherlands, 2007-2018

	GEN[a]	TAZ	FOT	CHL	TMP	SMX	AMP	TET	NAL	CIP
Cluster 1 (n=7566)	0.01	0	0	0	0.01	0.01	0.05	0.16	0	0
Cluster 2 (n=698)	0.08	0.02	0.02	0.06	0.05	0.12	0.33	0.18	0.96	0.99
Cluster 3 (n=3289)	0.04	0.04	0.04	0.26	0.77	0.94	0.66	0.81	0	0
Cluster 4 (n=1433)	0.16	0.12	0.13	0.41	0.90	0.98	0.88	0.80	0.97	0.99
Overall[b]	0.04	0.02	0.03	0.12	0.30	0.36	0.31	0.39	0.16	0.16

[a]GEN=gentamicin,TAZ=ceftazidime,FOT=cefotaxime,CHL=chloramphenicol,TMP=trimethoprim, SMX=sulfamethoxazole,AMP=ampicillin,TET=tetracycline,NAL=nalidixicacid,CIP=ciprofloxacin.
[b] Overall probability of resistance, i.e. the fraction, out of all isolates (N=12 986), resistant against an antimicrobial.

Table 2. Distribution of the clusters per animal species (with the overall distribution in the last row)

		Cluster			
	N[a]	1	2	3	4
Broilers	3 602	0.28[b]	0.18	0.22	0.31
Dairy cows	2 958	0.96	0.00	0.03	0.01
Slaughter pigs	3 491	0.53	0.00	0.46	0.01
Veal calves	2 935	0.62	0.01	0.28	0.08
Overall	12 986	0.58[c]	0.05	0.25	0.11

[a] Number of isolates tested per animal species, from 2007-2018
[b] Proportion, out of all isolates tested for this animal species, that belong to this cluster
[c] Relative cluster size, i.e. the proportion of all 12 986 isolates that belong to this cluster

Table 3A-B. Relation between the four clusters from model-based clustering and outcome indicators as proposed by ECDC, EFSA and EMA(4): pan-susceptibility (Pan-S), resistant to three or more classes (>=3), and ciprofloxacin resistance (CIP-R), shown as proportions of commensal *E. coli* isolates (N=12 986), of broilers, dairy cows, slaughter pigs and veal calves, the Netherlands, 2007-2018, for the dataset overall (Table 3A) and per animal species (Table 3B)

Table 3A						
Total (n=12 986)	**Indicator**	**Overall proportion[a]**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**
	Pan-S[b]	0.46	0.46	0.00	0.00	0.00
	>=3[c]	0.33	0.00	0.01	0.21	0.11
	CIP-R[d]	0.16	0.00	0.05	0.00	0.11
Table 3B						
Animal species	**Indicator**	**Proportion per animal species[e]**	**Cluster 1**	**Cluster 2**	**Cluster 3**	**Cluster 4**
Broilers (n=3 602)	**Pan-S[f]**	0.20	0.20	0.00	0.00	0.00
	>=3[g]	0.55	0.00	0.05	0.19	0.31
	CIP-R[h]	0.49	0.00	0.18	0.00	0.31
Dairy cows (n=2 958)	**Pan-S**	0.95	0.95	0.00	0.00	0.00
	>=3	0.02	0.00	0.00	0.02	0.01
	CIP-R	0.01	0.00	0.00	0.00	0.01
Slaughter pigs (n=3 491)	**Pan-S**	0.33	0.33	0.00	0.00	0.00
	>=3	0.38	0.00	0.00	0.36	0.01
	CIP-R	0.01	0.00	0.00	0.00	0.01
Veal calves (n=2 935)	**Pan-S**	0.46	0.46	0.00	0.00	0.00
	>=3	0.33	0.00	0.01	0.24	0.08
	CIP-R	0.09	0.00	0.01	0.00	0.08

[a] Overall proportion of isolates from the total number of isolates (N= 12 986), belonging to this outcome indicator by ECDC, EFSA and EMA(4). Note: these indicators are not mutually exclusive
[b] Proportion of pan-susceptible isolates per cluster
[c] Proportion of isolates resistant to three or more classes per cluster
[d] Proportion of ciprofloxacin resistant isolates per cluster
[e] Total proportion per animal species belonging to this outcome indicator
[f] Proportion of pan-susceptible isolates per animal species per cluster
[g] Proportion of isolates resistant to three or more classes per animal species per cluster
[h] Proportion of ciprofloxacin resistant isolates per animal species per cluster

Discussion

The purpose of this study was to use a data-driven method, model-based clustering, to summarize AMR in bacterial isolates over antimicrobial classes, in order to develop suitable outcome indicators of AMR monitoring based on objective arguments. Model-based clustering delivered four clusters as a dimension reduction of the complex data of 12 986 isolates tested with a

panel of 10 antimicrobials of eight antimicrobial classes. These four identified clusters have potential to serve as AMR monitoring outcome indicators. We show a proof-of-principle of how model-based clustering can be used to develop data-driven summary indicators to assess AMR monitoring outcome. In addition, this analysis resulted in a deeper understanding of the patterns in co-occurrence of resistance to more than one antimicrobial per isolate, as expressed phenotypically in commensal *E. coli*. These patterns were identified by 201 unique resistance combinations in this dataset, divided over the four clusters (Supplementary Tables S1-S5). The prevalence of these combinations are different for the animal populations tested. It goes beyond the extend of this study to investigate all multidrug resistance patterns individually. Here, we discuss our main results and remarkable findings: the method creates clusters that differentiate levels of multidrug resistance, with or without resistance to (fluoro)quinolones and 3rd generation cephalosporins.

Quinolone resistance splits the clusters: cluster 1 and 3 without CIP NAL and cluster 2 and 4 with CIP NAL resistance (Table 1, Figure 1). Furthermore, 3rd generation cephalosporin resistance also differs between clusters: cluster 3 and 4 contain isolates with resistance against FOT TAZ, but cluster 1 and 2 contain hardly any. Hence, this cluster analysis divides multidrug resistant isolates over three different categories: relatively susceptible but with the fluoro(quinolones) CIP NAL (cluster 2), multidrug resistant mostly without resistance to these critically important antimicrobials for human medicine (World Health Organization, 2019) (cluster 3), and multidrug resistance including resistance to critically important antimicrobials (cluster 4). Most isolates were assigned to a cluster with high certainty, but not all, as can be seen from a few examples of less certain posterior memberships in Supplementary Table S1.

We conclude from this analysis that the isolates of the four animal species are differently distributed over the resistance clusters, Table 2. As an example: almost all isolates of dairy cows are in cluster 1, whereas broiler isolates are distributed over all clusters. This is in line with AMU in dairy cows, which has for many years been much lower than in broilers (SDa, 2018). Regarding the broilers, most isolates belong to the multidrug resistant cluster 4 (0.31), corresponding with the relatively high level of resistance against critically important antimicrobials in these animals. In pigs, the proportion of isolates in the multidrug resistant cluster 3 (without resistance against critically important antimicrobials) is high (0.46), and in contrast, is low in cluster 4 (0.01). Veal calves have multidrug resistant isolates mainly belonging to cluster 3 (0.28) and to a lesser extend to cluster 4 (0.08).

We illustrate time trends of the four clusters in different animal reservoirs in Figure 2. Interestingly, the susceptible cluster 1 increases over time in all animal species. And in pigs, multidrug resistant cluster 3 decreases over time. In broilers, the highly multidrug resistant cluster 4 decreases over time. These findings are in line with the overall reduction in AMU in all animal species in the Netherlands since 2009 (Dorado-Garcia et al., 2016; SDa, 2018; MARAN, 2019), and more specifically a stop of 3rd generations cephalosporin use in broilers (SDa, 2018; Mevius et al., 2014).

The four clusters we found can be used as indicators to benchmark AMR: over time, over several countries or between animal sectors, either as a reflection of AMU or to assess the overall AMR situation. For benchmarking it is crucial to create transparency by robust metrics, preferably developed by quantitative methods (Bos et al., 2015). These clusters lead to transparency of AMR present in different reservoirs and this method is flexible for policy makers to make choices. Suggestions for benchmarking methods are for example to set an AMR benchmark threshold for the proportion of isolates in 'susceptible' cluster 1. Also the cluster 3 versus cluster 4 proportion could be of interest to benchmark over different reservoirs (Table 2, Figure 2).

Our results indicate that more than one indicator is needed to describe multidrug resistance, as shown in the comparison between the four clusters from this analysis and the indicators proposed by EFSA, ECDC and EMA(European Food Safety Authority, 2017), Table 3. For example, the proportion of multidrug resistant isolates in slaughter pigs versus veal calves (0.38 and 0.33 respectively, Table 3). In pigs, almost all of these isolates belong to the non-critical multidrug-resistant cluster 3, but in contrast a higher proportion of the multidrug-resistant isolates from veal calves belong to the more critical multidrug resistant cluster 4 compared to slaughter pigs (0.08 versus 0.01). Ciprofloxacin resistance, a separate outcome indicator for EFSA, is represented in cluster 2 and 4, differentiating ciprofloxacin resistance as part of multidrug resistance (cluster 4) or mostly without other resistances (cluster 2). Cluster 2 with the phenotype containing just CIP NAL resistance is almost exclusively present in broilers (Table 2, Table 3, Table S3). (Fluoro)quinolone resistance seems to persist in broiler flocks, as also described by other studies (Roth et al., 2019; Chantziaras et al., 2019; Taylor et al., 2016; Vieira et al., 2011).

Model-based clustering summarizes the data without loss of relevant information. As ECDC, EFSA and EMA mentioned in their recent report, for a more detailed analysis of causes for AMR in the agricultural sector, an in-depth

breakdown to the level of individual drug-microbe combinations by animal species and production sector is needed (European Food Safety Authority, 2017). Furthermore, Buyle et al.(2013) mentioned an unavoidable loss of information to occur when indicators are used to summarize large datasets. Using model-based clustering may tackle both of these problems, providing a solution to balance between reducing complexity and loss of information. The clusters reduce complexity, but can be broken down by composition to look up specific information (see the examples of the resistance combinations in Supplementary tables). Another advantage over the EFSA indicators is that these clusters are mutually exclusive, while the EFSA indicators are not mutually exclusive and therefore cannot give an overview of the whole dataset. This is illustrated in Table 3.

In comparison with the work of Havelaar et al.(2017), Dorado Garcia et al.(2016) and Laxmariyan et al. (2011) another important advantage of model-based clustering is that it avoids the making of arbitrary choices, i.e. on what basis groups are made, because this method is data-driven. Although the antimicrobial susceptibility panel included in the analysis will influence results, this probabilistic approach avoids weighing or prioritising with lack of quantitative arguments. And in the model no heuristic choices such as similarity coefficients have to be made, as explained in Methods.

So far, few studies have investigated multi-drug resistance patterns with a quantitative approach to summarize the data and reduce complexity. Most studies on clusters in AMR data describe hierarchical clustering of genetic data from the bacterial genome from which their genetic relatedness can be inferred. Kappell et al. (2015) applied a principal component analysis to both genetic and phenotypic AMR data of multidrug resistant strains, but describe only ordination to visualise patterns, not quantify them (i.e. leading to an output that can be interpreted, for ranking and prioritising specific AMR patterns). Multivariate analyses to quantify multidrug resistance in either genetic or phenotypic AMR data have hardly been performed. Now that whole genome sequencing is becoming available for routine diagnostics and surveillance activities, methods to reduce complexity in genetic AMR data are needed. We used phenotypic data, but this approach could also be interesting to apply on genetic data.

This study concerns ecological data: commensal *E. coli* is a sentinel organism from samples of healthy animals at slaughter, and multidrug resistance in a commensal organism from healthy animals is not directly a public health threat. However, the patterns we found reflect either direct selection or co-selection of

AMR by AMU, or other driving mechanisms in the animal populations. These patterns can consequently be subjected to further investigating the biological mechanisms behind. For example, besides AMU also an increased use of disinfectants (biocides, i.e. quaternary ammonium compounds) were positively associated with increased abundance of AMR genes on pig farms (Van Gompel et al., 2019).

The cluster output of this analysis is, apart from the variability in the data itself, dependent of the selected panel of antimicrobials. Two antimicrobials of the critically important classes 3rd generation cephalosporins and (fluoro) quinolones were included as variables in the analysis. Both FOT and TAZ are included in the EUVSEC susceptibility testing panel because that increases the sensitivity for ESBL-screening, and both CIP and NAL are included in that panel to monitor different types of quinolone resistance (European Food Safety Authority, 2012). In this analysis, these two classes partly determine the way the data is divided in these four clusters. For those classes, the isolates resistant to one of these antimicrobials are nearly always resistant to both antimicrobials (because of cross-resistance), therefore the model considers them to be a cluster. We checked for the influence of modelling only one antimicrobial per antimicrobial class (without TAZ and NAL, which are additional in the classes of 3rd generation cephalosporins and quinolones to FOT and CIP, respectively). This resulted in a solution of only two clusters: one with all almost completely susceptible isolates, and the other being all the multidrug resistant isolates (data not shown). Apparently, in this data, once an isolate is resistant to one antimicrobial, it has a high probability of being resistant to multiple antimicrobials. We considered the two cluster solution less informative and decided to include all 10 antimicrobials from the susceptibility testing panel. As a result of the characteristics of this data, the four clusters in our results have an intrinsic focus on (fluoro)quinolones and 3rd generation cephalosporins. This could be of practical use, since these antimicrobial classes are of specific interest to policy. However, this clustering method should be re-evaluated after analyzing a more diverse international dataset.

These clusters may be interesting benchmark indicators for EU member states, that monitor with the susceptibility panel as prescribed by EU legislation. This analysis was performed on Dutch data, so the question is whether the clusters would also be applicable for data from other countries. It could be dependent of specific AMR patterns, which may vary between ecologies of microbes, different animal sectors, and between regions and countries. To further develop this method, this analysis should be repeated for several other countries, such as

the monitoring data yearly reported by all EU member states to EFSA.

In this study, isolates from all animal species were included in one analysis instead of analyzing all animal species separately, this enables comparing the cluster outcomes for benchmarking purposes. However, the effect of this methodology on the cluster outcome should be further investigated. In addition, it could be interesting to use this model to investigate associations within one animal species over different European countries. Countries often have differences in food animal producing sectors, and other clusters may be found with different input data. However, in principle the method should perform the same: summarizing resistance data in a more easily understandable output than achieved so far.

In conclusion, model-based clustering identifies clusters that summarize resistance over antimicrobials or antimicrobial classes. The four clusters we found have potential to be used by policy makers as monitoring outcome indicators, as we showed for Dutch AMR monitoring data from livestock, 2007-2018. The composition of the clusters was determined by the co-occurrence of resistance to more than one antimicrobial per isolate, and these reflect selection and co-selection patterns by AMU or other determinants. This study concerns ecological data from a commensal microorganism from Dutch livestock reservoirs, but this analytical method has potential value to identify clusters as outcome indicators for data from other microorganisms (for example foodborne pathogens such as *Salmonella, Campylobacter*), or data from other reservoirs.

Acknowledgements

We thankfully acknowledge the technicians in the National Reference Laboratory at Wageningen Bioveterinary Research (WBVR), Joop Testerink, Marga Japing, Yvon Geurts and Alieda van Essen for determining the MIC. For help in data handling we would like to thank Alieda van Essen at WBVR.
Funding statement: This research was supported by the Ministry of Agriculture, Nature and Food Quality (WOT-01-001-003) and Wageningen Bioveterinary Research.

References

Biernacki, C., Celeux, G., & Govaert, G. (2000). Assessing a mixture model for clustering with the integrated completed likelihood. *IEEE Transactions on Pattern Analysis and Machine Intelligence, 22*(7), 719-725. doi:10.1109/34.865189

Bos, M. E. H., Mevius, D. J., Wagenaar, J. A., van Geijlswijk, I. M., Mouton, J. W., Heederik, D. J. J., & Authority, o. b. o. t. N. V. M. (2015). Antimicrobial prescription patterns of veterinarians: introduction of a benchmarking approach. *Journal of Antimicrobial Chemotherapy, 70*(8), 2423-2425. doi:10.1093/jac/dkv104

Buyle, F., Metz-Gercek, S., Mechtler, R., Kern, W., Robays, H., Vogelaers, D., & Struelens, M. (2013). Development and validation of potential structure indicators for evaluating antimicrobial stewardship programs in European hospitals. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology, 32*. doi:10.1007/s10096-013-1862-4

Chantziaras, I., Smet, A., Filippitzi, M. E., Damiaans, B., Haesebrouck, F., Boyen, F., & Dewulf, J. (2018). The effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal E. coli in broilers. *Avian Pathology, 47*(5), 443-454. doi:10.1080/03079457.2018.1486027

Commission implementing decision on the monitoring and reporting of antibmicrobial resistance in zoonotic and commensal bacteria, 2013/652/EU Stat. (2013).

Dorado-Garcia, A., Mevius, D. J., Jacobs, J. J., Van Geijlswijk, I. M., Mouton, J. W., Wagenaar, J. A., & Heederik, D. J. (2016). Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. *J Antimicrob Chemother, 71*(12), 3607-3619. doi:10.1093/jac/dkw308

European Food Safety Authority. (2017). ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. *EFSA Journal, 15*(10), e05017. doi:10.2903/j.efsa.2017.5017

European Food Safety Authority. (2012). *Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in Salmonella, Campylobacter and indicator Escherichia coli and Enterococcus spp. bacteria transmitted through food.* Retrieved from https://www.efsa.europa.eu/en/efsajournal/pub/2742

European Committee on Antimicrobial Susceptibility Testing. (2019). Antimicrobial wild type distributions of microorganisms. Retrieved from http://www.eucast.org/mic_distributions_and_ecoffs/

Havelaar, A. H., Graveland, H., van de Kassteele, J., Zomer, T. P., Veldman, K., & Bouwknegt, M. (2017). A summary index for antimicrobial resistance in food animals in the Netherlands. *BMC Vet Res, 13*(1), 305. doi:10.1186/s12917-017-1216-z

Hesp, A., Veldman, K., van der Goot, J., Mevius, D., & van Schaik, G. (2019). Monitoring antimicrobial resistance trends in commensal Escherichia coli from livestock, the Netherlands, 1998 to 2016. *Eurosurveillance, 24*(25), 1800438. doi:doi:https://doi.org/10.2807/1560-7917.ES.2019.24.25.1800438

Kappell, A. D., DeNies, M. S., Ahuja, N. H., Ledeboer, N. A., Newton, R. J., & Hristova, K. R. (2015). Detection of multi-drug resistant Escherichia coli in the urban waterways of Milwaukee, WI. *Frontiers in Microbiology, 6*(336). doi:10.3389/fmicb.2015.00336

Laxminarayan, R., & Klugman, K. P. (2011). Communicating trends in resistance using a drug resistance index. *BMJ Open, 1*(2), e000135. doi:10.1136/bmjopen-2011-000135

Leisch, F. (2004). FlexMix: A General Framework for Finite Mixture Models and Latent Class Regression in R. *Journal of Statistical Software, 011*(i08). Retrieved from https://EconPapers.repec.org/RePEc:jss:jstsof:v:011:i08

Mevius, D., & Heederik, D. (2014). Reduction of antibiotic use in animals "let's go Dutch". *Journal für Verbraucherschutz und Lebensmittelsicherheit, 9*(2), 177-181. doi:10.1007/s00003-014-0874-z

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/.

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., & Domig, K. J. (2019). The application of antibiotics in broiler production and the resulting antibiotic resistance in Escherichia coli: A global overview. *Poult Sci, 98*(4), 1791-1804. doi:10.3382/ps/pey539

O'Neill.(2016). *Tackling drug-resistant infections globally: Final report and recommendations. The review on antimicrobial resistance. Chaired by Jim O'Neill*. (2016). Retrieved from https://amr-review.org/Publications.html

Taylor, N. M., Wales, A. D., Ridley, A. M., & Davies, R. H. (2016). Farm level risk factors for fluoroquinolone resistance in E. coli and thermophilic Campylobacter spp. on poultry farms. *Avian Pathology, 45*(5), 559-568. doi:10.1080/03079457.2016.1185510

SDa. (2018). *Usage of Antibiotics in Agricultural Livestock in the Netherlands in 2018*. Retrieved from https://cdn.i-pulse.nl/autoriteitdiergeneesmiddelen/userfiles/Publications/2018-def-rapport1.pdf

Van Gompel, L., Luiken, R. E. C., Sarrazin, S., Munk, P., Knudsen, B. E., Hansen, R. B., . . . Smit, L. A. M. (2019). The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries. *Journal of Antimicrobial Chemotherapy, 74*(4), 865-876. doi:10.1093/jac/dky518

Veldman, K.T., Mevius, D.J., Wit, B., Van Pelt, W., Franz, E., Heederik, D. (2019). *Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2018*. Retrieved from https://www.wur.nl/upload_mm/a/7/9/89640bbc-53a2-40f0-ba4a-a9a34a7bf416_Nethmap%20Maran%202019.pdf

Vermunt J.K., editors Hagenaars, A., McCutcheon, Applied latent class analysis. Cambridge University Press. 2002. p. 89-106.

Vieira, A. R., Collignon, P., Aarestrup, F. M., McEwen, S. A., Hendriksen, R. S., Hald, T., & Wegener, H. C. (2011). Association Between Antimicrobial Resistance in Escherichia coli Isolates from Food Animals and Blood Stream Isolates from Humans in Europe: An Ecological Study. *Foodborne Pathogens and Disease, 8*(12), 1295-1301. doi:10.1089/fpd.2011.0950

World Health Organization. (2015). *Global action plan on antimicrobial resistance*. Retrieved from https://www.who.int/antimicrobial-resistance/publications/global-action-plan/en/

World Health Organization. (2017). *Critically important antimicrobials for human medicine, 5th revision*. Retrieved from http://www.who.int/foodsafety/publications/antimicrobials-fifth/en/

Supplementary data

Table S1-S5: See supplementary xlsx.file, available online.

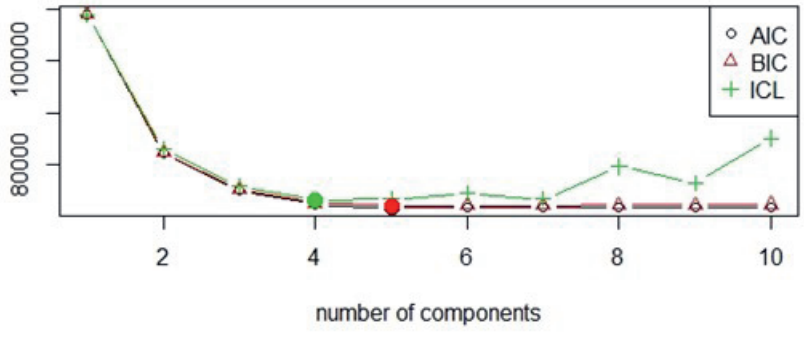


Figure S1. Model selection was based on the mean ICL[a] for all Flexmix models generated in the stepFlexmix model (1000 iterations) showing four clusters as ideal number to describe the data

[a] The number of components (clusters) is shown on the x-axis and the information criterion (for AIC, BIC and ICL) on the y-axis.

Appendix S1: Posterior membership of a resistance pattern

Appendix S1: Posterior membership of a resistance pattern

To assign an isolate to a cluster we need the resistance pattern of the isolate and the parameters of the cluster solution, namely

- the probability of resistance against each of the ten antimicrobials for each of the four clusters; these probabilities are given in Table 1 of the main text.
- the relative cluster sizes, given as last row in Table 2 of the main text.

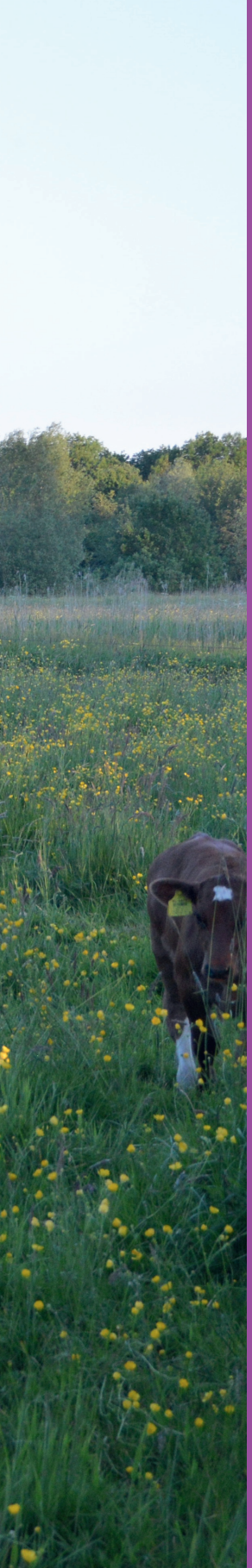
Notation. Let $\mathbf{y} = (y_1, \ldots, y_m)$ be the resistance pattern of an isolate, with y_i=1 for resistance against the j^{th} antimicrobial (j = 1,...,m) with m = 10, $\mathbf{p}_k = (p_{k1}, \ldots, p_{km})$ the probability vector containing the resistance probabilities against each of the ten antimicrobials, thus, p_{kj} is the resistance probability against the j^{th} antimicrobial for the k^{th} cluster (k = 1,..., K) with K = 4, and $\mathbf{w} = (w_1, \ldots, w_K)$ are the relative cluster sizes with w_k the relative size of the k^{th} cluster, with $\sum_k w_k = 1$.

The unnormalized posterior membership probability (q_k^*) that a resistance pattern $\mathbf{y}$ $\mathbf{y}$ belongs to cluster k is

$$q_k^* = w_k \prod_{j=1}^{m} p_{jk}^{y_j} \left(1 - p_{jk}\right)^{1-y_j}$$

The isolate is assigned to the cluster with the largest value of q_k^* (k = 1..., K). The posterior membership probability is obtained by normalization:

$$q_k = q_k^* / \sum_{l=1}^{K} q_l^* .$$



Chapter 5

Antimicrobial resistance prevalence in commensal *Escherichia coli* from broilers, fattening turkeys, fattening pigs, and veal calves in European countries and association with antimicrobial usage at country level

Ceccarelli D, Hesp A, van der Goot J, Joosten P, Sarrazin S, Wagenaar JA, Dewulf J, Mevius DJ, Effort Consortium OBOT. Antimicrobial resistance prevalence in commensal Escherichia coli from broilers, fattening turkeys, fattening pigs and veal calves in European countries and association with antimicrobial usage at country level. J Med Microbiol. 2020 Apr;69(4):537-547. doi: 10.1099/jmm.0.001176. PMID: 32186483.

https://doi.org/10.1099/jmm.0.001176

Abstract

The aim of this article is to report on antimicrobial resistance (AMR) in commensal *Escherichia coli* from livestock from several European countries. The relationships with antimicrobial usage (AMU) at country level and harmonized indicators to cover the most relevant AMR aspects for human health in animal production were also investigated.

Escherichia coli were isolated in faeces from broilers and fattening pigs (from nine countries), and fattening turkeys and veal calves (from three countries) and screened against a fixed antimicrobial panel. AMU data were collected at farm and average treatment incidences stratified by antimicrobial class, country and livestock species were calculated. Associations between AMR and AMU at country level were analysed.

Independent of animal species, the highest resistance was observed for ampicillin, sulphamethoxazole, tetracycline and trimethoprim. *E. coli* from broilers showed the highest resistance level for (fluoro)quinolones, and multidrug resistance peaked in broilers and fattening turkeys. Colistin resistance was observed at very low levels with the exception of fattening turkeys. High resistance to 3rd- and 4th- generation cephalosporins was detected in broilers and fattening turkeys. The lowest levels of resistance were for meropenem, azithromycin and tigecycline (<1%).

Significant correlations between resistance and usage at country level were detected in broilers for fluoroquinolones, polymyxins and aminoglycosides, and in fattening pigs for cephalosporins, amphenicols, fluoroquinolones and polymyxins. None of the correlations observed between AMR and AMU were statistically significant for fattening turkey and veal calves. The strength of the analysis performed here is the correlation of aggregated data from the same farms at country level for both AMU and AMR within antimicrobial classes.

Introduction

Antimicrobial resistance (AMR) has emerged globally in food-producing animals during the last decades, with consequent concerns for both veterinary and human medicine [1]. The AMR reservoir in bacteria from livestock has been increasingly investigated for its potential to transfer AMR to humans via direct contact, the environment or the food-chain [2-3]. AMR is not an issue only for pathogenic bacteria but also for commensal intestinal microbiota. *Escherichia coli* is commonly used as an indicator of the Gram-negative gut microbiota [4]. Most livestock carry *E. coli* as a commensal in their intestine and thus it can be regarded as a reservoir of acquired resistance determinants. Phenotypic assessment of *E. coli* is used as a proxy of AMR in the intestinal tract of healthy animals, including resistance determinants mediated by mobile genetic elements. This approach is crucial in monitoring activities in Europe as recommended by EFSA [5].

In the EU FP7 EFFORT project (http://www.effort-against-amr.eu/), a cross sectional analysis of antimicrobial resistance and antimicrobial usage (AMU) was conducted in a selection of broilers and fattening pigs farms in nine EU countries. In three countries, the occurrence and characteristics of AMR and AMU was also analysed in a selection of veal calf and fattening turkey farms. The strength of the EFFORT project relies on the fact that both AMR and AMU datasets were gathered from the same farms, making this data explicitly suitable to analyse overall correlations between AMR and AMU. Conversely, in monitoring activities, this is more difficult since AMR data are collected at slaughterhouse, while AMU data are available from different farms, therefore, not from the same epidemiological units. The data was also analysed considering harmonized indicators in food-producing animals proposed by EFSA and ECDC to estimate the progress made towards a reduction in bacterial resistance to key antimicrobials in livestock within the European Union [5]. One primary indicator (full susceptibility) and three secondary indicators (resistance to 3rd- and 4th-generation cephalosporins, multidrug resistance and ciprofloxacin resistance) can be used to provide a general assessment of the overall AMR situation in each nation and information on specific issues of a more restrict scope, respectively [5].

The aim of this paper is to report on antimicrobial resistance prevalence in indicator *E. coli*, as well as on the overall correlation between resistant proportions of isolates and mean AMU per country from farms sampled within the EFFORT project. Furthermore, the correlation of mean AMU per country with national harmonized indicators per animal species to cover the most relevant AMR aspects for human health in animal production at national level is analysed.

Methods

Farm selection and animal sampling

Farm selection and sampling has been previously described [6]. Briefly, for each of the participating countries (Belgium, Bulgaria, Denmark, France, Germany, Italy, the Netherlands, Poland and Spain) 20 conventional integrated farrow-to-finisher pig farms and 20 conventional broiler farms were included. Countries were anonymized (A to I) to ensure that results could not be traced back and because farm selections cannot be considered representative of an entire country. However, the sample size of species at country level does allow showing an approximation of the problem, including differences at species level and trends at country level when it comes to AMR and AMU. Furthermore, 20 conventional turkey farms with an all-in-all out system (countries B, E, and H) and 20 non-mixed white or rosé veal calf farms with an all-in all-out system at the compartment level (countries B, E and F) were included. These two datasets were incorporated in the original EFFORT project based on the relevance of these specific livestock production from a national point of view, and because of the scarse data available on AMU and AMR in veal calves and fattening turkeys, especially in a multi-country setting. In order to standardize methods and techniques during a pilot study, one additional farm for both fattening pigs and broilers (30 instead of 10 animals per farm, country A,) and veal calves (10 animals, country B) were included. All farms included in the study were epidemiologically unrelated, as the farms were required not to have contact with each other through trade and each farm had only one owner.

Faecal samples from ten animals per farm (1830 broilers, 1830 fattening pigs, 600 fattening turkeys and 610 calves) were collected within the last week before slaughter. To prevent seasonal influences farm sampling was distributed over the year, sampling 5 farms per season. Overall sampling in all countries was conducted between April 2014 and October 2016. Deviations from sampling protocol are described in detail by Munk and colleagues [6]. For each farm, faecal samples were suspended in Buffered Peptone Water (BPW) [1:10 (w/v)] with 20% glycerol and from each sample a volume of 2 g was stored in duplicate at -80 °C.

E. coli isolation and susceptibility testing

Faecal samples were individually inoculated on MacConkey agar without antibiotic selection at 37 °C. After overnight incubation, one randomly picked presumptive *E. coli* colony per faecal sample, per farm, was pure cultured and stored individually in BPW with 20% glycerol at -80 °C pending analysis. Colonies were confirmed as *E. coli* biochemically or alternatively by MALDI-TOF

MS (Microflex LT MALDI Biotyper; Bruker Biosciences) or sub-culturing onto CHROMagar (CHROMagarTM).

Minimum inhibitory concentrations (MIC) with broth microdilution were determined for a fixed panel of antimicrobials by commercially available microtitre plates (EUVSEC, Thermo Fisher Scientific). Quality assurance among laboratories was ensured by distribution of a Standard Operating Procedure according to ISO standard 20776-1-2006 [7], use of ATCC strains as control, and standardization of methodologies during a mandatory training organized before sample collection. Within EFFORT, no External Quality Assurance Services (EQAS) was organized because the majority of laboratories involved are National Reference Laboratories that already take part in the annual EQAS organized by the EU Reference Laboratory – Antimicrobial, hosted at DTU-Food (DK), also one of the participating laboratories in EFFORT.

EUCAST epidemiological cut-off values were used to differentiate between wild-type and non-wild-type susceptibility (henceforward referred to as resistant isolates). The epidemiological cut-off values (ECOFFs) used were: ampicillin (AMP) ≤8 mg/L, cefotaxime (FOT) ≤0.25 mg/L, ceftazidime (TAZ) ≤0.5 mg/L, meropenem (MERO) ≤0.125 mg/L, ciprofloxacin (CIP) ≤0.064 mg/L, nalidixic acid (NAL) ≤16 mg/L, azithromycin (AZI) ≤16 mg/L, chloramphenicol (CHL) ≤16mg/L, colistin (COL) ≤2 mg/L, gentamicin (GEN) ≤2 mg/L, sulphamethoxazole (SMX) ≤64 mg/L, trimethoprim (TMP) ≤2 mg/L, tetracycline (TET) ≤8 mg/L, and tigecycline (TGC) ≤0.5 mg/L [8]. Misinterpretation of sulphamethoxazole MIC-endpoints (overestimation of resistance) for country B led to the exclusion of these data from the analysis (Table S1, Supplementary data).

Antimicrobial usage data

Antimicrobial usage data for the following antimicrobial classes were obtained for all livestock (unless otherwise specified): aminoglycosides, aminopenicillins, amphenicols (fattening turkeys excluded); cephalosporins (fattening pigs and veal calves); fluoroquinolones, lincomycin-spectinomycin, lincosamides (fattening pigs and broilers only); macrolides, other quinolones, paromomycin (fattening pigs only); penicillin, pleuromutilins (fattening pigs only); polymyxins; tetracycline; sulphonamides (fattening pigs and veal calves only) and different combinations of trimethoprim-sulphonamides. Group treatment data were collected at farm level using specific questionnaires per animal species (questionnaire for broiler farms is provided as an example in (Table S2, Supplementary data) and were quantified with the treatment incidence (TI) indicator. Briefly, TI was calculated as the antimicrobial dose per defined daily animal doses (DDDvet) per 1000 animals at risk. As TI is expressed per 1000

animals at risk, this number, when divided by 10, represents the percentage of their lifetime that the animals received a daily dose of antimicrobials. TI results are described in more detail per 100 animals at risk elsewhere [9-10]. The TI formula adjusts the total amount of active substance administered for the average duration of one production cycle on country level, in case of the broiler farms [9], and on farm level in case of the veal calf and turkey farms. For pig farms, duration of a production cycle was age category-specific (sucklers, weaners and finishers). The TI of sucklers, weaned piglets and finishers were combined and recalculated into a standardised lifespan of 200 days to express AMU from birth to slaughter (TI200) [10]. In addition, TI takes into account a standardized dose and the number of animals at risk for being treated. The latter was derived from the questionnaire where we recorded the group size of the animals. Corrections for a standardized dose were made by using DDDvet values from ESVAC (European Surveillance of Antimicrobial Consumption) for quantification in broilers, pigs and veal calves. Whenever DDDvet was not available for a product, dosage mentioned in the SPC (summary of product characteristics) of that product was used. DDD_{turkey} was defined for all antimicrobials used on the participating turkey farms, using a similar approach as previously described [11]. In this study average TIs on participating farms, stratified by antimicrobial class, country and livestock species were used.

Statistical analysis

For all livestock species, MIC data were aggregated at country level in resistance proportions. AMU data were aggregated as mean treatment incidence on participating farms. The aggregated MIC data were correlated to the mean treatment incidence per country, over countries, giving insight in the overall correlations of AMU and AMR within antimicrobial classes.

The correlation between AMR proportions and mean AMU per country was tested using the Spearman rank correlation test (rho) in RStudio, version 1.1.423. Each antibiotic resistance proportion was tested against up to three different antimicrobial treatments (T1, T2, T3) depending on the available AMU data per animal species: AMP, FOT, TAZ: penicillins, aminopenicillins, cephalosporins (only fattening pigs); AZI: macrolides, lincomycin-spectinomycin, lincosamides; CHL: amphenicols; CIP, NAL: fluoroquinolones, other quinolones; COL: polymyxins; GEN: aminoglycosides, lincomycin-spectinomycin, paromomycin (only fattening pigs); SMX: trimethoprim-sulphonamides, sulphonamides; TET: tetracyclines; TGC: tigecyclines; TMP: trimethoprim-sulphonamides. All statistical analyses were performed in R version 3.4.3 [12] under the integrated development of R-studio.

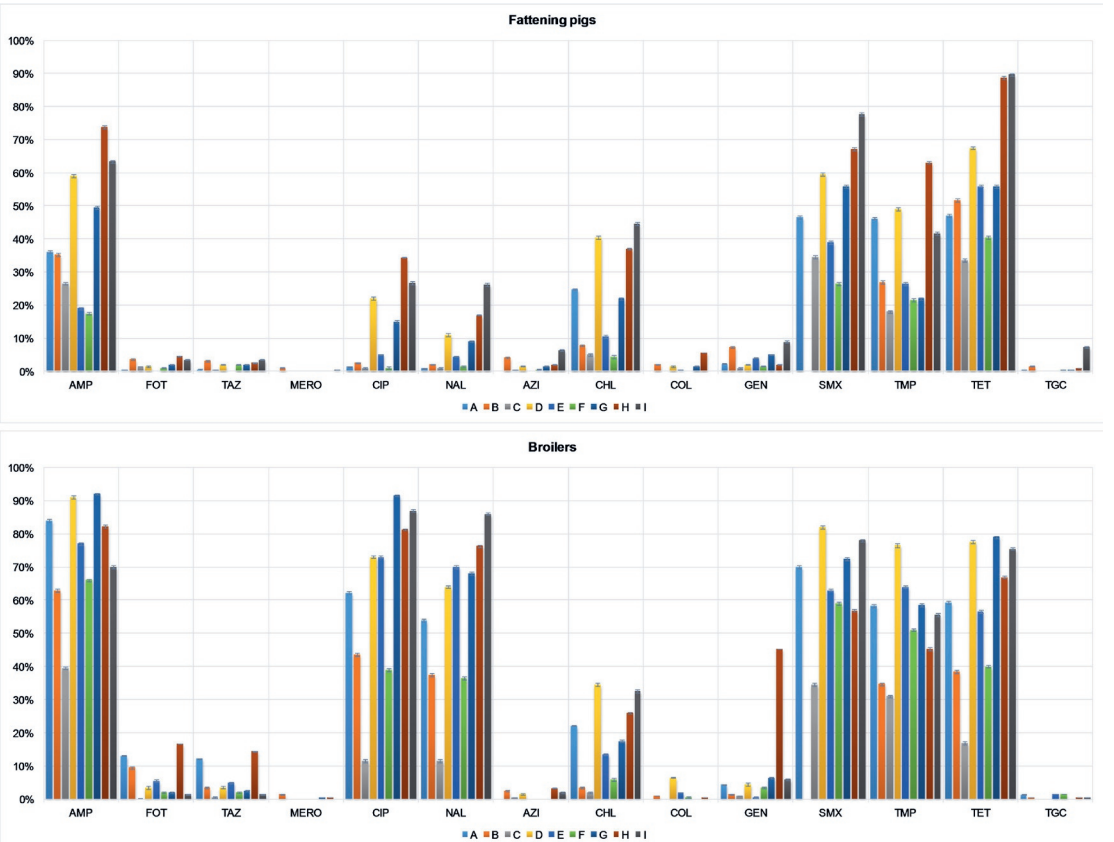


Figure 1. Antimicrobial resistance proportions (%) of *E. coli* isolated from broilers, fattening pigs, fattening turkeys and veal calves per country.

AMP: ampicillin; FOT: cefotaxime; TAZ: ceftazidime; MERO: meropenem; CIP: ciprofloxacin; NAL; nalidixic acid; AZI: azithromycin; CHL: chloramphenicol; COL: colistin; GEN: gentamicin; SMX: sulphamethoxazole; TMP: trimethoprim; TET: tetracycline; TGC: tigecycline.

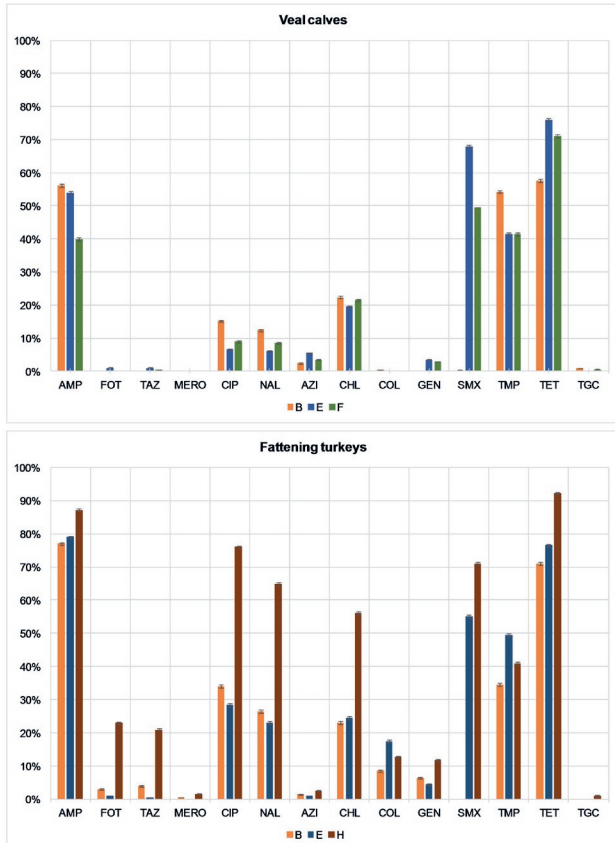


Figure 1. Antimicrobial resistance proportions (%) of *E. coli* isolated from broilers, fattening pigs, fattening turkeys and veal calves per country. (continue)

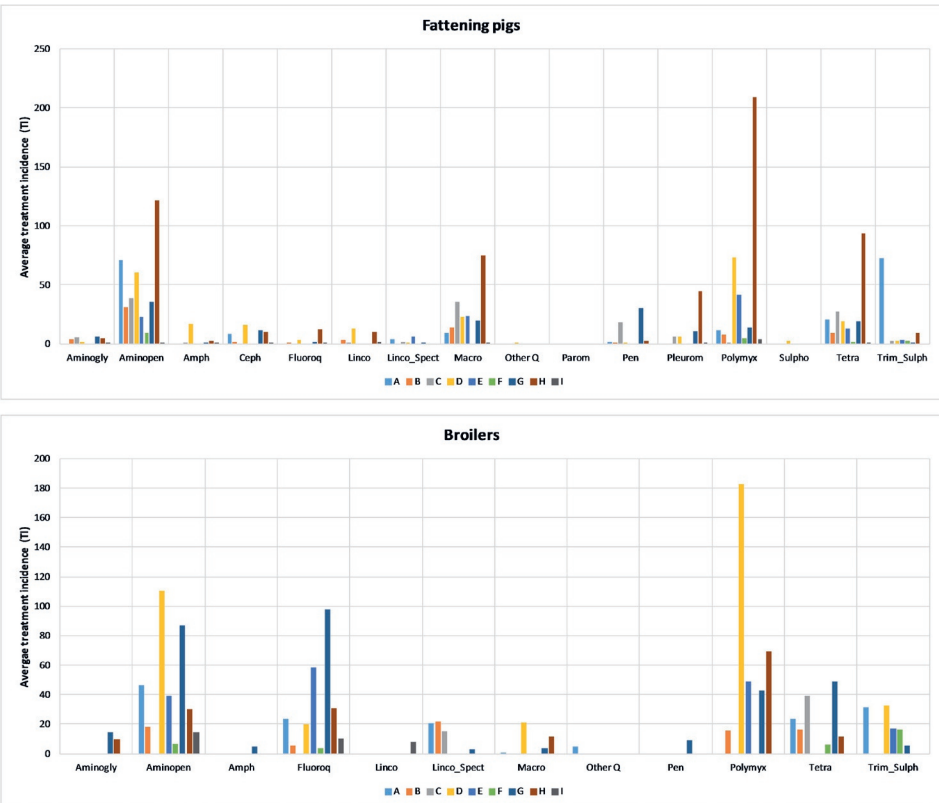


Figure 2. Average antimicrobial usage (TI/1000) in sampled farms for broilers, fattening pigs, fattening turkeys and veal calves per country.

Aminogly: Aminoglycosides; Aminopen: Aminopenicillins; Amph: Amphenicols; Ceph: Cephalosporins; Fluoroq: Fluoroquinolones; Linco: Lincosamides; Linco_Spect: Lincomycin-Spectinomycin; Macro: Macrolides; Other Q: Other quinolones; Parom: Paromomycin; Pen: Penicillins; Pleurom: Pleuromutilins; Polymix: Polymyxins; Sulpho: Sulphonamides; Tetra: Tetracyclines; Trim_Sulpha: Trimethoprim-Sulphonamides.

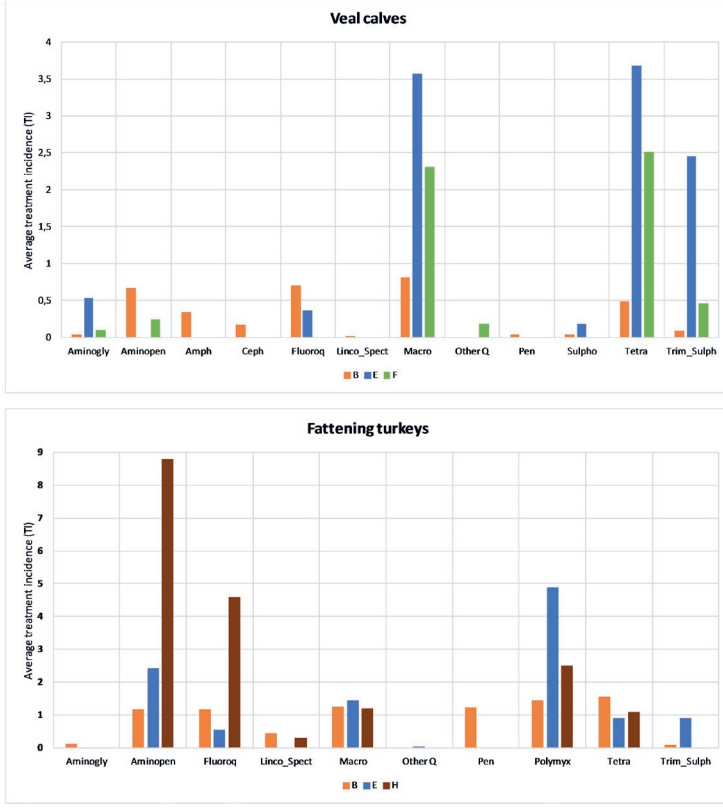


Figure 2. Average antimicrobial usage (TI/1000) in sampled farms for broilers, fattening pigs, fattening turkeys and veal calves per country. (continue)

Results and Discussion

E. coli isolation was successful for all but 34 out of 4860 faecal samples. MIC values for 4826 *E. coli* from broilers (n=1811), fattening pigs (n=1809), fattening turkeys (n=596) and veal calves (n=610) were included in this study (Table S1). A large variation in resistance proportions between antimicrobials, countries and animal species was observed (Figure 1). Substantial differences between countries were also observed in AMU (Figure 2).

Independent of the animal species, the highest levels of resistance were observed for ampicillin, sulphamethoxazole, tetracycline and trimethoprim, which is in line with previous studies [13-15]. Resistance to ampicillin in broilers varied between 39.5% and 92% (Figure 1) and strongly correlated (Spearman's rho= 0.917, p=0.001) with aminopenicillins usage over countries, but not with penicillin (Table 1). Resistance to ampicillin in fattening pigs (17.5-73.8%) did not correlate with either penicillins or aminopenicillins (Table 1). Because penicillin as such does not select for ampicillin resistance in *E. coli* it is not surprising that penicillin treatment (T1) does not correlate to ampicillin resistance in either animal species. As for the difference observed in correlation between ampicillin resistance and aminopenicillins use in broilers and fattening pigs, time could be the most likely explanation. The interval between amoxicillin use in young broilers and their slaughter age (6 weeks) is very short compared to the relatively long interval in pig production, with amoxicillin use in sows at delivery and in piglets to control bacterial infections (i.e. *Streptococcus suis*), and the slaughter age of pigs. Surprisingly, resistance to ampicillin in fattening pigs correlated significantly (Spearman's rho=0.729, p=0.026) with cephalosporins usage over countries. This association might be an artefact, since cephalosporins only select for resistance to extended spectrum cephalosporins and not to ampicillin (as for example in the case of TEM-1 beta-lactamases).

For simplicity, resistance to 3rd - and 4th -generation cephalosporins and ciprofloxacin are discussed later in the article in correlation with the harmonised indicators proposed by EFSA to assess the most relevant aspects of AMR of public health concern in food-producing animals.

Resistance proportions for sulphamethoxazole were highest in poultry (82%, country D) and fattening pigs (77.7%, country I). Tetracycline resistance recorded the highest proportions in broilers (89.6%, country I) and fattening turkeys (92.3%, country H). No significant correlations were observed between sulphamethoxazole and tetracycline resistance and their respective treatment incidents data over countries (Table 1). Despite the high resistance levels, tetracycline did not belong to the three most used antimicrobials neither in broilers nor in fattening pigs [9, 10]. However, part of the resistance could be explained by a historical built-up, due to

heavy use in the past [16], and/or the common occurrence of *tet* and *sul* resistance genes in *E. coli*, independently on antibiotic usage.

Trimethoprim resistance was highest in broilers (76.5%, country D) and significantly correlated (Spearman's rho=0.809, p=0.008) with trimethoprim-sulphamethoxazole usage over countries (Table 1). Although nalidixic acid resistance against the use of fluoroquinolones and/or quinolones reached its highest levels in broilers (86%, country I), no correlation was observed with fluoroquinolone usage. Conversely, fluoroquinolone usage significantly correlated (Spearman's rho=0.698, p=0.037) with resistance in fattening pigs (Table 1).

In spite of a ban on the use of chloramphenicol in animals used for food production since the early 1990s [17], resistance was high in fattening turkeys (up to 56.1%, country H) and fattening pigs (up to 44.5%, country I), with lower proportions in broilers (up to 34.5%, country D) and veal calves (up to 22.4%, country B) (Figure 1). In fattening pigs, chloramphenicol resistance significantly correlated (Spearman's rho=0.807, p=0.009) with phenicols usage over countries (Table 1). Gentamicin resistance in broilers was <10% in all countries, except for country H (45.3%) (Figure 1). This significantly correlated (Spearman's rho=0.713, p=0.031) to no or very low aminoglycosides usage at sampled farms over countries (Table 1). Very low resistance to tigecycline was observed, a drug not used in veterinary medicine, whose resistance mechanism is mostly due to efflux pump over-expression [18]. Azithromycin resistance was very low or absent in *E. coli* (Figure 1).

The highest prevalence (3.8%, country E) was observed in veal calves, where metaphylaxis with macrolides is commonly used to control bovine respiratory diseases, which may exert selective pressure on commensal intestinal microbiota [19]. A high negative correlation (Spearman's rho= -0.818, p=0.007) between azithromycin resistance and usage of lincomycin-spectinomycin in fattening pigs was observed, a phenomenon for which a biological explanation is not currently available.

Table 1. Correlation between antimicrobial usage (average treatment incidence on farm level stratified per country, species and antimicrobial class) and resistance (MIC data aggregated at country level in resistance proportions).

Animal	Antimicrobial	T1[#]		T2[#]		T3[#]	
		rho[§]	***p***	*rho*	***p***	*rho*	***p***
Broilers	AMP	0.548	0.127	0.917	**0.001**		
	AZI	0.173	0.656	0.019	0.961	0.217	0.576
	CHL	0.000	1.000				
	CIP	0.778	**0.014**	-0.138	0.724		
	COL	0.718	**0.029**				
	FOT	-0.279	0.468	0.424	0.256		
	GEN	0.713	**0.031**	-0.359	0.343		
	NAL	0.667	0.059	-0.137	0.725		
	SMX	0.574	0.106				
	TAZ	-0.138	0.723	0.588	0.096		
	TET	-0.153	0.695				
	TGC	-0.471	0.200				
	TMP	0.809	**0.008**				
Fattening pigs	AMP	0.271	0.480	0.417	0.270	0.729	**0.026**
	AZI	-0.145	0.709	-0.818	**0.007**	0.638	0.064
	CHL	0.807	**0.009**				
	CIP	0.806	**0.009**	0.279	0.468		
	COL	0.789	**0.011**				
	FOT	0.260	0.500	0.128	0.743	0.329	0.387
	GEN	-0.035	0.928	-0.315	0.408		
	NAL	0.698	**0.037**	0.276	0.472		
	SMX	0.151	0.699	0.274	0.476		
	TAZ	-0.035	0.928	-0.147	0.705	0.282	0.462
	TET	-0.126	0.748				
	TGC	-0.594	0.092				
	TMP	0.494	0.177				

[§]Spearman's *rho*
In bold, $p < 0.05$
[#]T1, T2 and T3 treatments correspond to the following classes per antimicrobial, respectively:
AMP, FOT, TAZ: Penicillins, Aminopenicillins, Cephalosporins*;
AZI: Macrolides (T1), Lincomycin-Spectinomycin (T2), Lincosamides (T3);
CHL: Amphenicols (T1);
CIP, NAL: Fluoroquinolones (T1), Other quinolones (T2);
COL: Polymyxins (T1);
GEN: Aminoglycosides (T1), Lincomycin-Spectinomycin (T2), Paromomycin* (T3);
SMX: Trimethoprim-Sulphamethoxazole (T1), Sulphonamides (T2);
TET, TGC: tetracyclines (T1);
TMP: Trimethoprim-Sulphamethoxazole (T1).
*only fattening pigs.

Low proportions were observed for colistin resistance in broilers, fattening pigs and veal calves (0-6.5%), conversely to fattening turkeys (8.5-17.5%). Although sales of polymyxin dropped in Europe between 2010 and 2016 [20], colistin was still substantially used in some of the countries involved in this study. In both broilers and fattening pigs, polymyxin usage corresponded significantly (Spearman's rho=0.718, p=0.029 and Spearman's rho=0.789, 0.011, respectively) with the mean resistant proportion of isolates over countries (Table 1). Meropenem resistance was absent in veal calves and only sporadically detected in other animal species (<1.5%) (Figure 1). Meropenem resistant *E. coli* isolates were confirmed to be negative for carbapenemase genes $bla_{\text{OXA-48}}$, bla_{NDM}, bla_{KPC}, bla_{VIM} and bla_{IMP} (data not shown). As carbapenems are not licensed for use in livestock, correlation between meropenem resistance and aminopenicillins and/or cephalosporin usage was tested. No significant correlation between antimicrobial usage and resistance was observed for broilers and fattening pigs (data not shown).

For fattening turkeys and veal calves no statistically significant correlation was observed between AMR and AMU; this is most probably due to the small sample size of only 3 countries.

Four harmonised indicators are proposed by EFSA to assess the most relevant aspects of AMR of public health concern in food-producing animals: 1) resistance to 3rd- and 4th-generation cephalosporins, 2) resistance to ciprofloxacin, 3) full susceptibility, and 4) multidrug resistance (Table 2) [5]. The proportion of indicator *E. coli* resistant to 3rd- and 4th- generation cephalosporins is prioritized since these antimicrobials are classified as the highest prioritized critically important for human medicine [21].

In general, the proportion of *E. coli* displaying non-wild type susceptibility to cefotaxime and/or ceftazidime varied by animal species and country (Figure 1). It was low in fattening pigs and veal calves (0.5-2.1%), but reached higher levels in fattening turkeys (23.5%, country H) and broilers (16.6%, country H) (Table 2). Occurrence and prevalence of extended spectrum β-lactamase-producing *E. coli* that may enter the food chain varies greatly by animal species and country. No significant correlation between resistance to cefotaxime and/or ceftazidime and cephalosporins use was observed in broilers and fattening pigs (Table 1), contrarily to what is reported in other countries [22]. Although specific selection for ESBL detection was not included in the EFFORT sampling and data collection (as for any other antibiotic), cefotaxime and ceftazidime resistant *E. coli* were used as a proxy for ESBL-producing *E. coli* proportion. However, the proportion of these isolates observed in the dataset does not resemble the proportion of samples or animals containing ESBL/AmpC producing *E. coli*, as indicated by EFSA, and this may explain why no significant associations with usage were observed.

Table 2. Proportions (%) of resistant *E. coli* expressed according to primary and secondary indicators per animal species per country.

Country	Broilers					Fattening pigs					Fattening turkeys					Veal calves				
	N	S[1]	F/T[2]	MDR[3]	CIP[4]	**N**	S	F/T	MDR	CIP	**N**	S	F/T	MDR	CIP	**N**	S	F/T	MDR	CIP
A	**230**	4.8	13.0	80.4	62.2	**219**	33.8	0.5	31.5	1.4										
B	**200**	11.5	10.5	42.5	43.5	**193**	31.6	3.6	21.8	2.6	**200**	11.5	4.0	54.0	34.0	**210**	0.0	0.0	53.3	15.2
C	**200**	49.0	0.5	32.5	11.5	**200**	50.5	0.5	19.0	1.0										
D	**200**	3.5	3.5	87.0	73.0	**200**	19.0	2.0	58.0	22.0										
E	**200**	7.5	5.5	81.5	73.0	**200**	31.0	0.0	18.5	5.0	**200**	11.5	1.0	61.0	28.5	**200**	21.0	1.0	58.0	6.5
F	**200**	17.5	2.0	58.5	39.0	**200**	45.5	2.0	12.5	1.0						**200**	25.5	0.5	34.5	9.0
G	**200**	2.0	2.5	90.5	91.5	**200**	25.0	2.0	47.0	15.0										
H	**181**	3.3	16.6	82.3	81.2	**195**	3.1	4.6	76.4	34.4	**196**	3.6	23.5	88.3	76.0					
I	**200**	1.5	1.5	85.5	87.0	**202**	2.5	3.5	69.3	26.7										
Total	**1811**					**1809**					**596**					**610**				

N= number of isolates.
[1]S: fully susceptible;
[2]F/T: resistance to cefotaxime and/or ceftazidime;
[3]MDR: resistance to ≥3 antimicrobial classes;
[4]CIP: resistance to ciprofloxacin.

Resistance to ciprofloxacin is used as a proxy for resistance to (fluoro) quinolones, included in the list of the highest prioritized critically important antimicrobials for human medicine [21,23]. Reduced susceptibility to ciprofloxacin in broilers varied by country between 11.5% and 91.5% (Table 2) and correlated significantly (Spearman's rho=0.778, p=0.014) with fluoroquinolone usage over countries. Reduced susceptibility to ciprofloxacin in pigs occurred less frequently and varied by country between 1% and 34.4% (Table 2). It also correlated significantly (Spearman's rho=0.778, p=0.014) with fluoroquinolone usage over countries. No correlation was observed with other quinolones for both broilers and fattening pigs. Fluoroquinolone resistance has been documented in commensal *E. coli* in livestock in Europe [24-25], and throughout turkey breeding and meat flocks in the UK [26], where biosecurity and responsible AMU were recognized as contributors to restrict AMR occurrence and spread [27].

The proportion of indicator *E. coli* fully wild-type susceptible to the entire panel of antimicrobials tested is used as a proxy of the overall selective pressure exerted by agricultural usage of antimicrobials [28]. In general, *E. coli* isolates from fattening pigs were more susceptible than from other livestock (Table 2). The highest prevalence of fully susceptible *E. coli* was observed in country C in both broilers and fattening pigs (49-50.5%, respectively). There was no significant correlation between the proportion of fully susceptible *E. coli* and AMU for broilers or pigs (Figure S1). Several speculations could be made for this correlation not being significant. Although the quality of the dataset is undisputable, this was not designed to prove an association between overall AMU and fully susceptible E. coli. Additionally, the proportion of fully susceptible *E. coli* is not a specific indicator for overall AMU. When applied to a very large dataset, as done by EFSA, this correlation will likely be very high. However, in a study like EFFORT with more diversity relative to the number of samples taken, this correlation is expected to be lower, a reason why also EFSA is re-evaluating how specific these composite indicators are. Finally, as previously reported in another publication by the EFFORT consortium [9], for some countries data collection on AMU was challenging, as the sample period was not always consistent over the year, and this may also influence this correlation.

The last indicator considered was multidrug resistance (MDR), i.e. resistance to three or more antimicrobials classes [29]. This indicator is informative in situations with high levels of resistance with very few isolates displaying full susceptibility [5]. MDR was observed in most of the countries, especially in broilers (58.5-90.5%) and fattening turkeys (54-88.3%) (Table 2). High MDR level in poultry is a well-known phenomenon, partially related to frequent use of antimicrobials as oral group treatments in fattening periods as short as six

weeks for broilers [30]. No significant correlation between AMU at country level and MDR in broilers and fattening pigs was observed (Figure S1). As for the fully susceptible *E. coli* indicator, the MDR indicator might better apply to very large datasets, in relation to the number of farms sampled here relative to the diversity in the dataset. However, correlations between high MDR levels and other AMU parameters as well as specific farm characteristics might be significant, as investigated in more in depth studies from the EFFORT consortium [6, 31-32]. At strain level, the most resistant *E. coli* of the entire collection was recovered from a fattening turkey of country H resistant to eleven antibiotics (AMP, FOT, TAZ, CIP, NAL, CHL, COL, GEN, SMX, TET, TGC) spanning eight different classes (Table S1). MDR profiles to seven different classes were sporadically observed in *E. coli* from fattening pigs (country I), broilers (countries H and I), and veal calves (country E), (see Table S1).

Conclusions

A wide collection of *E. coli* isolates and related MIC profiles from different animal reservoirs was produced and analysed in relation with harmonized AMR indicators and AMU at country level. Resistance proportions varied between antimicrobials and animal species, and correlation with usage was not always significant. Where possible the results gathered here were compared cautiously with AMR and AMU data from other studies, keeping in mind the use of different methodologies to describe the data.

The strength of the EFFORT project relies on AMR and AMU datasets gathered from the same farms. The analysis performed here correlates aggregated data from the same farms at country level for both AMU and AMR within antimicrobial classes. To the best of our knowledge, this was not performed before with data gathered from the same epidemiological unit, i.e. the farm, on such a scale. Even though the data is aggregated and was not analysed at farm level, there is still a stronger correlation than when correlating AMR monitoring data to AMU data at farm level, as regularly done in numerous reports and studies [33, 34]. Although it is accepted generally that AMU will cause AMR [34, 35], the extent to which this happens differs enormously per antimicrobial class, as is clearly shown by these results, also depending on other factors like farm management and characteristics. Since Spearman's rank correlation is a quantitative association test, we get a feeling of the extent of the correlations per antimicrobial class.

Author statements

Authors Contributions

Conceptualization, Resources, Funding: DM, JD, JW; Methodology: AH, JvdG, PJ, SS; Formal Analysis: DC, AH, JvdG, PJ; Writing – Original Draft Preparation: DC; Writing- Review and Editing: AH, JvdG, PJ, DM, JD, JW. All authors approved the final paper.

Conflict of Interest

The author(s) declare that there are no conflicts of interest.

Funding

The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement number: 613754 (http://www.effort-against-amr.eu/).

Ethical Approval

Ethical approval for sampling was not required in any of the participating countries since fresh faecal droppings were collected according to good veterinary practice and without invasive procedures on the animals.

Disclaimer

Daniela Ceccarelli is currently employed by the Research Executive Agency, European Commission. The views expressed are purely those of the author(s) and may not in any circumstances be regarded as stating an official position of the European Commission.

Acknowledgements

We would like to thank all farmers and veterinarians who agreed to participate in the study, and everyone involved in sampling and lab work in the different contributing countries.

Members of the EFFORT Consortium: H. Graveland[3], H. Schmitt[4], L. Van Gompel[4], R.E.C. Luiken[4], D.J.J. Heederik[4], A. van Essen[1], B. Gonzalez-Zorn[5], G. Moyano[5], P. Sanders[6], C. Chauvin[6], J. David[6], A. Battisti[7], A. Caprioli[7], T. Blaha[8], K. Wadepohl[8], M. Brandt[8], F. Aarestrup[9], T. Hald[9], S. Duarte[9], D. Wasyl[10], M. Skarzyńska[10], M. Zajac[10], H. Daskalov[11], H. Saatkamp[12], K. Stärk[13].

[4]Institute for Risk Assessment Sciences, Utrecht University, Utrecht, the Netherlands; [5]Complutense University of Madrid, Madrid, Spain; [6]French Agency for Food, Environmental and Occupational Health & Safety, Fougères, France; [7]Istituto Zooprofilattico Sperimentale del Lazio e della Toscana, Rome,

Italy; [8]University of Veterinary Medicine Hannover, Bakum, Germany; [9]National Food Institute, Technical University of Denmark, Kongens Lyngby, Denmark; [10]National Veterinary Research Institute, Pulawy, Poland; [11]National Diagnostic Research Veterinary Institute, Sofia, Bulgaria; [12]Wageningen University and Research, Wageningen, the Netherlands; [13]SAFOSO AG, Liebefeld, Switzerland.

References

Mcdermott PF, Zhao S, Wagner DD, Simjee S, Walker RD, *et al.* The food safety perspective of antibiotic resistance. *Anim Biotechnol* 2002; 13:71-84.

Hoelzer K, Wong N, Thomas J, Talkington K, Jungman E, *et al.* Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? *BMC Vet Res* 2017;13: 211.

Scott AM, Beller E, Glasziou P, Clark J, Ranakusuma RW, *et al.* Is antimicrobial administration to food animals a direct threat to human health? A rapid systematic review. *Int J Antimicrob Agents 2018;*52:316-323.

Szmolka A, Nagy B. Multidrug resistant commensal *Escherichia coli* in animals and its impact for public health. *Front Microbiol 2013;*4:258.

European Centre for Disease Control, European Food Safety Authority - Panel on Biological Hazard (BIOHAZ), and European Medicines Agency - Committee for Medicinal Products for Veterinary Use (CVMP). ECDC, EFSA and EMA Joint Scientific Opinion on a list of outcome indicators as regards surveillance of antimicrobial resistance and antimicrobial consumption in humans and food-producing animals. *EFSA J 2017;*15:e05017.

Munk P, Knudsen BE, Lukjancenko O, Duarte ASR, Van Gompel L, *et al.* (2018). Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. *Nat Microbiol* 2018;3:898-908.

European Food Safety Authority. Report from the Task Force on Zoonoses Data Collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. *EFSA J 2008*;6: 141r.

Kahlmeter G, Brown DFJ, Goldstein FW, Macgowan AP, Mouton JW, *et al.* European harmonization of MIC breakpoints for antimicrobial susceptibility testing of bacteria. *J Antimicrob Chemother 2003;*52:145-148.

Joosten P, Sarrazin S, Van Gompel L, Luiken REC, Mevius DJ, *et al.* Quantitative and qualitative analysis of antimicrobial usage at farm and flock level on 181 broiler farms in nine European countries. *J Antimicrob. Chemother 2018;*74:798-806.

Sarrazin S, Joosten P, Van Gompel L, Luiken REC, Mevius DJ, *et al.* Quantitative and qualitative analysis of antimicrobial usage patterns in 180 selected farrow-to-finish pig farms from nine European countries based on single batch and purchase data. *J Antimicrob Chemother 2019;*74:807-816.

Postma M, Sjolund M, Collineau L, Losken S, Stark KD, *et al.* Assigning defined daily doses animal: a European multi-country experience for antimicrobial products authorized for usage in pigs. *J Antimicrob Chemother* 2015;70: 294-302.

R Core Team. R: A language and environment for statistical computing. 2017, R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Hanon JB, Jaspers S, Butaye P, Wattiau P, Meroc E, *et al.* A trend analysis of antimicrobial resistance in commensal *Escherichia coli* from several livestock species in Belgium (2011-2014). *Prev Vet Med 2015;*122:443-452.

Wasyl D, Hoszowski A, Szulowski K, Zając M. Antimicrobial resistance in commensal *Escherichia coli* isolated from animals at slaughter. *Front Microbiol 2013;*4: 221.

European Food Safety Authority, European Centre for Disease Control. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2017. *EFSA J* 2019;17 (2):5598, 278 pp.

Grossman T. H. Tetracycline Antibiotics and Resistance. *Cold Spring Harbor Perspect Med 2016*;*6*(4),a025387.

European Commission. Commission Regulation (EC) No 1430/94 amending annexes I, II, III and IV of Council Regulation (EEC) No 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin. *OJ 1994*; L156/6.

Pournaras S, Koumaki V, Spanakis N, Gennimata V, Tsakris A. Current perspectives on tigecycline resistance in *Enterobacteriaceae*: susceptibility testing issues and mechanisms of resistance. *Int J Antimicrob Agents 2016;*48:11-18.

Nickell JS, White BJ. Metaphylactic antimicrobial therapy for bovine respiratory disease in stocker and feedlot cattle. *Vet Clin North Am Food Anim Pract 2010*; 26:285-301.

European Medicines Agency. Sales of veterinary antimicrobial agents in 30 European countries in 2016. Trends from 2010 to 2016. *8th ESVAC report, 2018*.

World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO AGISAR). Critically important antimicrobials for human medicine, 6th Revision, (ed.) World Health Organization. (Geneva), 2019.

Randall LP, Lemma F, Rogers JP, Cheney TE, Powell LF, *et al.* Prevalence of extended-spectrum-β-lactamase-producing *Escherichia coli* from pigs at slaughter in the UK in 2013*. J Antimicrob Chemother 2014*;69:2947–2950.

Collignon PJ, Conly JM, Andremont A, Mcewen SA, Aidara-Kane A, *et al.* World Health Organization ranking of antimicrobials according to their importance in human medicine: a critical step for developing risk management strategies to control antimicrobial resistance from food animal production. *Clin Infect Dis 2016;*63:1087-1093.

De Jong A, Stephan B, Silley P. Fluoroquinolone resistance of *Escherichia coli* and *Salmonella* from healthy livestock and poultry in the EU. *J Appl Microbio 2012;*112:239-245.

Falgenhauer L, Imirzalioglu C, Ghosh H, Gwozdzinski K, Schmiedel J, *et al.* Circulation of clonal populations of fluoroquinolone-resistant CTX-M-15-producing *Escherichia coli* ST410 in humans and animals in Germany. *Int J Antimicrob Agents 2016;*47:457-465.

Gosling RJ, Clouting CS, Randall LP, Horton RA, Davies RH. Ciprofloxacin resistance in *E. coli* isolated from turkeys in Great Britain. *Avian Pathol 2012;*41:83-89.

Jones EM, Snow LC, Carrique-Mas JJ, Gosling RJ, Clouting C, *et al.* Risk factors for antimicrobial resistance in *Escherichia coli* found in GB turkey flocks. *Vet Rec 2013;*173:422.

Economou V, Gousia P. Agriculture and food animals as a source of antimicrobial-resistant bacteria. *Infect Drug Res 2015;*8: 49-61.

Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, *et al.* Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin Microbiol Infect 2012;*18:268-281.

Simoneit C, Burow E, Tenhagen BA, Kasbohrer A. Oral administration of antimicrobials increase antimicrobial resistance in *E. coli* from chicken - a systematic review. *Prev Vet Med 2015;*118:1-7.

Luiken REC, Van Gompel L, Munk P, Sarrazin S, Joosten P, *et al.* Associations between antimicrobial use and the faecal resistome on broiler farms from nine European countries. *J Antimicrob Chemother* 2019;74:2596-2604.

Van Gompel L, Luiken REC, Sarrazin S, Munk P, Knudsen BE, *et al.* The antimicrobial resistome in relation to antimicrobial use and biosecurity in pig farming, a metagenome-wide association study in nine European countries. *J Antimicrob Chemother* 2019;74:865-876.

Dorado-García A, Mevius DJ, Jacobs JJ, Van Geijlswijk IM, Mouton JW, *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;*71:3607-3619.

European Centre for Disease Control, European Food Safety Authority, European Medicines Agency. 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 – Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA J* 2017;15:4872.

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

Supplementary Material (available online)

Table S1. MIC raw data for all *E. coli* isolates from broilers, fattening pigs, fattening turkeys and veal calves included in this study.

Table S2. Overview table on the collection of antimicrobial consumption data in poultry farms.

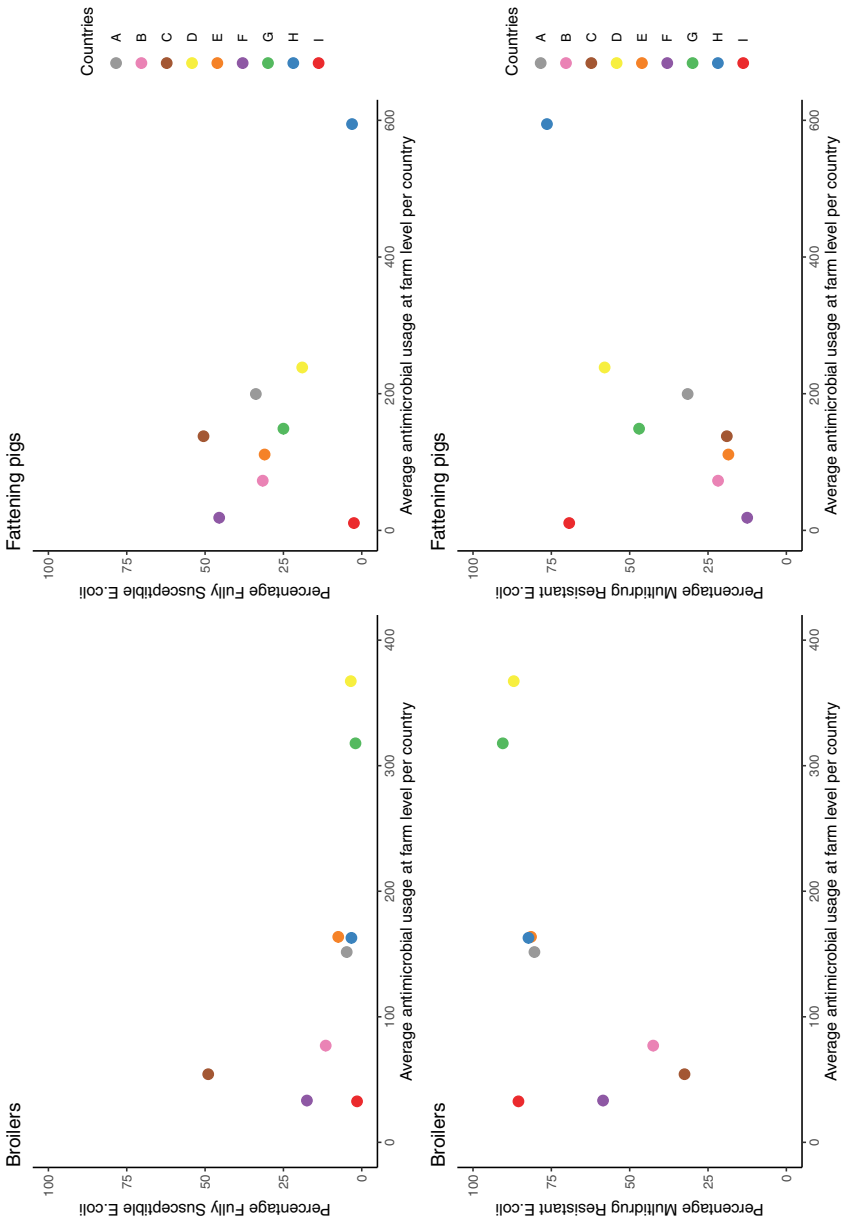


Figure S1. Average antimicrobial usage at farm level per country versus percentage of fully susceptible *E. coli* and percentage of multidrug resistant *E. coli* for broilers and fattening pigs.

Chapter 6

Antimicrobial resistance monitoring in commensal and clinical *Escherichia coli* from broiler chickens: differences and similarities

Ayla Hesp, Gerdien van Schaik, Jeanine Wiegel, Annet Heuvelink, Dik Mevius, Kees Veldman

Submitted

Abstract

Background: In the Netherlands, antimicrobial resistance (AMR) is monitored in commensal indicator *Escherichia coli* from healthy broilers at slaughter as part of a European monitoring programme. In a separate programme for poultry health, AMR is monitored in veterinary pathogens from diseased broilers. The differences and similarities of the information obtained in the two monitoring approaches are unknown.

Aims: This study compares non-wildtype susceptibility in commensal *E. coli* isolated from healthy broilers with clinical resistance in *E. coli* isolated from diseased broilers.

Methods: Data acquired by broth microdilution was analysed for commensal indicator *E. coli* and clinical *E. coli* from the Netherlands, 2014-2019. An additive generalised linear model (Poisson regression) was used to determine time trends and identify differences in mean resistant proportions.

Results: Despite the differences in the monitoring approach, mean resistant proportions were similar in commensal indicator *E. coli* and clinical *E. coli* for most antimicrobials. The random sample of commensal *E. coli* isolated from healthy animals was more suitable for monitoring time trends in AMR. The selected sample of clinical isolates resulted in a higher chance to detect low-prevalent resistance: i.e. cefotaxime and colistin. The clinical *E. coli* data showed more fluctuation over time, and more data is needed to quantify the association between the two types of monitoring data over time.

Conclusions: We conclude that the two monitoring strategies are complementary and that it is therefore necessary to monitor AMR both in commensal *E. coli* from healthy broilers and in clinical *E. coli* from diseased broilers.

Introduction

Antimicrobial resistance (AMR) in livestock as public health hazard is monitored in commensal indicator organism *Escherichia coli (E. coli)*, and food-borne pathogens *(Salmonella, Campylobacter*). In the European Union, it is mandatory to sample food producing animals in slaughterhouses, isolate *E. coli* and determine their susceptibility for antimicrobials with a standardized susceptibility panel, as prescribed by EU legislation (1). Results are reported yearly in Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals (MARAN) (2) in the Netherlands and by the European Food Safety Authority (EFSA) on European level (3).

Several studies have shown that monitoring of AMR in commensal indicator organism *E. coli* is useful to determine AMR occurrence or trends as a potential public health threat in food producing animals (4-6). Veterinary prescription guidelines are based on AMR trends in commensal *E. coli* as well as on AMR trends in clinical isolates. Commensal *E. coli* are no direct health threat, but considered a potential source of resistance genes for pathogenic bacteria. Currently, the association between resistance in commensal (non-clinical) *E. coli* isolates and clinical isolates in livestock is mostly unknown.

In the Netherlands, next to the national AMR monitoring in commensal *E. coli* isolates from slaughter animals, AMR monitoring in veterinary pathogens from clinical submissions and post-mortem examinations of livestock is performed by Royal GD (GD) in Deventer (7, 8), The Netherlands. These results are reported as part of the national farm animal health surveillance system, by order of the Dutch government and the animal industry. In broiler chickens, among other bacterial species, *E. coli* isolated from diseased broilers is tested for susceptibility.

To optimize the interpretation of AMR monitoring data, the similarities and differences need to be determined between the two types of monitoring: in commensal and clinical bacteria. This study aims to compare the results of monitoring of non-wildtype (NWT) susceptibility in commensal *E. coli* isolated from healthy animals at slaughter with clinical resistance in *E. coli* isolated from diseased broilers. For that purpose, resistance data acquired by broth microdilution were analyzed of commensal indicator *E. coli* from MARAN and clinical *E. coli* from GD monitoring, the Netherlands, 2014-2019.

Methods

Sample collection

Sample collection in MARAN was equal to described in Hesp *et al* (4). This consisted of a stratified random sampling strategy of caecal samples, each originating from a unique flock, collected at slaughterhouses. As defined by EFSA, this stratified sampling of caecal samples 'accounted for slaughterhouses processing at least 60% of the domestic annual production of the broiler population, with proportionate allocation to the slaughterhouse production' (Commission Implementing Decision (EU) 2013/652, Annex Technical Requirements 2.3).

In GD monitoring, clinical *E. coli* isolates were obtained from lesions of diseased broilers submitted for pathology to GD, as well as a random selection of isolates cultured in private practice laboratories, also obtained from lesions of diseased broilers. For the clinical *E. coli*, most isolates (88%) were considered to be sampled in animals before treatment was applied, as part of good veterinary practice when performing bacteriology. From the clinical isolates, 12% were known to come from treated animals and marked as such by the veterinarian. A comparison was made between resistant proportions in all clinical *E. coli* versus only the isolates marked as 'no treatment', indicating no differences of resistant proportions as confidence intervals overlapped (results not shown). Hence, all clinical *E. coli* data were included in the analysis.

Bacterial isolation and susceptibility testing

Isolation of *E. coli* in MARAN is described in Hesp *et al* (4). Commensal *E. coli* were isolated on MacConkey agar. Clinical *E. coli* isolates were isolated on sheep blood agar.

Susceptibility testing in MARAN was performed by broth microdilution, determining minimum inhibitory concentrations (MIC) according to ISO 20776-1 using commercially available microtiter plates (Sensititre EUVSEC by Thermo Scientific, East Grinsted, United Kingdom). GD performed broth microdilution with customized microtiter plates (Merlin Diagnostics, Bornheim-Hersel, Germany). In both monitoring programs, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF)(Bruker Daltonik GmbH, Bremen, Germany) was used to confirm that the isolates were *E. coli* (in MARAN this method was introduced in 2015, in the year 2014 isolates were biochemically identified).

MIC distributions were scrutinized of the antimicrobials present in both susceptibility testing panels (Supplementary Figures S1-S7). This was to detect any methodological differences and to determine which antimicrobial classes

could be compared. These concerned: ampicillin representing aminopenicillins, gentamicin representing aminoglycosides, cefotaxime as representative of cephalosporins, tetracycline representing tetracyclines, colistin representing polymyxins, ciprofloxacin and enrofloxacin representing fluoroquinolones. Ciprofloxacin resistance in commensal isolates was compared to enrofloxacin resistance in clinical isolates. For the folate pathway inhibitors trimethoprim and sulfamethoxazole, the proportion of isolates resistant to both of these antimicrobials in commensal *E. coli* was compared to resistance to the combination trimethoprim/sulfamethoxazole in the clinical *E. coli* isolates. Susceptibility testing panels and concentrations ranges used in the two monitoring programs are summarized in Supplementary Table S1. MIC data from both Dutch monitoring programs were available from 2014-2019, for commensal *E. coli* isolates in MARAN (N=1,992) and clinical *E. coli* isolates in GD (N=1,253). The terms 'commensal' and 'clinical' are used in the rest of the paper to indicate the isolates from the two monitoring programs.

Breakpoints

Table 1 presents the breakpoints used to calculate resistant proportions of the two populations. To determine proportions of NWT susceptibility in commensal indicator *E. coli,* internationally standardized epidemiological cut-off values (ECOFFs) were used (9). These were compared with resistant proportions determined with clinical breakpoints (CBP) used for clinical *E. coli* (10, 11). ECOFFs and CBPs defined by EUCAST were used, wherever available. For fluoroquinolones, the CBP for ciprofloxacin was applied in commensal *E. coli* in addition to the ECOFF to show the difference in resistance proportion between NWT susceptibility and clinical resistance for that specific example. For enrofloxacin no EUCAST CBP was available, hence a CLSI breakpoint for poultry was used (11). For tetracycline the ECOFF was used for the clinical isolates in absence of a CBP (Table 1).

Statistical analysis

Trends were evaluated by plotting the observed resistant proportions with 95% confidence intervals (CI) of the two monitoring datasets, as well as with Poisson regression models. Using the MIC data and selected breakpoints, yearly resistant isolate proportions were calculated for each antimicrobial, and exact 95% CIs were calculated, using yearly resistant proportions and the total numbers of isolates tested (N). All statistical analyses were performed in R version 3.3.3 (R Foundation, Vienna, Austria) (12). Regression models were selected by comparison of lowest values for Akaike's Information Criterion (AIC), model fit was assessed by the scaled deviance.

Table 1. Breakpoints used to determine non-wildtype susceptibility (NWT) in commensal *E. coli* and resistance (R) in clinical *E. coli* [a]

Antimicrobial	Testing range commensal *E. coli* MARAN (mg/L)	Testing range clinical *E. coli* GD (mg/L)	ECOFF (mg/L)	Clinical Breakpoint EUCAST (mg/L)	Clinical Breakpoint CLSI (mg/L)
			NWT (>)	R (>)	R (>)
Ampicillin	1 - 64	0.25 - 32	8	***8***[a]	-
Gentamicin	0.5 - 32	2 - 8	2	***2***	-
Cefotaxime	0.25 - 4	1 - 4	0.25	***2***	-
Tetracycline	2 - 64	0.25 - 16	***8***	-	-
Colistin	1 - 16	0.5 - 16	2	***2***	-
Trimethoprim	0.25 - 32	0.5 - 16	4	***4***	-
Sulfamethoxazole	8 - 1024	64 - 256	64	-	-
Ciprofloxacin (commensal) and enrofloxacin (clinical)	0.015 - 8	0.25 - 2*	0.064	0.5 (ciprofloxacin)	***1 (enrofloxacin)***

[a] Breakpoints in bold italic show criteria used for determining resistance in the clinical *E. coli* isolates

In the analysis, an additive generalised linear model was used with Poisson distribution and a log link function (Poisson regression) for yearly resistance counts (n), with the log of the total number of strains per year (N) as offset. Two explanatory variables were used: the first for the years one to six (2014-2019), the second was a binary variable for the monitoring program (0 for commensal *E. coli*, 1 for clinical *E. coli*). By using these explanatory variables in the additive model, time trends for both monitoring datasets were determined and quantified by the incidence rate ratio (IRR). Next to that, the model indicated whether the level of resistance differed between the two monitoring programs.

Results

In this study, resistant counts were modelled of commensal *E. coli* from healthy broilers and of clinical *E. coli* isolated from diseased broilers. An overview of resistant counts and totals per year of both datasets is presented in Supplementary Table S2. MIC distributions showed that there were no methodological differences as a potential hurdle for analytical comparison (Figures S1-S7). Observed resistant proportions of the commensal *E. coli* and

clinical *E. coli* were similar with overlapping CI for many of the time points for ampicillin, gentamicin, cefotaxime, tetracycline, colistin and trimethoprim/sulfonamide (Figure 1).

Figure 1. Proportions with 95% confidence intervals of antimicrobial resistance[a] in commensal *E. coli* from healthy broilers at slaughter versus in clinical *E. coli* from diseased broilers for ampicillin, gentamicin, cefotaxime, tetracycline, colistin and trimethoprim/sulfonamide from broilers, the Netherlands, 2014-2019

[a] Observed resistant proportions in commensal *E. coli* (dots) and clinical *E. coli* (triangles)

Model results for ampicillin resistance showed a decrease over time in the commensal *E. coli*: IRR per year was 0.91 (CI 0.87-0.94) (Table 2). In contrast, clinical *E. coli* showed stable resistant proportions over time (Figure 1-A) with no trend observed (Table 2).

Table 2. Poisson regression estimates for time trends and difference between the mean prevalence of antimicrobial resistance in commensal *E. coli* from healthy broilers at slaughter versus in clinical *E. coli* from diseased broilers, the Netherlands, 2014-2019

Antimicrobial	Time trend commensal *E. coli*		Time trend clinical *E. coli*		Difference in resistant proportions (commensal versus clinical *E. coli*)	
	Incidence rate ratio (95% CI)	P value*	Incidence rate ratio (95% CI)	P value	Incidence rate ratio (95% CI)	P value**
Ampicillin	**0.91 (0.87-0.94)**	**0.00**	1.02 (0.97-1.08)	0.39	0.98 (0.89-1.09)	0.72
Gentamicin	0.98 (0.88-1.10)	0.75	0.88 (0.74-1.04)	0.13	1.07 (0.78-1.45)	0.69
Cefotaxime	**0.68 (0.53-0.86)**	**0.00**	**0.65 (0.50-0.82)**	**0.00**	**1.94 (1.21-3.11)**	**0.01**
Tetracycline	**0.91 (0.88-0.95)**	**0.00**	0.98 (0.92-1.04)	0.53	**1.14 (1.01-1.29)**	**0.03**
Colistin	-	-	**0.67 (0.50-0.82)**	**0.04**	-	-
Trimethoprim/sulfonamide	**0.89 (0.85-0.93)**	**0.00**	0.99 (0.93-1.06)	0.79	0.96 (0.84-1.09)	0.50
Ciprofloxacin[a] and enrofloxacin	**0.89 (0.80-0.98)**	**0.02**	1.01 (0.88-1.16)	0.90	1.20 (0.92-1.57)	0.17

[a]The EUCAST clinical breakpoint for ciprofloxacin (0.5 mg/L) was used for commensal *E. coli* to determine resistance and compare time trends, using the CLSI clinical breakpoint for enrofloxacin resistance (1.0 mg/L) in clinical *E. coli*

* Values in bold indicate significant time trends (the P value is <0.05)

** Values in bold indicate a significant difference in the mean resistance prevalence (the P value is <0.05)

The means of ampicillin resistance were similar in both datasets (Table 2), also reflected by overlapping CI for most of the observed resistant proportions (Figure 1-A).

Gentamicin resistance prevalence was low in both commensal and clinical *E. coli* (Figure 1-B, Supplementary Table S2). In both programs, no time trends were observed and the means of the data did not differ (Table 2).

A decrease over time of already low prevalent cefotaxime resistance was observed in both commensal and clinical *E. coli* (Figure 1-C, Table 2). However, the mean cefotaxime resistance in the clinical *E. coli* was estimated to be higher than in commensal *E. coli*, indicated by the IRR of 1.94 (CI 1.21-3.11, clinical relative to commensal *E. coli*, Table 2).

Resistance to tetracycline decreased over time in commensal *E. coli* but fluctuated in clinical *E. coli* (Table 2, Figure 1-D). The clinical data had a slightly higher mean (IRR 1.14, CI 1.01-1.29, relative to commensal *E. coli*, Table 2).

Colistin resistance was not detected in commensal indicator *E. coli* but few resistant isolates were detected in clinical *E. coli* (Figure 1-E, Supplementary Table S2). The model estimated a decrease over time for the clinical data (IRR 0.67, CI 0.50-0.82, Table 2).

Findings for trimethoprim/sulfonamide resistance were similar to those for ampicillin. In commensal *E. coli* a decrease over time was detected, but not in clinical *E. coli*, and the means of both datasets were not different (Table 2). This was also observed in overlapping CI for resistant proportions per year (Figure 1-F).

For fluoroquinolones, application of the EUCAST CBP instead of the ECOFF resulted in lower ciprofloxacin resistant proportions in commensal *E. coli*, which were comparable to enrofloxacin in clinical *E. coli* (Table 2, Figure 2). For these related antimicrobials, a decrease of resistance over time was observed for ciprofloxacin in the commensal *E. coli*, but not for enrofloxacin in clinical *E. coli* isolates.

Discussion

Methodology is different in the two monitoring systems as they have different aims. To begin with, the sampling strategies differ. For the commensal isolates, a stratified random sample (active surveillance) from healthy animals at slaughter versus a selected sample from diseased animals (enhanced passive surveillance) from the same broiler population. The genetic background of the sampled *E. coli* populations is unknown and possible relatedness was not determined. Despite the differences in the monitoring, mean resistant proportions are similar for

most antimicrobials in this study.

The test panel for monitoring in commensal *E. coli* includes antimicrobials relevant to human healthcare, with long concentration ranges to determine NWT susceptibility. The panel for monitoring in clinical *E. coli* consists of antimicrobials relevant for veterinary use in livestock species, with shorter ranges to determine clinical resistance (Table S1).

The breakpoints used also differ between the two monitoring systems. In monitoring in commensal indicator *E. coli*, ECOFFs are used to early detect evolution of NWT susceptibility in gut bacteria (13). In the monitoring in clinical *E. coli* isolates, CBP are used, which are generally higher, and indicate clinical resistance for treatment strategy in veterinary practice. ECOFFs are internationally standardized (9), whereas CBP are not available for all veterinary antimicrobials (14)(Table 1). This did not harm our analysis, since the MIC distributions showed that the available internationally standardized CBP could be applied to the MIC of the clinical isolates (Supplementary Figure S1-S7). For fluoroquinolones and tetracyclines, standardized EUCAST veterinary CBP were absent, this was solved by using the CLSI CBP and ECOFF for the clinical isolates for these classes, respectively (Table 1).

Similarities in AMR proportions

The mean resistant proportions were similar for the majority of the antimicrobials. Except for cefotaxime and tetracycline, for which the mean resistant proportion was higher in clinical isolates (Table 2, Figure 1). Mesa-Varona *et al*. also found higher resistance to cefotaxime in clinical compared to commensal *E. coli* in German broilers 2014-2017 (15). Contrarily to expectations, Mesa-Varona *et al.* (16) found for ampicillin and tetracycline lower resistance levels for clinical isolates compared to non-clinical isolates in France and Germany. This does not correspond to our findings. This may be due to differences in sampling of clinical versus non-clinical isolates, or differences in antimicrobial use between countries (17, 18).

Monitored resistant proportions are influenced by the breakpoints used (19). Especially when there is a gap between the CBP and the ECOFF, as shown in the example of fluoroquinolones in these data. The ECOFF (0.064 mg/L) aims at detecting NWT susceptibility to monitor acquired resistance, these strains do not have to be clinically resistant. When the CBP for ciprofloxacin (0.5 mg/L) is applied to commensal *E. coli*, the resistant proportion is similar to the proportion for enrofloxacin in clinical *E. coli* (Figure 2). Therefore, data from different AMR monitoring programs have to be interpreted with care. To enhance standardization of AMR monitoring, it is worth considering to include both the ECOFF and the CBP in the concentration range of the susceptibility

panel, for a complete view on the AMR situation.

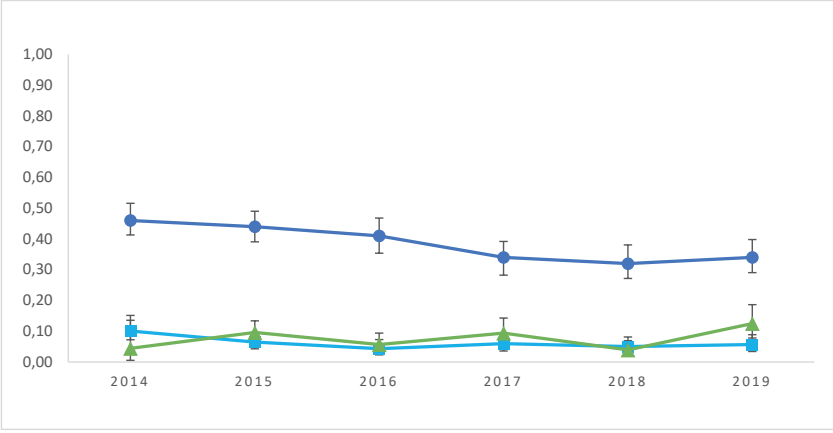


Figure 2. Proportions with 95% confidence intervals of antimicrobial resistance in commensal *E. coli* from healthy broilers at slaughter for ciprofloxacin[a] versus for enrofloxacin in clinical *E. coli* from diseased broilers, the Netherlands, 2014-2019

[a] Observed resistant proportions calculated with the EUCAST clinical breakpoint for ciprofloxacin of 0.5 mg/L to determine resistance (squares) and calculated with the epidemiological cut-off value of 0.064 mg/L (dots). To the GD data, the CLSI clinical breakpoint for enrofloxacin was applied of 1.0 mg/L (triangles).

AMR trend analysis

In commensal *E. coli,* decreasing trends in time are detected for the majority of antimicrobials (Table 2). Since 2009, resistant proportions in Dutch animals have decreased for many antimicrobial classes as a result of antimicrobial use interventions (2, 5, 20) as was observed in commensal *E. coli* in the present study (Table 2).

In contrast, the observed resistant proportions in clinical isolates fluctuate more and no time trends are statistically significant for the majority of antimicrobials (Figure 1, Table 2). However, in clinical isolates resistant *E. coli* were detected for two low prevalent and relevant antimicrobials: colistin and cefotaxime. Colistin resistance decreased over time in the clinical *E. coli* and was not detected in commensal *E. coli* (Table 2, Supplementary Table S2). The decrease over time for colistin resistance in the clinical isolates should be interpreted with care, since it concerns a limited number of resistant isolates (Supplementary Table S2) and selection bias cannot be excluded. For cefotaxime decreasing trends were observed in both commensal and clinical *E. coli* (Table 2).

Apparently, the stratified sample of commensal *E. coli* isolated from healthy animals at slaughter is more suitable to monitor time trends in AMR in this animal population. Randomization of a sample in active surveillance helps to detect trends (21). The selected sample of clinical isolates can be considered risk-based. Risk-based surveillance results in a higher chance to detect low prevalent incidents (22). Since the clinical *E. coli* data have shown to fluctuate over time (Table 2, Figure 1), more data is needed to quantify the association with commensal *E. coli.* Especially, because the observed decrease over time was small (Figure 1). Regarding the added value of both monitoring strategies, we conclude that it is necessary to monitor both in commensal *E. coli* from healthy broilers as well as in clinical *E. coli* from diseased broilers.

Acknowledgements

We thank the Ministry of Agriculture, Nature and Food Quality for the funding of the monitoring in commensal *E. coli* (WOT-01-002-003). We thankfully acknowledge Yvon Geurts, Alieda van Essen, Joop Testerink and Marga Japing for their technical assistance in determining the MIC in the commensal *E. coli*. We also acknowledge the financers of the poultry health surveillance system of GD, the Ministry of Agriculture, Nature and Food Quality and the Dutch poultry sector.

Funding

This research was supported by Wageningen Bioveterinary Research.

References

Commission implementing decision on the monitoring and reporting of antibmicrobial resistance in zoonotic and commensal bacteria, Stat. 2013/652/EU (2013).

Veldman KT MD, Wit B, Van Pelt W, Franz E, Heederik D. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2018. 2019.

EFSA (European Food Safety Authority) and ECDC (European Centre for DiseasePrevention and Control). The European Union summary report on antimicrobial resistance inzoonotic and indicator bacteria from humans, animals and food in 2016. EFSA Journal 2018;16(2):5182, 270pp. https://doi.org/10.2903/j.efsa.2018.5182. 2018.

Hesp A, Veldman K, van der Goot J, Mevius D, van Schaik G. Monitoring antimicrobial resistance trends in commensal Escherichia coli from livestock, the Netherlands, 1998 to 2016. Eurosurveillance. 2019;24(25):1800438.

Dorado-Garcia A, Mevius DJ, Jacobs JJ, Van Geijlswijk IM, Mouton JW, Wagenaar JA, 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(12):3607-19.

Hanon JB, Jaspers S, Butaye P, Wattiau P, Meroc E, Aerts M, et al. A trend analysis of antimicrobial resistance in commensal Escherichia coli from several livestock species in Belgium (2011-2014). Preventive veterinary medicine. 2015;122(4):443-52.

Antibioticumgevoeligheid pluimvee [Available from: https://www.gddiergezondheid.nl/nl/Diergezondheid/Antibioticumgevoeligheid/Pluimvee]

Wiegel J, Heuvelink, A., Veldhuis, A. , editor AAVM. Proceedings of the Ninth International Conference on Antimicrobial Agents In Veterinary Medicine (AAVM); 2018; Rome: Int. J. of Health, Animal science and Food safety

EUCAST. Antimicrobial wild type distributions of microorganisms 2019 [Available from: http://www.eucast.org/mic_distributions_and_ecoffs/]

EUCAST. Clinical Breakpoints and Dosing by European Committee on Antimicrobial Susceptility Testing 2020 [Available from: https://www.eucast.org/clinical_breakpoints/.

CLSI. 2020. [Available from: https://clsi.org/standards/products/free-resources/access-our-free-resources/]

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Frimodt-Moller N. Microbial Threat--The Copenhagen Recommendations initiative of the EU. J Vet Med B Infect Dis Vet Public Health. 2004;51(8-9):400-2.

Toutain PL, Bousquet-Mélou A, Damborg P, Ferran AA, Mevius D, Pelligand L, et al. En Route towards European Clinical Breakpoints for Veterinary Antimicrobial Susceptibility Testing: A Position Paper Explaining the VetCAST Approach. Front Microbiol. 2017;8:2344.

Mesa-Varona O, Kaspar H, Grobbel M, Tenhagen BA. Phenotypical antimicrobial resistance data of clinical and non-clinical Escherichia coli from poultry in Germany between 2014 and 2017. PLoS One. 2020;15(12):e0243772.

Mesa-Varona O, Mader R, Velasova M, Madec J-Y, Granier SA, Perrin-Guyomard A, et al. Comparison of Phenotypical Antimicrobial Resistance between Clinical and Non-Clinical E. coli Isolates from Broilers, Turkeys and Calves in Four European Countries. Microorganisms. 2021;9(4):678.

Joosten P, Sarrazin S, Van Gompel L, Luiken REC, Mevius DJ, Wagenaar JA, et al. Quantitative and qualitative analysis of antimicrobial usage at farm and flock level on 181 broiler farms in nine European countries. The Journal of antimicrobial chemotherapy. 2019;74(3):798-806.

Sarrazin S, Joosten P, Van Gompel L, Luiken REC, Mevius DJ, Wagenaar JA, et al. Quantitative and qualitative analysis of antimicrobial usage patterns in 180 selected farrow-to-finish pig farms from nine European countries based on single batch and purchase data. J Antimicrob Chemother. 2019;74(3):807-16.

Schwarz S, Silley P, Simjee S, Woodford N, van Duijkeren E, Johnson AP, et al. Editorial: assessing the antimicrobial susceptibility of bacteria obtained from animals. J Antimicrob Chemother. 2010;65(4):601-4.

Usage of Antibiotics in Agricultural Livestock in the Netherlands in 2018. Netherlands Veterinary Medicines Institute (SDa); 2018.

Bisdorff B, Schauer B, Taylor N, Rodríguez-Prieto V, Comin A, Brouwer A, et al. Active animal health surveillance in European Union Member States: gaps and opportunities. Epidemiol Infect. 2017;145(4):802-17.

Alban L, Rugbjerg H, Petersen JV, Nielsen LR. Comparison of risk-based versus random sampling in the monitoring of antimicrobial residues in Danish finishing pigs. Preventive veterinary medicine. 2016;128:87-94.

Table S1. Susceptibility testing panels and testing ranges used in antimicrobial resistance monitoring in commensal *E. coli* (MARAN) and in clinical *E. coli*[a] (GD)

Antimicrobial	MARAN (mg/L)	GD (mg/L)
Azithromycin	2-64	x
Amoxicillin / clavulanic acid	x	0.25/0.125 -32/16
Ampicillin	1-64	0.25 - 32
Apramycin	x	8-16
Cefepime	x	1-32
Cefotaxime	0.25-4	1-4
Ceftazidime	0.5-8	x
Chloramphenicol	8-128	x
Colistin	1-16	0.5 - 16
Enrofloxacin	x	0.25 - 2
Ciprofloxacin	0.015-8	x
Nalidixic acid	4-128	x
Florfenicol	x	2-8
Flumequine	x	2-16
Gentamicin	0.5-32	2-8
Neomycin	x	4-16
Spectinomycin	x	8-128
Streptomycin	x	2-64
Sulfamethoxazole	8-1024	64-256
Tetracycline	2-64	0.25-16
Tiamulin	x	8-32
Tilmicosin	x	2-32
Trimethoprim	0.25-32	0.5 - 16
Trimethoprim/Sulfamethoxazole	x	0.25/4.75 - 4/76
Tylosin	x	0.25 - 4
Tigecycline	0.25-8	x
Meropenem	0.03-16	x

[a] In the susceptibility panel of clinical *E. coli* (GD), antimicrobials for veterinary use in other animal species than broilers are included

Table S2. Resistant counts in commensal *E. coli* (MARAN) and clinical *E. coli* (GD) monitoring in broilers, the Netherlands, 2014-2019

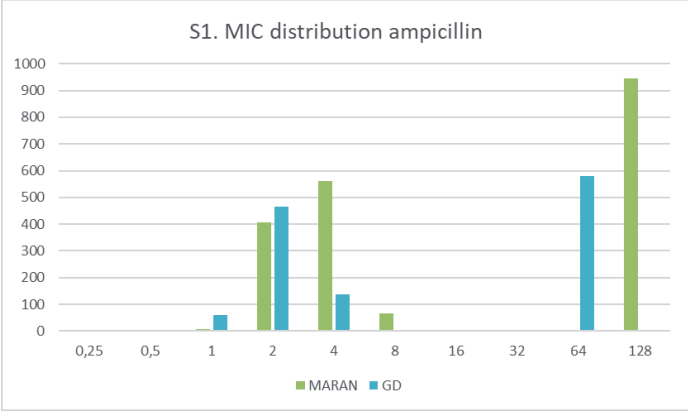
Antimicrobial	Year	Resistant count MARAN	Total MARAN	Resistant count GD	Total GD	Remarks
Ampicillin	2014	234	377	18	45	
	2015	213	400	151	322	
	2016	141	300	114	245	
	2017	109	301	89	202	
	2018	131	299	126	279	
	2019	121	315	88	160	
Gentamicin	2014	24	377	2	45	
	2015	16	400	18	322	
	2016	13	300	21	245	
	2017	17	301	10	202	
	2018	14	299	10	279	
	2019	16	315	6	160	
Cefotaxime	2014	11	377	3	45	
	2015	10	400	16	322	
	2016	3	300	12	245	
	2017	5	301	2	202	
	2018	3	299	5	279	
	2019	0	315	1	160	
Tetracycline	2014	160	377	15	45	
	2015	143	400	110	322	
	2016	91	300	114	245	
	2017	75	301	81	202	
	2018	85	299	77	279	
	2019	86	315	63	160	
Colistin	2014	0	377	0	45	
	2015	0	400	5	322	
	2016	0	300	8	245	
	2017	0	301	1	202	
	2018	0	299	1	279	
	2019	0	315	0	160	
Trimethoprim/ sulfonamide*	2014	159	377	11	45	
	2015	159	400	117	322	
	2016	100	300	71	245	
	2017	77	301	55	202	
	2018	78	299	81	279	
	2019	79	315	58	160	

						Data compared**	
Ciprofloxacin and enrofloxacin	2014	175	377	2	45	38	
	2015	176	400	31	322	26	
	2016	123	300	14	245	13	
	2017	101	301	19	202	18	
	2018	97	299	11	279	15	
	2019	108	315	20	160	18	

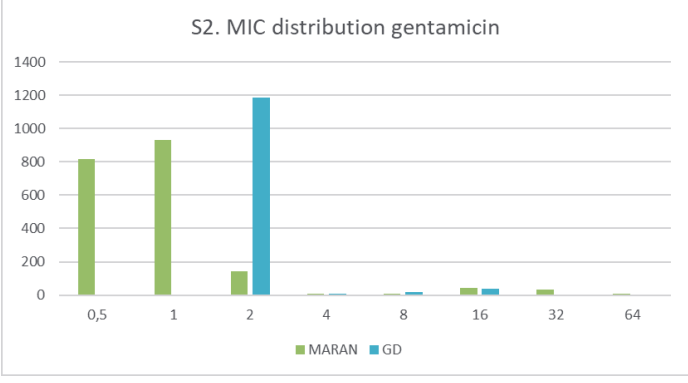
*Resistant count of MARAN are isolates non-wildtype susceptible to both trimethoprim and sulfonamide

**Resistant count of MARAN isolates when applying the CBP instead of the ECOFF

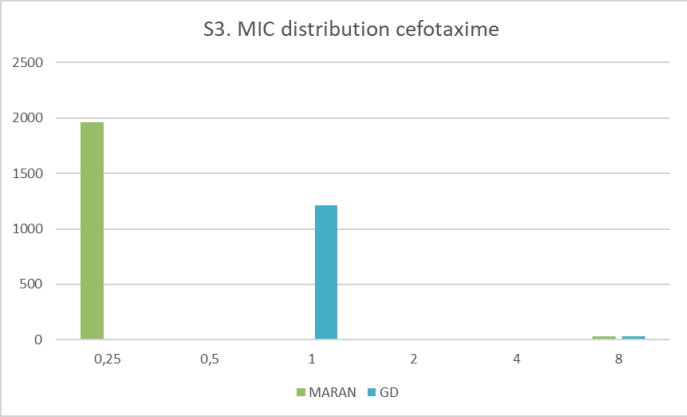
Figure S1-S7. Distributions of minimum inhibitory concentrations (MIC) of commensal *E. coli* isolates (MARAN) and clinical *E. coli* isolates (GD) tested for antimicrobial susceptibility, the Netherlands, 2014-2019



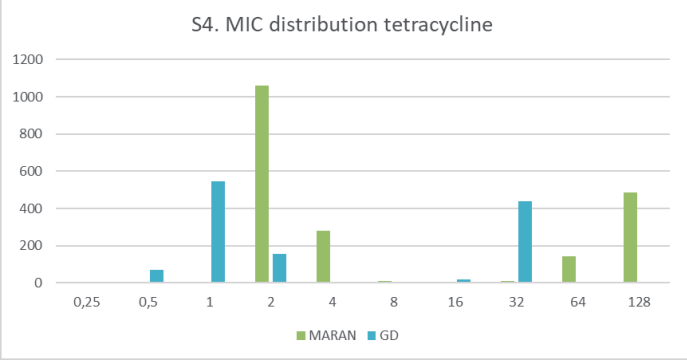
Concentration range MARAN 1-64 mg/L, range GD 0.25-32 mg/L
MARAN: >8 is resistant (ECOFF)
GD: >8 is resistant (EUCAST CBP)



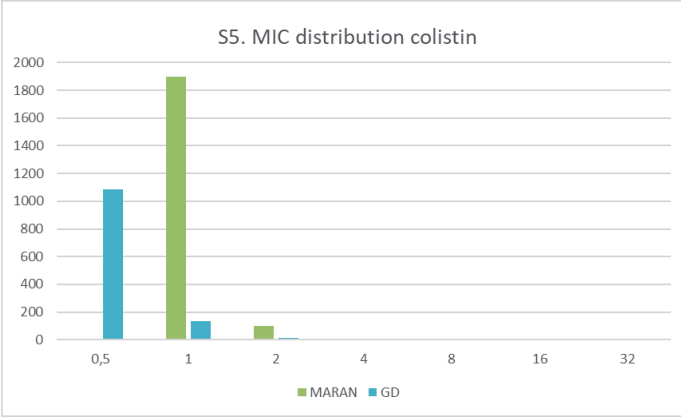
Concentration range MARAN 0.5-32 mg/L, range GD 2-8 mg/L
MARAN: >2 is resistant (ECOFF)
GD: >2 is resistant (EUCAST CBP)



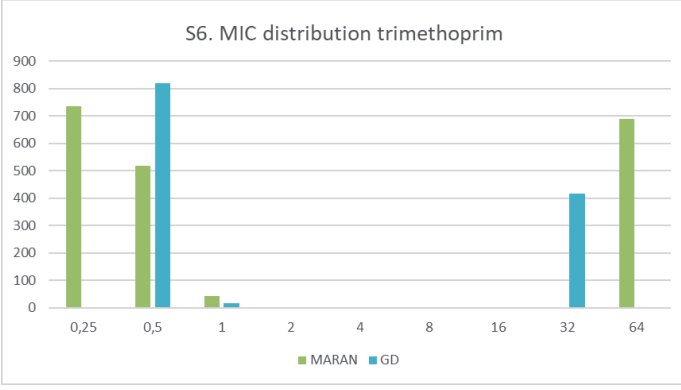
Concentration range MARAN 0.25-4 mg/L, range GD 1-4 mg/L
MARAN: >0.25 is resistant (ECOFF)
GD: >2 is resistant (EUCAST CBP)



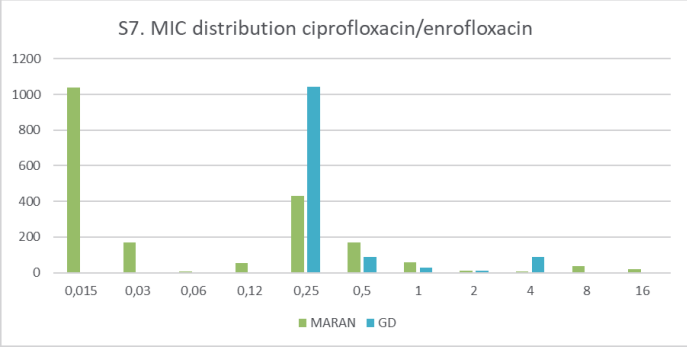
Concentration range MARAN 2-64 mg/L, range GD 0.25-16 mg/L
MARAN: >8 is resistant (ECOFF)
GD: >8 is resistant (ECOFF)



Concentration range MARAN 1-16 mg/L, range GD 0.5-16 mg/L
MARAN: >2 is resistant (ECOFF)
GD: >2 is resistant (EUCAST CBP)



Concentration range MARAN 0.25-32 mg/L, range GD 0.5-16 mg/L
MARAN: >4 is resistant for TMP (ECOFF)
GD data: >4 is resistant (EUCAST CBP)



Concentration range MARAN 0.015-8 mg/L, range GD 0.25-2 mg/L
MARAN: >0.064 is resistant (ECOFF)
MARAN CBP: >0.5 is resistant (EUCAST CBP ciprofloxacin)
GD: >1 is resistant (CLSI CBP enrofloxacin)

III

Part III

The use of whole-genome sequencing to monitor antimicrobial resistance

Chapter 7

Latent class analysis to assess whole-genome sequencing versus broth microdilution for monitoring antimicrobial resistance in livestock

Hesp A, Veldman K, Brouwer MSM, Wagenaar JA, Mevius D, van Schaik G. Latent class analysis to assess whole-genome sequencing versus broth microdilution for monitoring antimicrobial resistance in livestock. Prev Vet Med. 2021 Jun 4;193:105406. doi: 10.1016/j.prevetmed.2021.105406. Epub ahead of print. PMID: 34147959.

https://doi.org/10.1016/j.prevetmed.2021.105406

Abstract

Antimicrobial resistance (AMR) monitoring in animals is performed in commensal *Escherichia coli*, and other microorganisms relevant for human or veterinary health. Due to advances in the field and major reductions in cost, it is expected that whole-genome sequencing (WGS)-based antimicrobial susceptibility testing (AST) will (partly) replace culture-based AST. So far, no studies have been performed without using culture-based AST as the gold standard. Our aim was to use Bayesian latent class analysis to evaluate the accuracy of susceptibility testing of commensal *E. coli* by WGS-based AST versus culture-based AST as this test does not assume a gold standard. OpenBUGS was used to model two independent tests in three animal populations (N=150, 50 bacterial isolates per population): veal calves, pigs, and broilers. This resulted in the first estimation of sensitivity and specificity of WGS-based AST versus culture-based AST to detect AMR without a gold standard. Both methods had high sensitivity (>0.92, lowest limit probability interval: 0.76) and specificity was generally high for both methods for all antimicrobial classes except for aminoglycosides and macrolides. We compared WGS results for different length and identity settings (%) of gene alignment and found few differences between the 60/90, 90/90 and 95/95 settings. We recommend to further investigate sensitivity and specificity of WGS-based AST by means of latent class analysis, especially for low-prevalent resistance.

Introduction

As part of global efforts to control antimicrobial resistance (AMR) (O'Neill, 2016), monitoring AMR in animals is performed in sentinel organisms such as commensal *Escherichia coli* (Frimodt-Moller, 2004; EFSA, 2019). Currently, this is mostly done with culture-based antimicrobial susceptibility testing (AST) methods such as broth microdilution, determining minimum inhibitory concentrations (MIC) for pre-defined panels of antimicrobials. Epidemiological cut-off values (ECOFFs) or clinical breakpoints are used to determine if bacterial isolates have non-wildtype susceptibility or resistance, respectively. Recently, whole-genome sequencing (WGS) is becoming more widely available for routine AMR monitoring, and it is the expectation that WGS will mostly replace culture-based phenotypic typing in the future (Ellington et al., 2017). This paper aims to determine the validity of WGS for AMR monitoring purposes in the commensal indicator organism *E. coli.*

In the European Union, AMR monitoring in food-borne pathogens and indicator organisms from food animals is mandatory by EU legislation (2013/652/EU), and prescribed by guidelines of the European Food Safety Authority (EFSA, 2012). As part of the recently revised EFSA guidelines (EFSA, 2019), WGS is implemented for monitoring of Extended Spectrum Beta-Lactamase producing *E. coli* in European member states from 2021 onwards as a first step towards the transfer to WGS-based AMR monitoring. Many studies have shown that WGS performs well in identifying acquired resistance genes and point mutations that lead to phenotypic resistance (McDermott et al., 2016; Shelburne et al., 2017; Hendriksen et al., 2019; Bortolaia et al., 2020; Mahfouz et al., 2020). Next to information on AMR genes, WGS provides additional information, which is considered to enhance AMR monitoring (McDermott et al., 2016; Hendriksen et al., 2019). WGS elucidates the genetic relatedness of resistant strains, as well as information on virulence factors, and potentially the genetic link between AMR genes and mobile genetic elements. When these are linked, resistance genes can spread among bacteria, for example from commensal organism *E. coli* to veterinary pathogens. Therefore, information on virulence and genetic links with mobile genetic elements is relevant from a public health perspective, for zoonotic potential, and for (veterinary) clinical interest. Furthermore, WGS has other advantages over culture-based antimicrobial susceptibility typing: the potential to store sequence data indefinitely, data is easier to share with other laboratories and stakeholders, and it solves the lack of reproducibility across different laboratories described for broth microdilution (Bortolaia et al., 2020).

So far, no studies have been performed without culture-based susceptibility testing as the gold standard (Mahfouz et al., 2020). Most existing studies

focus on estimating the concordance of WGS-based AST to culture-based AST (Hendriksen et al., 2019), in which an objectivity bias may exist when comparing sensitivity and specificity of WGS-based AST to these other methods. Bayesian latent class analysis enables the estimation of sensitivity and specificity of diagnostic tests without a gold standard (Johnson et al., 2019). The purpose of this work is to use Bayesian latent class analysis to evaluate the sensitivity and specificity of WGS-based AST and culture-based AST to test commensal *E. coli.*

Methods

Sample collection

Included in the analysis were 150 commensal *E. coli* isolates collected on broiler, pig, and veal calf farms in the Netherlands in the EFFORT project (EFFORT, 2020) from October 2014 to December 2015, 10 isolates from five farms for each animal population (Ceccarelli et al., 2020). To include the diversity of the Dutch livestock sector in the sample, the farms in EFFORT were selected by different levels of antimicrobial use on farms (low to high). Faecal isolates from individual animals were randomly collected on these farms. It was part of the EFFORT sampling protocols that all animals should be sampled as close to slaughter age as possible. The EFFORT sampling protocols are described extensively in the Supplementary material of Munk et al. (2018).

Antimicrobial susceptibility testing: WGS-based

From the 150 randomly isolated E. coli strains, bacterial DNA was isolated using the Qiagen Pure Gene kit, sequencing libraries were prepared using the Illumina TruSeq kit and sequenced with Illumina HiSeq. The average genome coverage resulted between 48.4 to 301 times coverage. Raw sequence data have been deposited at ENA, a list of accession numbers (EFFORT ID) is available in Supplementary Table S2. High-quality trimmed reads (BBmap, version 38.87 (2020)) were assembled using Unicycler (version 0.4.5) and screened for resistance genes using ResFinder 3.0 and PointFinder (Bortolaia et al., 2020) on a local Linux server (databases downloaded April 2020). Isolates were considered resistant by WGS-based AST conform the ResFinder 3.0 and Pointfinder definitions of resistance genes that encode resistance to specific antimicrobial classes (Bortolaia et al., 2020). Results were compared between all resistance genes belonging to the class aminoglycosides as positive for WGS-based AST, versus only the two genes that encode gentamicin resistance (*aac(3')-IId* and *aac(3')-IV*), to show the effect on sensitivity and specificity. Similarly, phenotypical azithromycin resistance was compared to detection

of only azithromycin resistance genes (*mph*(A)) versus the complete class of resistance genes for macrolides.

WGS-based AST results were compared for different settings of gene alignment to the ResFinder 3.0 database of length and identity of the resistance genes: length 60% and identity 90%, length 90% and identity 90%, length 95% and identity 95%, length 99% and identity 99%, and length 100% and identity 100% (Table 2). For the latent class analysis, test results of ResFinder default settings for length/identity 60/90 were used, and cross-classified with the culture-based AST results, since the aim was to evaluate WGS-based AST for routine AMR monitoring purposes. To further investigate discordant results, the WGS-based AST results of the other length/identity settings summarized in Table 2 were scrutinized.

Antimicrobial susceptibility testing: Culture-based

Culture-based AST for AMR monitoring in *E. coli* was performed using the broth micro-dilution reference method according to ISO standards (ISO 20776-1) with a fixed panel of antimicrobials relevant to human healthcare according to EU legislation and European Food Safety Authority (EFSA) guidelines (Sensititre, EUVSEC antimicrobial panel). This was performed within the EFFORT project (Ceccarelli et al., 2020). The terms 'resistant' and 'resistance' in this study refer to non-wild type susceptibility, based on epidemiological cut-off (ECOFF) values as defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). Singular culture-based AST results were used in this latent class analysis. For a set of isolates with discordant results between culture-based AST and WGS-based AST, culture-based AST was repeated to verify the results, and identify possible explanations for discordance of the WGS-based AST results.

Bayesian latent class analysis

Counts of positive and negative isolates for resistance by WGS-based AST and culture-based AST were cross-classified in tables, per antimicrobial class (Supplementary Table S1). Latent class analysis was performed in OpenBUGS software (version 3.2.3, download September 2020). Based on the difference between the two test methodologies, it was assumed the two tests were conditionally independent. Culture-based AST detects expression of resistance genes by culturing in broth while WGS detects resistance genes in the bacterial genome. A model was used comparing two independent tests in three animal populations (50 isolates for each animal population) that differed in expected prevalence of AMR. Code for the OpenBUGS model was adapted from a previous publication (Johnson et al., 2019). In all models, 1000 iterations were used as

burn-in and discarded, and summary statistics were based on the next 10,000 iterations. Convergence of each model was assessed by standard diagnostic procedure for latent class analysis (Benedict et al., 2014).

Prior probability distributions

The prior probability distributions of resistance prevalence in the three animal populations were based on data from the Dutch National monitoring program (MARAN) in which culture-based AST is performed (MARAN, 2016). The dataset of MARAN is large (300 commensal *E. coli* isolates per animal species/year) and consists of isolates from random samples of animals at slaughter. Given that sample selection differed for MARAN (random representative) and our samples (10 random animals in five selected herds), weak-informative priors for resistance prevalence were used similar to the methodology of Benedict et al. (2014). The priors were based on the MARAN data of 2015 (MARAN, 2016) for each antimicrobial class in broilers, veal calves, and pigs. Beta distributions (Table 1) were calculated with Betabuster 1.0 freely available software (Betabuster 1.0, accessed September 2020). For sensitivity and specificity of both tests, weak-informative priors for culture-based AST in *E. coli* (Table 1) were used from Benedict et al. (2014). In a sensitivity analysis, model results were compared with a non-informative, uniform prior distributions (Beta(1,1)) for sensitivity and specificity.

Results

In this study a latent class model was used to determine the sensitivity and specificity of WGS-based AST versus culture-based AST without a gold standard. Convergence of the latent class model was good, based on history and auto-correlation plots (examples for gentamicin, beta-lactams and phenicols presented in Supplementary Figure S1). Model results showed that, in this data, the sensitivity and specificity of WGS-based AST and culture-based AST were similar. This corresponded to the cross-classified test outcomes of WGS-based AST and culture-based AST, in which relatively few differences were found (Supplementary Table S1). For tetracyclines, test results were identical for WGS-based AST and culture-based AST. For the other antimicrobial classes, only a small number of isolates (n=13) were found to be discordant between the two tests (Table 4, Table S1). Discordance was much higher for the complete classes of aminoglycosides and macrolides if all genes which encode resistance to any aminoglycoside or macrolide were considered as positive for resistance (Table S1).

Table 1. Prior probability distributions for prevalence of antimicrobial resistance and for sensitivity and specificity of culture-based antimicrobial susceptibility testing by broth microdilution (culture-based AST) and whole-genome sequenced based AST to detect antimicrobial resistance in livestock

Antimicrobial class		Beta distribution parameters (a, b)	Mode (%)	95% PI[a]
Gentamicin / Aminoglycosides	Veal calves	(1.2, 25.7)	1.0	0.2-15
	Pigs	(1.2, 25.7)	1.0	0.2-15
	Broilers	(3.0, 50.1)	4.0	1.2-13
Beta-lactams (ampicillin)	Veal calves	(14.0, 60.0)	19.0	11-30
	Pigs	(22.6, 53.8)	29.0	20-40
	Broilers	(2.8, 2.6)	53.0	15-88
Phenicols	Veal calves	(5.9, 38.4)	11.5	5-24.5
	Pigs	(3.7, 26.6)	9.4	3-26
	Broilers	(5.2, 34.9)	11.0	4.6-25
Trimethoprim	Veal calves	(3.4, 17.7)	12.7	4-34
	Pigs	(5.4, 8.9)	35.9	16-63
	Broilers	(3.5, 4.5)	41.5	14-76
Azithromycin / Macrolides	Veal calves	(1.0, 21.85)	0.0	0-15
	Pigs	(1.25, 25.7)	1.0	0.2-15
	Broilers	(1.85, 34.5)	2.5	0.5-14
Quinolones	Veal calves	(2.9, 27.8)	6.7	2-22
	Pigs	(1.1, 13.0)	0.7	0.3-25
	Broilers	(47.3, 60.0)	44.0	34-54
Sulfonamides	Veal calves	(19.0, 60.2)	23.3	15-34
	Pigs	(8.0, 11.3)	40.3	21-63
	Broilers	(4.5,4.9)	47.0	19-77
Tetracyclines	Veal calves	(10.2, 14.2)	41.0	23-61
	Pigs	(6.4, 7.5)	45.3	22-71
	Broilers	(27.4, 48.4)	35.8	26-47
Culture-based AST (broth microdilution)	Sensitivity	(4.8, 1.2)	83.3	43.1–99.0
	Specificity	(4.8, 1.2)	83.3	43.1–99.0
Whole-genome sequenced based AST	Sensitivity	(4.8, 1.2)	83.3	43.1–99.0
	Specificity	(4.8, 1.2)	83.3	43.1–99.0

[a] Probability interval

Results of the comparison of WGS-AST methodology regarding different settings for the gene alignment are presented in Table 2. The differences between the settings 60/90, 90/90, and 95/95 were few (Table 2). The highest number of resistant isolates (both with culture-based AST and WGS-based AST

with length/ID: 60/90 respectively) were found for tetracyclines (n=94, n=94), sulfonamides (n=74, n=75), trimethoprim (n= 64, n=67) and beta-lactams (n=76, n=77) (Table 2). Lower numbers of resistant isolates were identified for quinolones (n=22, n=23) (Table 2). Resistance for gentamicin (n=2, n=1) and azithromycin (n=3, n=3) was rarely detected (Table 2). Overall, the difference between culture-based AST and WGS-based AST (length/ID: 60/90) was small (Table 2). The difference remained small with more strict settings, but substantially increased when using the 100/100 settings (Table 2).

Table 2. Results of culture-based antimicrobial susceptibility testing (AST) by broth microdilution versus different gene alignment settings for whole-genome sequenced based AST to detect antimicrobial resistance in livestock (N=150)

Antimicrobial class	MIC[a]	60/90[b]	90/90[c]	95/95[d]	99/99[e]	100/100[f]
Gentamicin	2	1	0	0	0	0
Beta-lactams	76	77	76	76	75	66
Phenicols	22	22	21	21	14	0
Trimethoprim	64	67	67	67	67	18
Azithromycin	3	3	3	3	3	3
Quinolones	22	23	23	23	19	2
Sulfonamides	74	75	75	75	73	70
Tetracyclines	94	94	94	94	94	79

[a] Number of isolates found resistant by broth microdilution (MIC) out of a total of 150 isolates
[b] Number of isolates found resistant by WGS (N=150) with length/identity setting 60/90 % for the alignment
[c] Number of isolates found resistant by WGS (N=150) with length/identity setting 90/90 %
[d] Number of isolates found resistant by WGS (N=150) with length/identity setting 95/95 %
[e] Number of isolates found resistant by WGS (N=150) with length/identity setting 99/99 %
[f] Number of isolates found resistant by WGS (N=150) with length/identity setting 100/100 %

Regarding the latent class analysis results as shown in Table 3, estimated prevalence was low for gentamicin with 1%, 2% and 2% in veal calves, pigs, and broilers, respectively. Azithromycin resistance amounted 5%, 2% and 2% in veal calves, pigs, and broilers, respectively (Table 3). For sulfonamides, prevalence was moderate in veal calves (26%) and low in pigs (6%) and broilers (4%) (Table 3). For both culture-based AST and WGS-based AST, the sensitivity and specificity for most antimicrobial classes was high, with the exception of sensitivity of the complete class of aminoglycosides and gentamicin, and the complete class of macrolides and azithromycin (Table 3). For all other antimicrobial classes, the sensitivity was >0.92 (lowest probability interval limit: 0.76) and the specificity was generally high for both WGS-based AST and culture-based AST (Table 3).

Table 3. Latent class analysis estimates (median and 95% probability interval) for sensitivity and specificity of culture-based antimicrobial susceptibility testing (AST) versus whole-genome sequenced based AST to detect antimicrobial resistance in veal calves (n=50), pigs (n=50) and broilers (n=50)

Antimicrobial class	Prevalence[a]			Sensitivity[b]		Specificity[c]	
	Veal calves	Pigs	Broilers	Culture-based AST	WGS-based AST	Culture-based AST	WGS- based AST
Gentamicin	0.01	0.02	0.03	0.77 (0.36-0.98)	0.76 (0.35-1.00)	0.98 (0.95-1.00)	0.99 (0.97-1.00)
Aminoglycosides	0.02	0.03	0.03	0.73 (0.29-0.98)	0.79 (0.40-0.98)	0.99 (0.96-1.00)	0.46 (0.38-0.54)
Beta-lactams (ampicillin)	0.34	0.29	0.67	0.99 (0.94-1.00)	0.99 (0.95-1.00)	0.99 (0.95-1.00)	0.98 (0.93-1.00)
Phenicols	0.45	0.24	0.53	0.92 (0.76-0.99)	0.92 (0.76-0.99)	0.99 (0.95-1.00)	0.99 (0.95-1.00)
Trimethoprim	0.08	0.03	0.41	0.97 (0.90-1.00)	0.99 (0.94-1.00)	0.99 (0.95-1.00)	0.97 (0.92-1.00)
Azithromycin	0.03	0.04	0.04	0.84 (0.43-0.99)	0.83 (0.44-0.99)	0.83 (0.43-0.99)	0.83 (0.43-1.00)
Macrolides	0.03	0.02	0.02	0.77 (0.36-0.99)	0.88 (0.54-0.99)	0.99 (0.95-1.00)	0.07 (0.04-0.12)
Quinolones	0.38	0.34	0.55	0.96 (0.84-1.00)	0.97 (0.86-1.00)	0.99 (0.97-1.00)	0.99 (0.96-1.00)
Sulfonamides	0.25	0.08	0.08	0.98 (0.94-1.00)	0.99 (0.95-1.00)	0.99 (0.95-0.99)	0.99 (0.94-1.00)
Tetracyclines	0.70	0.55	0.40	0.99 (0.96-1.00)	0.99 (0.96-1.00)	0.98 (0.93-1.00)	0.98 (0.93-1.00)

[a] Median for estimated prevalence
[b] Median for sensitivity, the 95% probability intervals are listed in parenthesis.
[c] Median for specificity, the 95% probability intervals are listed in parenthesis.

In case of discordant results, culture-based AST was repeated (Table 4). For most isolates (n=10), resistance found was identical to the first test, with exception of three isolates. Isolates initially tested resistant for gentamicin (n=2) or azithromycin (n=1) were found susceptible after repeating the test (Table 4). These results were then concordant with WGS-based AST.

Scrutinizing the other length/identity settings of the WGS-based AST results clarified more discordant results (Table 2, Table 4). For phenicols, for example,, one *catA1* gene was not detected with higher length/identity setting i.e. 90/90 (Table 2). Another discordant isolate with a *floR* gene was still found positive for this gene with a setting of 95/95, but not anymore with settings of 99/99. For trimethoprim resistance, resistance genes in the three discordant isolates were

not found with length/identity of 100/100, corresponding with the repeated culture-based AST (Table 2). The isolate with a *aac(3')IId* gene was detected with 60/90 but not detected with the 90/90 alignment setting (Table 2, Table 4).

Table 4. Discordant isolates (n=13) in results of broth microdilution (culture-based AST) versus whole-genome sequenced based AST to detect antimicrobial resistance in livestock

Antimicrobial class	Antimicrobial	Isolate ID	MIC[a]	Repeated MIC	ECOFF	Resistance gene
Aminoglycosides	Gentamicin	100302010	4	1	2	None for gentamicin
		101702014	8	2		None for gentamicin
		103003004	1	0.5		*aac(3')-IId*
Beta-lactams	Ampicillin	110704022	2	2	8	$bla_{\text{TEM-1C}}$
Phenicols	Chloramphenicol	110004010	32	32	16	None for phenicols
		110004014	32	32		None for phenicols
		110504004	8	8		*catA1*
		111604014	8	8		*floR*
Trimethoprim	Trimethoprim	110504004	0.5	0.5	2	*dfrA1*
		111604014	0.25	0.25		*dfrA1*
		111804010	0.25	0.25		*dfrA7*
Macrolides	Azithromycin	110504020	8	8	16	*mph*(A), *mph*(B)
		102702012	128	8		None for azithromycin
Quinolones	Ciprofloxacin	102302012	0.015	0.015	2	*parC* p.A56T
Sulfonamides	Sulfamethoxazole	111604014	8	8	16	*sul1*, *sul2*

[a] Minimum inhibitory concentration (MIC) determined by broth microdilution (culture-based AST)

Discussion

The purpose of this study was to evaluate the sensitivity and specificity of WGS-based AST versus culture-based AST to monitor AMR in livestock, without a gold standard, by means of latent class analysis. The estimated sensitivity and specificity across antimicrobial classes are similar for WGS-based AST and culture-based AST. The latent class analysis allowed the test validity of both tests to be determined relative to the latent class, the true resistance for antimicrobials.

Test validity

For some antimicrobial classes, the sensitivity of WGS-based AST is slightly higher than of culture-based AST, although probability intervals overlap (Table

3). Also, there is some indication that the overall specificity of WGS-based AST is lower than of culture-based AST, but these probability intervals also overlap, indicating that the specificity of both methods is similar. Few differences were found in the outcomes between the two methods, resulting in low numbers of discordant isolates. The finding that WGS-based AST performs at least as well as culture-based AST is in line with previous studies using WGS-based AST as the gold standard. The review paper of Hendriksen et al. (2019) includes an overview of WGS-based AST versus culture-based AST comparisons, showing that many studies report high concordance of WGS-based AST and culture-based AST. A study by McDermott et al. (2016) in Salmonella from retail meat reached similar conclusions as this study, reporting high sensitivity and specificity for WGS-based AST.

Advantages and disadvantages of culture-based AST and WGS-based AST

The advantage of culture-based AST is that the phenotype is measured (Ellington et al., 2017) as a cumulative result of all resistance mechanisms present in a bacterial cell. For example, less specific resistance mechanisms like efflux pumps leading to resistance to multiple antimicrobial classes (Swick et al., 2011). A limitation of broth microdilution is the lack of reproducibility of end-point-reading (Bortolaia et al., 2020; Mahfouz et al., 2020).

In WGS-based AST, the database used determines the outcomes, and defines strains resistant versus susceptible. The choice of database may influence the sensitivity and specificity of WGS-based AST (Mahfouz et al., 2020). We used ResFinder 3.0, considering it is well curated and performs well compared to other resistance databases (Hendriksen et al., 2019; Mahfouz et al., 2020). The results presented here and previously by McDermott et al. (2016) show that sensitivity and specificity of WGS-based AST versus culture-based AST is mostly antimicrobial class specific and not so much database specific. Therefore, we expect that re-analysis using for instance ResFinder 4.0 or CARD will not result in major differences in the estimated sensitivity and specificity of WGS-based AST.

The comparison of the aminoglycoside and macrolide antimicrobial classes illustrates the importance of the definition of specific resistance phenotypes of AMR genes in the interpretation of WGS-based AST. Aminoglycosides are represented in culture-based AST by gentamicin but many aminoglycoside resistance genes do not lead to gentamicin resistance, potentially leading to a high number of false-positive results when this distinction is not made (specificity of WGS: 0.46, Table 3). Similarly for macrolides, the efflux pump encoding gene, *mdfA* (Edgar and Bibi, 1997) does not always lead to phenotypic

azithromycin resistance, resulting in a high number of false positives (specificity of WGS: 0.07, Table 3).

Explanation of discordant results

For almost all discordant results, we identified the cause of the mismatch. Some isolates were found resistant to culture-based AST, without detection of specific resistance mechanisms by WGS-based AST (Table 4). In three cases the causes were 'skips' or other issues with reproducibility of MICs, resulting in a match between culture-based and WGS-based AST after repeating the MIC. The test results repetitively susceptible to culture-based AST despite being resistant by WGS-based AST (Table 4, Table S1) are partly explained by the relatively low length/ID settings of 60/90 used for ResFinder (Table 2). Genes could have mutations, and may therefore not be expressed as phenotypic resistance (Bortolaia et al., 2019). In a systematic review, Mahfouz et al. (2020) discuss that it may be advisable to revise the default settings for ResFinder of 60/90 length/identity. In our data, only minor differences were found between the 60/90, 90/90 and 95/95 settings (Table 2). In general, the 60/90 setting seems well suited for routine AMR monitoring, although in some cases resistance genes are identified which do not lead to phenotypic expression (Table 2). Using higher-length settings then prevents a false-negative result. AMR genes can also be detected in raw sequence data instead of assemblies, it is expected this will not influence the estimated sensitivity and specificity.

For some discordant isolates we found a very low sequence depth as the cause, possibly due to contamination or spill-over between multiplexed samples. The standard depth-filter of Unicycler is 25% sequence depth compared to the chromosomal sequence depth, but this was turned off for these assemblies, as some plasmid encoded resistance genes were previously missed due to this depth-filter. For routine AMR monitoring, a setting between 10 and 25% is advisable, to prevent false-positive findings.

Two phenicol resistant isolates in veal calves are rare examples for which we did not find an explanation for the difference in test outcomes (Table 4). This may be the rare situation where the detected phenotype concerns new (variations of) resistance genes, or results could be different using a different database.

Assumptions of latent class analysis

The latent class model estimates the true resistance prevalence by combining the data with the prior information and estimating how both tests identify the true resistance prevalence in the different animal populations (Johnson et. al 2019). Consequently, the prevalence of resistance in the data will influence the precision

of the estimated sensitivity and specificity. Gentamicin and azithromycin resistance both have a low prevalence in all three animal populations (Table S1) and in the prior information (Table 1). This results in lower estimates of sensitivity (with wider probability intervals) for those two antimicrobial classes of both culture-based and WGS-based AST (Table 3). Interestingly, in another study with culture-based AST as the gold standard for WGS-based AST, also a lower sensitivity of WGS-based AST for gentamicin resistance (0.93) than for other antimicrobial classes was found (McDermott et al., 2016). To evaluate our findings, this analysis should be repeated in populations where resistance prevalence is higher (although this will be difficult due to the general low prevalence of these resistance mechanisms in *E. coli*) or with more data, should these become available.

In this latent class model, it was assumed that resistance prevalence differs in the different animal populations. However, for some antimicrobials, the prevalence was almost equal in the three populations, which potentially affects the accuracy of the sensitivity and specificity estimates. Others investigated the impact of breaching the prevalence assumption and found that for tests with high sensitivity or specificity this was of little influence (Toft et al., 2005).

The sample size in our study was relatively small (10 isolates from five farms) and resistance prevalence was low for some antimicrobial classes. Latent class analyses can deal with such limitations as long as there are sufficient degrees of freedom to estimate the posterior distributions of the parameters (Johnson et al., 2019). The models for the different antimicrobials all converged fairly rapidly (Supplementary Figure S1). This is presumably because an important requirement for test validation was met: all results were generated in the same laboratory with experienced staff and a high level of standardization. To improve external validity, it is advised to repeat this analysis with more, and preferably less clustered data.

Conclusions for AMR monitoring purposes

From the results of this Bayesian latent class analysis, we conclude that WGS-based AST is just as suitable for monitoring AMR in livestock as culture-based AST. Our findings highlighted some genetic variation of resistance genes and their phenotypic expression, compared to traditional AMR monitoring generated by culture-based AST. This can be of aid in future interpretation, when WGS will be further implemented to monitor AMR in livestock.

Acknowledgements

We thank the Ministry of Agriculture, Nature and Food Quality for funding the WOT-O project (WOT-01-002-003) for the sequencing of the bacterial isolates, and the EFFORT consortium for funding the susceptibility testing of the bacterial strains. Roosmarijn Luiken and Liese van Gompel we would like to thank for organizing the sampling of the EFFORT strains. We thankfully acknowledge Yvon Geurts, Alieda van Essen, Joop Testerink, Marga Japing and Albert de Boer for their technical assistance. We also acknowledge the contribution of Jeanet van der Goot to this work and Daniela Ceccarelli for analysing the MIC dataset within EFFORT, and Haitske Graveland for the project management within EFFORT. Finally, we would like to thank Bas Engel for his aid in optimizing the OpenBUGS coding.

Funding

This research was supported by the Ministry of Agriculture, Nature and Food Quality (WOT-01-002-003) and Wageningen Bioveterinary Research. The susceptibility testing of the bacterial strains was part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, funded by the European Commission, 7th Framework Program for Research and Innovation (FP7-KBBE-2013–7, grant agreement: 613754).

References

BBmap, 2020. BBMap short read aligner, and other bioinformatic tools. https://sourceforge.net/projects/bbmap/ (accessed April 2020)

Benedict, K.M., Gow, S.P., Reid-Smith, R.J., Booker, C.W., McAllister, T.A., Morley, P.S., 2014. Latent class comparison of test accuracy when evaluating antimicrobial susceptibility using disk diffusion and broth microdilution to test Escherichia coli and Mannheimia haemolytica isolates recovered from beef feedlot cattle. Epidemiol Infect 142, 2314-2325.

Betabuster 1.0. BetaBuster to obtain specific Beta prior distributions. https://betabuster.software.informer.com/ (accessed September 2020)

Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J.K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B.B., Malhotra-Kumar, S., Westh, H., Pinholt, M., Anjum, M.F., Duggett, N.A., Kempf, I., Nykäsenoja, S., Olkkola, S., Wieczorek, K., Amaro, A., Clemente, L., Mossong, J., Losch, S., Ragimbeau, C., Lund, O., Aarestrup, F.M., 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother.

Ceccarelli, D., Hesp, A., van der Goot, J., Joosten, P., Sarrazin, S., Wagenaar, J.A., Dewulf, J., Mevius, D.J., Effort Consortium, O., 2020. Antimicrobial resistance prevalence in commensal Escherichia coli from broilers, fattening turkeys, fattening pigs and veal calves in European countries and association with antimicrobial usage at country level. J Med Microbiol 69, 537-547.

Edgar, R., Bibi, E., 1997. MdfA, an Escherichia coli multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. J Bacteriol 179, 2274-2280.

EFFORT, 2020. Ecology from Farm to Fork of microbial drug Resistance and Transmission. http://www.effort-against-amr.eu/ (accessed December 14, 2020)

EFSA, 2012. Technical specifications on the harmonised monitoring and reporting of antimicrobial resistance in Salmonella, Campylobacter and indicator Escherichia coli and Enterococcus spp. bacteria transmitted through food. EFSA Journal 2012; 10(6):2742. [64 pp.] doi:10.2903/j.efsa.2012.2742. Available online: www.efsa.europa.eu/efsajournal

EFSA, 2019. Aerts, M., Battisti, A., Hendriksen, R., Kempf, I., Teale, C., Tenhagen, B.-A., Veldman, K., Wasyl, D., Guerra, B., Liébana, E., Thomas-López, D., Belœil, P.-A., 2019. Technical specifications on harmonised monitoring of antimicrobial resistance in zoonotic and indicator bacteria from food-producing animals and food. EFSA Journal 17, e05709. Available online: www.efsa.europa.eu/efsajournal

Ellington, M.J., Ekelund, O., Aarestrup, F.M., Canton, R., Doumith, M., Giske, C., Grundman, H., Hasman, H., Holden, M.T.G., Hopkins, K.L., Iredell, J., Kahlmeter, G., Köser, C.U., MacGowan, A., Mevius, D., Mulvey, M., Naas, T., Peto, T., Rolain, J.M., Samuelsen, Ø., Woodford, N., 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect 23, 2-22.

EUCAST, 2019. Antimicrobial wild type distributions of microorganisms. https://eucast.org/mic_distributions_and_ecoffs/ (accessed December 14, 2020)

Frimodt-Moller, N., 2004. Microbial Threat--The Copenhagen Recommendations initiative of the EU. J Vet Med B Infect Dis Vet Public Health 51, 400-402.

Hendriksen, R.S., Bortolaia, V., Tate, H., Tyson, G.H., Aarestrup, F.M., McDermott, P.F., 2019. Using Genomics to Track Global Antimicrobial Resistance. Front Public Health 7, 242.

Johnson, W.O., Jones, G., Gardner, I.A., 2019. Gold standards are out and Bayes is in: Implementing the cure for imperfect reference tests in diagnostic accuracy studies. Preventive veterinary medicine 167, 113-127.

Mahfouz, N., Ferreira, I., Beisken, S., von Haeseler, A., Posch, A.E., 2020. Large-scale assessment of antimicrobial resistance marker databases for genetic phenotype prediction: a systematic review. Journal of Antimicrobial Chemotherapy 75, 3099-3108.

MARAN, 2016. Veldman, K., Mevius, DJ, Wit, B, Van Pelt, W, Heederik, D. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2015.

McDermott, P.F., Tyson, G.H., Kabera, C., Chen, Y., Li, C., Folster, J.P., Ayers, S.L., Lam, C., Tate, H.P., Zhao, S., 2016. Whole-Genome Sequencing for Detecting Antimicrobial Resistance in Nontyphoidal Salmonella. Antimicrob Agents Chemother 60, 5515-5520.

Munk, P., Knudsen, B.E., Lukjancenko, O., Duarte, A.S.R., Van Gompel, L., Luiken, R.E.C., Smit, L.A.M., Schmitt, H., Garcia, A.D., Hansen, R.B., Petersen, T.N., Bossers, A., Ruppé, E., Lund, O., Hald, T., Pamp, S.J., Vigre, H., Heederik, D., Wagenaar, J.A., Mevius, D., Aarestrup, F.M., 2018. Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries. Nat Microbiol 3, 898-908.

O'Neill, 2016. Tackling drug-resistant infections globally: Final report and recommendations. The review on antimicrobial resistance. Chaired by Jim O'Neill. . Available online: https://amr-review.org/

OpenBUGS. BUGS software package for **Ba**yesian inference **U**sing **G**ibbs **S**ampling http://www.openbugs.net/w/Downloads (accessed September 2020)

Shelburne, S.A., Kim, J., Munita, J.M., Sahasrabhojane, P., Shields, R.K., Press, E.G., Li, X., Arias, C.A., Cantarel, B., Jiang, Y., Kim, M.S., Aitken, S.L., Greenberg, D.E., 2017. Whole-Genome Sequencing Accurately Identifies Resistance to Extended-Spectrum β-Lactams for Major Gram-Negative Bacterial Pathogens. Clin Infect Dis 65, 738-745.

Swick, M.C., Morgan-Linnell, S.K., Carlson, K.M., Zechiedrich, L., 2011. Expression of multidrug efflux pump genes acrAB-tolC, mdfA, and norE in Escherichia coli clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrob Agents Chemother 55, 921-924.

Toft, N., Jørgensen, E., Højsgaard, S., 2005. Diagnosing diagnostic tests: evaluating the assumptions underlying the estimation of sensitivity and specificity in the absence of a gold standard. Preventive veterinary medicine 68, 19-33.

Supplementary Table S1. Cross-classified test results of culture-based antimicrobial susceptibility testing by broth microdilution determining minimum inhibitory concentrations (MIC) versus whole-genome sequenced based antimicrobial susceptibility testing (WGS) to detect antimicrobial resistance in livestock (N=150)

Antimicrobial class		Veal calves (n=50)		Pigs (n=50)		Broilers (n=50)	
		WGS+	WGS-	WGS+	WGS-	WGS+	WGS-
Gentamicin	MIC+	0	0	0	2	0	0
	MIC-	0	50	0	48	1	49
Aminoglycosides	MIC+	0	0	1	1	0	0
	MIC-	36	14	32	16	14	36
Beta-lactams (ampicillin)	MIC+	28	0	14	0	34	0
	MIC-	1	21	0	36	0	16
Phenicols	MIC+	15	2	3	0	2	0
	MIC-	2	31	0	47	0	48
Trimethoprim	MIC+	27	0	10	0	27	0
	MIC-	3	20	0	40	0	23
Azithromycin	MIC+	2	0	0	1	0	0
	MIC-	1	47	0	49	0	50
Macrolides	MIC+	2	0	1	0	0	0
	MIC-	46	2	46	3	49	1
Quinolones	MIC+	4	0	1	0	17	0
	MIC-	0	46	1	48	0	33
Sulfonamides	MIC+	30	0	16	0	28	0
	MIC-	1	19	0	34	0	22
Tetracyclines	MIC+	42	0	29	0	23	0
	MIC-	0	8	0	21	0	27

Supplementary Figure S1 (available online):

https://ars.els-cdn.com/content/image/1-s2.0-S0167587721001501-mmc3.pdf


Universiteit Utrecht

Chapter 8

Whole-genome phylogeny, resistance genes and plasmids in commensal *Escherichia coli* from livestock

Ayla Hesp, Michael S.M. Brouwer, Gerdien van Schaik, Jaap A. Wagenaar, Dik Mevius, Kees Veldman

In preparation

Abstract

The purpose of this study is to explore the benefits of Illumina whole genome sequencing (WGS) for AMR monitoring in commensal *E. coli* and show examples of how the results can be interpreted. We analysed the genetic relatedness and presence of plasmids and resistance genes in 150 *E. coli* isolates of veal calves (n=50), pigs (n=50) and broilers (n=50) collected from farms in the Netherlands in 2014-2015. In general, there is no spread of specific resistant bacterial clones in these three animal species. On some farms, genetically identical strains were found in multiple animals, showing resistance to the same antimicrobial classes and plasmids of the same replicon types. This indicates that sampling not more than one animal per farm ensures the representativeness of the isolate collection monitored. As a consequence of the method (short read sequencing), plasmid location could only be confirmed for few resistance genes. However, significantly elevated levels of plasmid replicon types in animal populations could warrant further investigation by long-read sequencing: in veal calves IncHI2 (36%), and in poultry: IncI1 (46%) IncI2 (22%) and p0111 (46%). Col plasmids were more frequent in poultry (44%) and pigs (34%), compared to veal calves (12%). Combining advantages of short read sequencing with long read sequencing is advised to apply WGS in AMR monitoring.

Introduction

In the monitoring of antimicrobial resistance (AMR) in animals as a public health hazard, molecular methods are constantly scrutinised and updated. Whole genome sequencing (WGS) of bacteria has become available for AMR monitoring programs (Ellington et al., 2017; Oniciuc et al., 2018; Collineau et al., 2019). As part of the new European legislation, since 2021, WGS is allowed as alternative method to broth micro dilution for the specific monitoring of Extended-Spectrum Beta Lactamase (ESBL)- or AmpC- or carbapenemase-producing *E. coli* (2020/1729/EU). By sequencing the whole genome of bacteria, similar information on AMR prevalence can be acquired as by culture-based susceptibility testing (McDermott et al., 2016; Shelburne et al., 2017; Bortolaia et al., 2020; Mahfouz et al., 2020). Next to detecting resistance genes, WGS delivers data with characteristics of these genes and their genetic environment (for example location on mobile genetic elements), valuable to antimicrobial resistance monitoring (Hendriksen et al., 2019). In addition, WGS reveals the genetic relatedness of bacteria carrying resistance genes: crucial information by which the spreading potential of resistance genes is determined. For zoonotic pathogens such as *Salmonella*, *Campylobacter* and *Escherichia coli* O157 it may also reveal information on virulence characteristics.

In a previous study, we have shown by latent class analysis in a set of 150 bacterial isolates (*E. coli*) that AMR monitoring by WGS is just as sensitive and specific as by the culture-based method broth microdilution (Hesp et al., 2021b). The purpose of the present study is to explore the added value of WGS to monitor AMR in these *E. coli*, show how it can be used and interpreted, and discuss how this compares to AMR monitoring by previously used methods. In this paper, we focus on the genetic relatedness and the combined presence of resistance genes and plasmids, of 150 *E. coli* isolates of veal calves, pigs and broilers from farms in the Netherlands (2014-2015).

Methods

The sampling and isolation of the analysed *E. coli* isolates was previously described (Ceccarelli et al., 2020). In summary, the study included a total set of 150 commensal indicator *E. coli* which consisted of 10 isolates per farm, isolated from individual faecal samples from each of five veal calf, slaughter pig and broiler farms (n=50 for each animal population). Randomly selected fresh faecal samples were collected at (or close to) slaughter age from which *E. coli* strains (one per sample) were isolated on MacConkey agar. Bacterial DNA was

isolated using the Qiagen Pure Gene kit, sequencing libraries were prepared using the Illumina TruSeq kit and sequenced with Illumina HiSeq, the average read length of the run was 150 bp. The average genome coverage resulted between 48.4 to 301 times coverage. Raw sequence data have been deposited at ENA, a list of accession numbers (EFFORT ID) is available in Table S4.

High quality filtered reads were assembled using Unicycler (version 0.4.5) and screened *in silico* for resistance genes using ResFinder 3.0 and PointFinder (Bortolaia et al., 2020) on a local Unix server (databases downloaded April 2020). In this study, we used as length/identity settings the ResFinder default 60% length and 90% identity for detecting resistance genes. Plasmids were detected with PlasmidFinder (CGE, 2020) to determine plasmid replicons. Links between resistance genes and plasmids could be inferred in approximately 5-10% of the cases, due to presence on the same contig.

A custom core genome phylogeny scheme was used, based on the core genome of the sequences using cano-wgMLST_BacCompare (Liu et al., 2019). A phylogenetic tree was visualised in interactive Tree Of Life (iTOL, 2020), presenting the metadata (animal species, farms), resistance- and plasmid data grouped per antimicrobial class and replicon type, respectively.

Plasmid data per replicon type were cross-classified per animal species to indicate occurrence in the different subpopulations of all whole-genome sequenced *E. coli*. Differences between proportions of plasmid replicon type presence in different animal species were determined using logistic regression with a random farm effect to correct for possible clustering of data from the same farm. The overall correlation between the number of resistance genes present in the data (the absolute number and the number of classes) and the number of plasmids per replicon type present in these isolates was determined with a regression model. Models were selected by comparison of lowest values for Akaike's Information Criterion (AIC), model fit was assessed by the scaled deviance. All statistical analyses were performed in R version 3.3.3 (R Foundation, Vienna, Austria).

Results and discussion

This study presents examples of how plasmid data and phylogeny of strains acquired by high throughput WGS can be used to monitor antimicrobial resistance in livestock. In the interpretation, a few challenges have to be addressed. These are the characteristics of short read sequencing, and how to translate the data in AMR monitoring output.

Genetic clustering of bacterial strains is visualized in a phylogram (Figure 1).

This shows that in general, there is no spread of specific resistant bacterial clones in the three animal species (Figure 1). However, in some occasions, genetically identical strains were found in multiple animals on the same farm (Figure 1). These isolates show resistance to the same antimicrobial classes and contain plasmids of the same replicon types (Figure 1). Two examples concern *E. coli* which are resistant to three or more antimicrobial classes (Figure 1). One such case in poultry consists of three genetically identical strains (based on their bacterial core genome MLST) of the same farm with resistance by the same genes encoding resistance to beta-lactams ($bla_{\text{TEM-1B}}$), sulfonamide (*sul2*), trimethoprim (*dfrA1*) and quinolones (*parC mutation*) and furthermore contained *aph(3)-Ib* and *aph(6)-Id* genes (Table S5). These strains harbour the same plasmid types: IncI1(Gamma), IncI2, Col156 and p0111 plasmids (Table S6). The other example concerns *E. coli* in veal calves, also three identical strains from the same farm with the same resistance genes to tetracyclines (*tet*(B)), beta-lactams ($bla_{\text{TEM-1B}}$), sulfonamides (*sul2*) and trimethoprim (*dfrA7*), also contained *aph(3)-Ib* and *aph(6)-Id* and *mdf*(A) genes (Table S5), and all contain IncHI2 plasmids (Table S6). In the previous publication, we found that phenotypical resistance was identical to genotypical resistance for these strains (Hesp et al., 2021b)

In the current monitoring system by EU legislation (2013), one randomly isolated *E. coli* is derived from a caecal sample of an animal collected at slaughter from a unique farm (for veal calves and slaughter pigs) or flock (for broilers). In the present study, multiple animals were sampled on farms, resulting in inclusion of genetically identical isolates on several farms. These data show that a sample of one animal per farm is indeed representative for multiple animals in the farm. When the aim is to monitor AMR trends in the general animal population, it is therefore better to sample just one animal per farm.

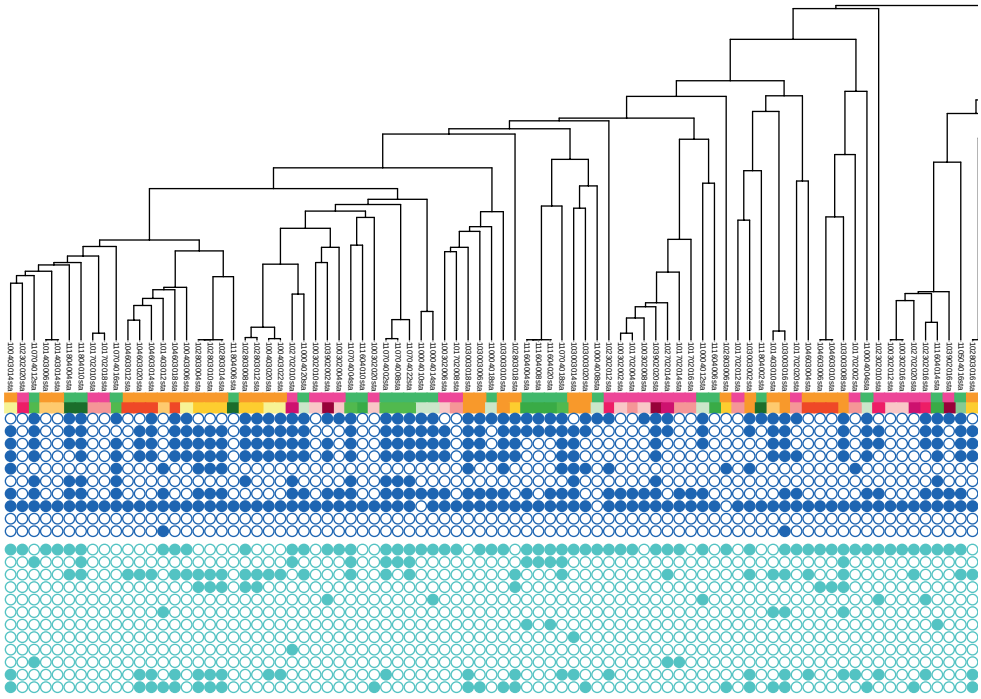
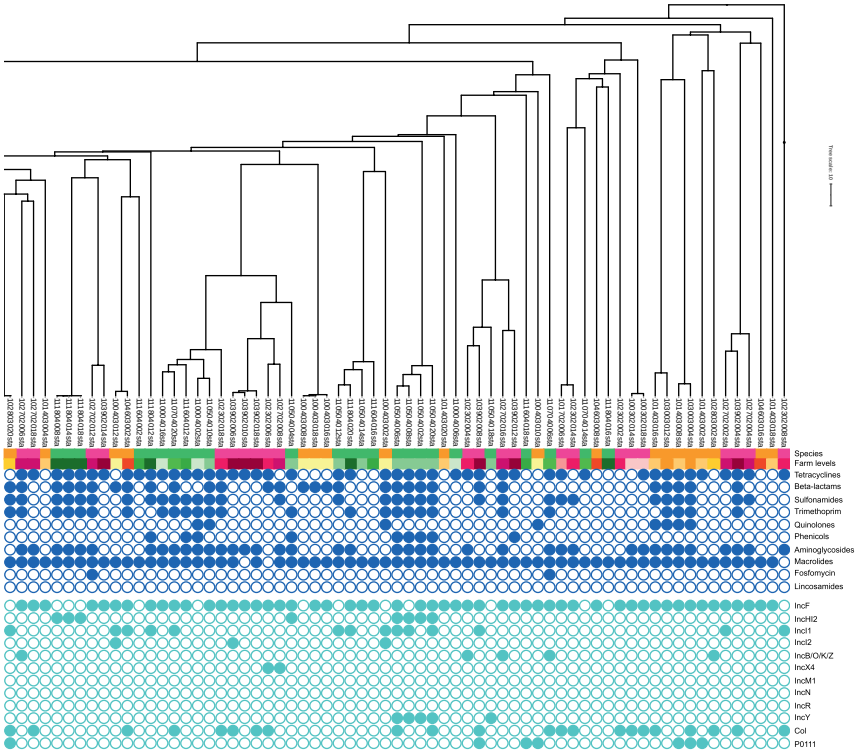


Figure 1. Whole-genome sequenced *E. coli* from livestock (N=150), based on whole-genome MLST, presenting the metadata (animal species, farms) and resistance genes and plasmids per antimicrobial class and replicon type



Species: animal species, green for veal calves, pink for slaughter pigs, yellow for broiler chickens.
Farm levels: a different shade of the same color for each farm.

The advantage of WGS by Illumina sequencing compared to long read sequencing techniques like PacBio or Nanopore, is that the accuracy is high and that the method is suitable for high-throughput application, as in monitoring activities. However, by the nature of this method, the reads are relatively short compared to genomic repeats and due to contig breaks it is often impossible to determine if a resistance gene is located on a certain plasmid. As a consequence, plasmid location could only be confirmed for a minority of the resistance genes and plasmids (only for 12 out of 70 genes, in 47 isolates) (Table S3). But because the presence of replicon types can be detected reliably with PlasmidFinder in data from Illumina sequencing (Carattoli et al., 2014), detection of trends in the plasmid replicon type distributions could be an indication to further investigate such isolates with long-read sequencing (acquired with PacBio or Nanopore). Long-read sequencing has lower accuracy but enables to determine if a resistance gene is located on a certain plasmid or on the chromosome. Combining the two methods, by hybrid assembly (Chen et al., 2020), forms a potential solution for future application of WGS in AMR monitoring.

In Table 1 we illustrate findings of significantly elevated levels of plasmid replicon types compared to the other animal populations, which could warrant further investigation. The IncHI2 replicon type was most abundant in veal calves compared to the other animal species (36%)(Table 1). In poultry IncI1 was frequent (46%), as was IncI2 (22%), compared to the other two animal populations (Table 1). Col plasmids were present often in poultry (44%) and relatively often in pigs (34%), compared to veal calves (12%). The p0111 plasmids were often present in poultry (46%)(Table 1). Next to these patterns for specific sub populations, an overall positive correlation was found between the resistance genes present in the data and the number of plasmid replicon types present in all 150 isolates (0.44 for the absolute number of resistance genes and 0.46 per antimicrobial class).

The challenge of relating data in the present study is that most plasmid typing in literature concerns bacterial isolates typed to determine the location of a specific target resistance gene (hazard-specific surveillance). The data in the present study are randomly isolated commensal *E. coli.* The finding that IncF is the most common plasmid replicon type in all animal species corresponds to several other studies (Yang et al., 2015; Madec and Haenni, 2018). The frequent finding of IncI1 plasmids in poultry (Table 1) corresponds to findings in hazard-specific surveillance for Extended Spectrum Beta-Lactamases (ESBL)(Ceccarelli et al., 2019), although ESBL-genes were not detected in the isolate collection used for this study.

The cross-classified results of presence of resistance genes per antimicrobial class and plasmids per replicon type show patterns in resistance and presence

of plasmids for a number of classes and replicon types (Table S2), also visualised in the phylogenetic tree (Figure 1). However, in the interpretation it must be considered that the genetic link between resistance gene and plasmid is unknown in most isolates and not necessarily present. An example of this is the finding of high co-occurrence of quinolone resistance in broilers with Col and p0111 plasmids (Figure 1, Table S2). This finding is unlikely to be related, since all findings of quinolone resistance in this data concern chromosomal point mutations (Table S5).

Table 1. Presence (n, p) of different plasmid replicon types per animal species for whole-genome sequenced *E. coli* from Dutch livestock (N=150)

	Plasmid replicon type											
	IncF	**Inc HI2**	**IncI1**	**IncI2**	**IncB/O/ K/Z**	**IncX4**	**IncM1**	**Inc N**	**Inc R**	**IncY**	**Col**	**P 0111**
	Isolates (n) with plasmids per replicon type											
All isolates (N=150)	110	20	43	12	10	6	3	1	1	8	45	28
Veal calves (n=50)	34	18	15	0	3	0	3	0	0	6	6	2
Pigs (n=50)	42	1	5	1	6	2	0	0	1	2	17	3
Poultry (n=50)	34	1	23	11	1	4	0	1	0	0	22	23
	Isolates (p) with plasmids per replicon type											
All isolates (N=150)	**0.73***	**0.13**	**0.29**	**0.08**	**0.07**	**0.04**	**0.02**	**0.01**	**0.01**	**0.05**	**0.30**	**0.19**
Veal calves (n=50)	0.68	***0.36[a]***	0.30	0.00	0.06	0.00	0.06	0.00	0.00	0.12	***0.12[b]***	0.04
Pigs (n=50)	0.84	0.02	0.10	0.02	0.12	0.04	0.00	0.00	0.02	0.04	0.34	0.06
Poultry (n=50)	0.68	0.02	***0.46[a]***	***0.22[a]***	0.02	0.08	0.00	0.02	0.00	0.00	0.44	***0.46[a]***

* The observed mean presence of this plasmid replicon type in all *E. coli* isolates from veal calves, pigs and poultry together

[a] The observed presence of this plasmid replicon type (in bold italic) was significantly higher in this animal population compared to the other animal populations

[b] The observed presence of this plasmid replicon type (in bold italic) was significantly lower in this animal population compared to the other animal populations

In this study, we used cross-classified tables next to a phylogenetic tree presenting metadata, resistance- and plasmid data. Alternatively, multivariate analyses could be used like principal component analysis (Dorado-García et al., 2018) or other methods as in previous studies (Hesp et al., 2021a). Multivariate analysis are useful in large datasets with many observations so that significant correlations can be based on the data, but results are complex to interpret and translate into practical knowledge. In general, this is a challenge for the use of WGS in monitoring, since it results in a large amount of data per resistance observation. However, the advantage of WGS is that the genetic data can be stored indefinitely: when future questions arise, or new methods for analysis, data is already available (Bortolaia et al., 2020).

To conclude, this study shows the challenges, but also the potential of WGS to monitor AMR in livestock, especially when more data to compare will become available and the analytical methods for WGS data will increase.

Acknowledgements

We thank the Ministry of Agriculture, Nature and Food Quality for funding the WOT-O project (WOT-01-002-003) for the sequencing of the bacterial isolates, and the EFFORT consortium for funding the sampling of the bacterial strains. Roosmarijn Luiken and Liese van Gompel we would like to thank for organizing the sampling of the EFFORT strains. We thankfully acknowledge Yvon Geurts, Alieda van Essen, and Albert de Boer for their technical assistance.

Funding

This research was supported by the Ministry of Agriculture, Nature and Food Quality (WOT-01-002-003) and Wageningen Bioveterinary Research. The sampling of the bacterial strains was part of the Ecology from Farm to Fork Of microbial drug Resistance and Transmission (EFFORT) project, funded by the European Commission, 7th Framework Program for Research and Innovation (FP7-KBBE-2013–7, grant agreement: 613754).

References

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

2013. Commission implementing decision on the monitoring and reporting of antibmicrobial resistance in zoonotic and commensal bacteria. European Union.

Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J.K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B.B., Malhotra-Kumar, S., Westh, H., Pinholt, M., Anjum, M.F., Duggett, N.A., Kempf, I., Nykäsenoja, S., Olkkola, S., Wieczorek, K., Amaro, A., Clemente, L., Mossong, J., Losch, S., Ragimbeau, C., Lund, O., Aarestrup, F.M., 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother.

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Møller Aarestrup, F., Hasman, H., 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58, 3895-3903.

Ceccarelli, D., Hesp, A., van der Goot, J., Joosten, P., Sarrazin, S., Wagenaar, J.A., Dewulf, J., Mevius, D.J., Effort Consortium, O., 2020. Antimicrobial resistance prevalence in commensal Escherichia coli from broilers, fattening turkeys, fattening pigs and veal calves in European countries and association with antimicrobial usage at country level. J Med Microbiol 69, 537-547.

Ceccarelli, D., Kant, A., van Essen-Zandbergen, A., Dierikx, C., Hordijk, J., Wit, B., Mevius, D.J., Veldman, K.T., 2019. Diversity of Plasmids and Genes Encoding Resistance to Extended Spectrum Cephalosporins in Commensal Escherichia coli From Dutch Livestock in 2007-2017. Front Microbiol 10, 76.

CGE, 2020. PlasmidFinder.

Chen, Z., Erickson, D.L., Meng, J., 2020. Benchmarking hybrid assembly approaches for genomic analyses of bacterial pathogens using Illumina and Oxford Nanopore sequencing. BMC Genomics 21, 631.

Collineau, L., Boerlin, P., Carson, C.A., Chapman, B., Fazil, A., Hetman, B., McEwen, S.A., Parmley, E.J., Reid-Smith, R.J., Taboada, E.N., Smith, B.A., 2019. Integrating Whole-Genome Sequencing Data Into Quantitative Risk Assessment of Foodborne Antimicrobial Resistance: A Review of Opportunities and Challenges. Front Microbiol 10, 1107.

Dorado-García, A., Smid, J.H., van Pelt, W., Bonten, M.J.M., Fluit, A.C., van den Bunt, G., Wagenaar, J.A., Hordijk, J., Dierikx, C.M., Veldman, K.T., de Koeijer, A., Dohmen, W., Schmitt, H., Liakopoulos, A., Pacholewicz, E., Lam, T., Velthuis, A.G., Heuvelink, A., Gonggrijp, M.A., van Duijkeren, E., van Hoek, A., de Roda Husman, A.M., Blaak, H., Havelaar, A.H., Mevius, D.J., Heederik, D.J.J., 2018. Molecular relatedness of ESBL/AmpC-producing Escherichia coli from humans, animals, food and the environment: a pooled analysis. J Antimicrob Chemother 73, 339-347.

Ellington, M.J., Ekelund, O., Aarestrup, F.M., Canton, R., Doumith, M., Giske, C., Grundman, H., Hasman, H., Holden, M.T.G., Hopkins, K.L., Iredell, J., Kahlmeter, G., Köser, C.U., MacGowan, A., Mevius, D., Mulvey, M., Naas, T., Peto, T., Rolain, J.M., Samuelsen, Ø., Woodford, N., 2017. The role of whole genome sequencing in antimicrobial susceptibility testing of bacteria: report from the EUCAST Subcommittee. Clin Microbiol Infect 23, 2-22.

Hendriksen, R.S., Bortolaia, V., Tate, H., Tyson, G.H., Aarestrup, F.M., McDermott, P.F., 2019. Using Genomics to Track Global Antimicrobial Resistance. Front Public Health 7, 242.

Hesp, A., Ter Braak, C., van der Goot, J., Veldman, K., van Schaik, G., Mevius, D., 2021a. Antimicrobial resistance clusters in commensal Escherichia coli from livestock. Zoonoses Public Health 68, 194-202.

Hesp, A., Veldman, K., Brouwer, M.S.M., Wagenaar, J.A., Mevius, D., van Schaik, G., 2021b. Latent class analysis to assess whole-genome sequencing versus broth microdilution for monitoring antimicrobial resistance in livestock. Preventive veterinary medicine 193, 105406.

iTOL, 2020. Retried from: https://itol.embl.de/, accessed December 2020

Liu, Y.-Y., Lin, J.-W., Chen, C.-C., 2019. cano-wgMLST_BacCompare: A Bacterial Genome Analysis Platform for Epidemiological Investigation and Comparative Genomic Analysis. Frontiers in Microbiology 10.

Madec, J.-Y., Haenni, M., 2018. Antimicrobial resistance plasmid reservoir in food and food-producing animals. Plasmid 99, 72-81.

Mahfouz, N., Ferreira, I., Beisken, S., von Haeseler, A., Posch, A.E., 2020. Large-scale assessment of antimicrobial resistance marker databases for genetic phenotype prediction: a systematic review. Journal of Antimicrobial Chemotherapy 75, 3099-3108.

McDermott, P.F., Tyson, G.H., Kabera, C., Chen, Y., Li, C., Folster, J.P., Ayers, S.L., Lam, C., Tate, H.P., Zhao, S., 2016. Whole-Genome Sequencing for Detecting Antimicrobial Resistance in Nontyphoidal Salmonella. Antimicrob Agents Chemother 60, 5515-5520.

Oniciuc, E.A., Likotrafiti, E., Alvarez-Molina, A., Prieto, M., Santos, J.A., Alvarez-Ordóñez, A., 2018. The Present and Future of Whole Genome Sequencing (WGS) and Whole Metagenome Sequencing (WMS) for Surveillance of Antimicrobial Resistant Microorganisms and Antimicrobial Resistance Genes across the Food Chain. Genes (Basel) 9.

Shelburne, S.A., Kim, J., Munita, J.M., Sahasrabhojane, P., Shields, R.K., Press, E.G., Li, X., Arias, C.A., Cantarel, B., Jiang, Y., Kim, M.S., Aitken, S.L., Greenberg, D.E., 2017. Whole-Genome Sequencing Accurately Identifies Resistance to Extended-Spectrum β-Lactams for Major Gram-Negative Bacterial Pathogens. Clin Infect Dis 65, 738-745.

Yang, Q.E., Sun, J., Li, L., Deng, H., Liu, B.T., Fang, L.X., Liao, X.P., Liu, Y.H., 2015. IncF plasmid diversity in multi-drug resistant Escherichia coli strains from animals in China. Front Microbiol 6, 964.

Table S1. Presence (n, p) of resistance genes to different antimicrobial classes, per animal species in whole-genome sequenced *E. coli* from Dutch veal calves (n=50), pigs (n=50) and broilers (n=50)

	Tetracyclines	Beta-lactams	Sulfonamides	Trimethoprim	Quinolones	Phenicols	Aminoglycosides	Macrolides	Fosfomycin	Lincosamides
Total (N=150)	94	77	75	67	23	22	83	144	2	2
Veal calves (n)	42	29	31	30	4	17	36	46	1	0
Pigs (n)	29	14	16	10	2	3	32	46	1	0
Broilers (n)	23	34	28	27	17	2	14	49	0	2
Veal calves (p)	0.84	0.58	0.62	0.60	0.08	0.34	0.00	0.06	0.02	0.00
Pigs (p)	0.58	0.28	0.32	0.20	0.04	0.06	0.00	0.00	0.02	0.00
Broilers (p)	0.46	0.68	0.56	0.54	0.34	0.04	0.02	0.00	0.00	0.04

Table S2. Presence (n, p) of plasmid replicon types in combination with resistance genes per antimicrobial class in whole-genome sequenced *E. coli* from Dutch livestock (N=150)

Antimicrobial class	Isolates (n) within this class with plasmids	Isolates (n) within this antimicrobial class with plasmids per replicon type											
		IncF	IncHI2	IncI1	IncI2	Inc B/O/ K/Z	IncX4	IncM1	IncN	IncR	IncY	Col	P 0111
All isolates (N=150)		**110**	**20**	**43**	**12**	**10**	**6**	**3**	**1**	**1**	**8**	**45**	**28**
Tetracyclines	94	73	20	33	6	8	4	3	0	1	6	30	15
Beta-lactams	77	56	19	37	9	6	5	3	1	1	6	31	21
Sulfonamides	75	53	16	34	8	8	4	1	1	1	5	32	19
Trimethoprim	67	46	17	31	8	5	3	1	1	1	5	26	18
Quinolones	23	16	1	6	4	0	1	0	1	0	0	12	13
Phenicols	22	18	12	10	1	0	0	1	1	1	5	4	0
Aminoglycosides	79	68	18	21	4	9	3	3	1	1	6	28	12
Macrolides	128	105	20	42	12	10	5	3	1	1	8	43	27
Fosfomycin	2	2	0	0	0	1	0	0	0	0	0	1	0
Lincosamides	2	2	0	1	0	0	0	0	0	0	0	1	2
		Isolates (p) within this antimicrobial class with plasmids per replicon type											
All isolates (N=150)		**0.73**	**0.13**	**0.29**	**0.08**	**0.07**	**0.04**	**0.02**	**0.01**	**0.01**	**0.05**	**0.30**	**0.19**
Tetracyclines		0.78	0.21	0.35	0.06	0.09	0.04	0.03	0.00	0.01	0.06	0.32	0.16
Beta-lactams		0.73	0.25	0.48	0.12	0.08	0.06	0.04	0.01	0.01	0.08	0.40	0.27
Sulfonamides		0.71	0.21	0.45	0.11	0.11	0.05	0.01	0.01	0.01	0.07	0.43	0.25
Trimethoprim		0.69	0.25	0.46	0.12	0.07	0.04	0.01	0.01	0.01	0.07	0.39	0.27
Quinolones		0.70	0.04	0.26	0.17	0.00	0.04	0.00	0.04	0.00	0.00	0.52	0.57
Phenicols		0.82	0.55	0.45	0.05	0.00	0.00	0.05	0.05	0.05	0.23	0.18	0.00
Aminoglycosides		0.86	0.23	0.27	0.05	0.11	0.04	0.04	0.01	0.01	0.08	0.35	0.15
Macrolides		0.82	0.16	0.33	0.09	0.08	0.04	0.02	0.01	0.01	0.06	0.34	0.21
Fosfomycin		1.00	0.00	0.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.50	0.00
Lincosamides		1.00	0.00	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.50	1.00

Table S3. Links between resistance genes and plasmid replicon types, in whole-genome sequenced *E. coli* from livestock (N=150)

Antimicrobial class	Resistance gene	Number of links per gene (n = 47)	Replicon type
Aminoglycosides	*aph(3'')-Ib*	4	IncFII (3), IncFIB
	aph(6)-Id	2	IncFIB, IncFII
Beta-lactams	$bla_{\text{TEM-1A}}$	2	IncBOKZ, IncFII
	$bla_{\text{TEM-1B}}$	4	IncI1 (2), IncFIA, IncBOKZ
	$bla_{\text{TEM-1D}}$	3	IncX1
Phenicols	*floR*	2	IncQ1
Trimethroprim	*dfra1*	5	IncQ1 (4), IncFIC
Sulfonamides	*sul2*	13	IncQ1 (11), IncFIC (2)
Tetracyclines	*tet*(A)	9	IncI1 (5), IncX1, Inc L/M, IncFII, IncFIC
	tet(M)	1	IncX1
	tet(C)	1	ColE10
Lincosamide	*lnu*(G)	1	IncX4

Chapter 9

General discussion

General discussion

Antimicrobial resistance (AMR) is an urgent global health threat that needs to be monitored (Walker et al., 2009; Ventola, 2015; WHO, 2015; O'Neill, 2016). Production animals are a relevant reservoir to monitor, because antimicrobial use (AMU) in livestock causes selective pressure, and AMR may be transmitted to humans directly, or indirectly via food or the environment (Michael et al., 2014; Chang et al., 2015; Hoelzer et al., 2017). This thesis is about the evaluation and interpretation of AMR monitoring in livestock in indicator organism *Escherichia coli*.

Commensal *E. coli* is used as sentinel organism to monitor AMR (Frimodt-Moller, 2004). It belongs to the order of Enterobacterales including *Salmonella* and *Klebsiella* (Adeolu et al., 2016). Commensal *E. coli* is a minor fraction of the gut microbiota (Munk et al., 2017), but is selected as indicator because of its characteristics. *E. coli* is present in all faecal samples from animals, enabling randomisation of sampling. The wildtype is susceptible to the antimicrobials which should be monitored, relevant to human healthcare. Isolation and antimicrobial susceptibility testing (AST) methods for *E. coli* can be easily standardised.

The international legislation in the European Union (2013/652/EU) has led to harmonisation and standardisation of AMR monitoring. Elements such as sampling protocol and AST methods are prescribed. To achieve international standardisation, proficiency tests are performed by the EU Reference Laboratory for Antimicrobials (EURL-AR, 2021), and audits by the EU. Elements not prescribed create room for improvement. The evaluation and quantitative interpretation of AMR monitoring results is not prescribed but is challenging and will be more complex when data increase. So far, no methods were described to statistically assess whole-genome sequencing (WGS) as alternative method to culture-based AST in AMR monitoring (2020/1729/EU). Ideally, the effects of interventions such as reductions in antimicrobial use are reflected in AMR monitoring data. Analyses can be improved for optimal evaluation of these effects. Therefore, the first aim of this thesis is to evaluate AMR monitoring results with statistical methods. The second aim is to enhance the interpretation of AMR monitoring outcome in commensal *E. coli*. The third aim is to assess WGS versus culture-based AST to monitor AMR.

Evaluation of antimicrobial resistance monitoring

In Chapter 2, AMR trends are quantified in commensal *E. coli* data from broiler chickens, slaughter pigs, and veal calves in the Netherlands, 1998 to 2016. This study aims to model these time trends, and to evaluate if any

trends and trend changes as effect of interventions were observed. The rates of increase or decrease of AMR over time are captured in a log-linear model (Poisson regression). Since 2009, as a likely effect of AMU interventions (Mevius and Heederik, 2014), a decrease over time for most antimicrobials is found in broilers and pigs, but some decrease faster than others. Based on these findings, hypotheses can be formed on the evolution of AMR in these reservoirs, such as the relatively slow decrease of ciprofloxacin resistance in broilers not corresponding to the decrease of AMU (Netherlands Veterinary Medicines Institute, 2018). Furthermore, the model allows one to assess in which year the trend change occurred. This shows that in broilers, the effect of the interventions is visible in the monitoring data from 2010 onwards, corresponding to the timing of AMU interventions (Mevius and Heederik, 2014). From this evaluation, we conclude that monitoring data from *E. coli* is suitable to quantify AMR trends over time, to follow AMR in animal populations and measure the effects of interventions.

Chapter 3 covers an entirely different perspective of AMR monitoring evaluation. Tools are assessed which can be used to evaluate AMR monitoring, by applying these tools to case studies in different countries. Regular evaluation is necessary of integrated surveillance systems for AMR and AMU in animals, humans and the environment (WHO, 2015), but there is a gap of knowledge on the evaluation of integrated surveillance systems (Aenishaenslin et al., 2019; Bennani et al., 2020). An integrated, 'One Health' approach provides a better understanding of the epidemiology of AMR and enhances intervention strategies. However, current programmes do not all address the necessary sectors and are rarely fully integrated (Johnson et al., 2018; Aenishaenslin et al., 2019; Aenishaenslin et al., 2021). Existing evaluation tools may not cover required aspects or have different focus and terminology. For example, tools designed to evaluate animal health surveillance focus on the aim 'detection of disease outbreaks' while AMR monitoring focuses on the aim 'detection of AMR trends' in healthy animals. In the present study, the applicability was assessed of six different evaluation tools for evaluation of integrated surveillance systems, by performing case studies in eight countries. Although some tools cover relevant aspects better than others, there is not one best tool: the suitability of the tool depends on the evaluation objective. We advise to start with formulating evaluation objectives and to consequently select the most fitting tool. In general, more scientific expertise on evaluation of AMR monitoring from an integrated perspective is needed. To do so, the Co-Eval-AMR network launched a platform to develop and share evaluators experience (https://guidance.fp7-risksur.eu/).

Interpretation of antimicrobial resistance monitoring data

AMR monitoring results in complex data, which are only interpretable by experts. Policymakers need a clear overview of the development of AMR in relevant reservoirs. Policy informing agencies such as the European Food Safety Authority (EFSA) have expressed the need for outcome indicators of AMR monitoring (EFSA, 2017). These outcome indicators are meant to summarize AMR for multiple antimicrobial classes in the population sampled. So far, objective (i.e. quantitative) arguments for how to compose suitable AMR monitoring outcome indicators were lacking.

To develop these, in Chapter 4 we performed a model-based cluster analysis on a dataset of minimum inhibitory concentrations (MIC) recoded to binary variables for 10 antimicrobials of commensal *E. coli* isolates (N=12,986) derived from four animal species (broilers, pigs, veal calves, and dairy cows) in AMR monitoring, the Netherlands, 2007-2018. Model-based cluster analysis is a data-driven method that summarizes resistance, based on the co-occurrence of resistance to more than one antimicrobial per isolate. Four clusters were found containing 201 resistance combinations, reflecting selection and co-selection patterns by AMU or other determinants. These clusters are potential monitoring outcome indicators, because they differentiate multidrug resistance: with or without resistance to (fluoro)quinolones and third- generation cephalosporins. Multidrug resistant (i.e. resistant to three or more antimicrobial classes) isolates are divided over three different categories: resistant to fluoro(quinolones) but with few other resistance (cluster 2), multidrug resistant but mostly without resistance to critically important antimicrobials for human medicine (WHO, 2019) (cluster 3), and multidrug resistance including resistance to critically important antimicrobials (cluster 4). The rest of the isolates, in cluster 1, are either pan-susceptible or with resistance to a single antimicrobial class that is common. The prevalence of these clusters and combinations are different for the animal populations tested, and over time. This makes them suitable as benchmarks of AMR in animal populations for risk managers, to design policy and assess effects of interventions. Compared to the indicators by EFSA, ECDC and EMA (EFSA, 2017), model-based cluster analysis may be a preferable method, because the outcome clusters are mutually exclusive. Other advantages are that no arbitrary choices have to be made, i.e. on what basis groups are made. The clusters reduce data complexity but can be broken down to search for specific details, avoiding loss of information. Like the model results in Chapter 2, trends observed in these outcome clusters could initiate other research to better understand AMR developments in animal populations. However,

compared to that model, this method gives insight in the co-occurrence of AMR within animal species, and in the similarity of resistance trends observed in Chapter 2. Outcome indicators for other AMR data than analysed here could also be identified with this approach. Since the method is data-driven, the AMR in the data determines the outcome clusters. To use them for benchmarking, the clusters should therefore be verified over a more diverse population, with data from other countries and animal sectors.

In Chapter 5, the relation between AMR prevalence and the EFSA indicators (EFSA, 2017) was investigated, and the correlation between AMR and AMU in several European countries. *E. coli* was isolated in faeces collected at farms from broilers and fattening pigs (from nine countries), and fattening turkeys and veal calves (from three countries). AMU data were collected at these farms and average treatment incidences were calculated. A large variation between countries in resistant proportions for antimicrobials and AMU was observed. Applying the EFSA outcome indicators (EFSA, 2017) showed that the proportion of multidrug resistant isolates (as indicator) was high in broilers. This study indicates that this indicator is not specific, since it overlaps with resistance to ciprofloxacin and cefotaxime, and no correlation between multidrug resistance and overall AMU was found in broilers and pigs. Likewise, no correlation was found between the proportion fully susceptible *E. coli* (the primary indicator by EFSA) (EFSA, 2017) and AMU for broilers or pigs. A few interesting correlations between AMR and AMU for specific antimicrobials were found, for example between use of fluoroquinolones and ciprofloxacin resistance in broilers and pigs. For broilers this correlation (Spearman's rho=0.778, p=0.014) seems remarkably high next to findings in Chapter 2 and 4. In Chapter 2, we found a relatively high persistence of fluoroquinolone resistance in broilers, not corresponding to AMU, worrisome because fluoroquinolones are critically important antimicrobials for human medicine (WHO, 2019). This persistence was attributed to (fluoro)quinolone resistance being mainly encoded in the bacterial chromosome and not in plasmids, the latter can be lost by bacteria due to fitness cost (Machuca et al., 2014). Fluoroquinolone resistance was found persistent in broilers in other studies (Vieira et al., 2011; Taylor et al., 2016; Chantziaras et al., 2018; Roth et al., 2019). In the cluster analysis in Chapter 4, we found that part of the isolates in broilers (cluster 2, only present in broilers) are only resistant to ciprofloxacin and nalidixic acid, and not to other antimicrobials. Apparently, for (fluoro)quinolone resistance this persistence, independent of AMU, exists next to a strong correlation between AMR and AMU. This is in contrast to sulfamethoxazole and tetracycline resistance, found persistent in broilers and pigs from different countries, for which no correlations between

AMR and AMU were found. Correlations found between AMR and AMU are not always easy to explain. Although it is generally accepted that AMU selects for AMR (Chantziaras et al., 2014) the extent to which this happens differs. This analysis over countries gives a rough estimate, but indicates the extent of the association per antimicrobial class (i.e. the extent of the selective pressure). The strength of this study is that commensal *E. coli* were collected from the same epidemiological units, i.e. the farms, in different countries. With regular AMR monitoring data (by EU legislation collected at slaughter) AMR and AMU cannot be correlated at farm level, although associations exist between AMR in *E. coli* from animals at slaughter and AMU at animal population level (Dorado-Garcia et al., 2016).

In Chapter 6, we compared monitoring in commensal *E. coli* isolated from healthy broilers with *E. coli* isolates of clinical submissions and post-mortem examinations of diseased broilers, the Netherlands, 2014-2019. Results of culture-based AST by broth microdilution were analysed. Monitoring methodology in the two programs is different as they have different aims. For the commensal isolates, a stratified random sample from healthy animals at slaughter is taken (active surveillance) versus a convenience sample from submissions of diseased animals from the same broiler population (passive surveillance). The test panels also differ: for commensal *E. coli* it includes antimicrobials relevant to human healthcare to detect early evolution of non-wildtype susceptibility, using epidemiological cut-off values (ECOFF). The test panel for clinical *E. coli* consists of antimicrobials for veterinary use, and clinical breakpoints instead of ECOFF are used to determine clinical resistance. Remarkably, despite these differences, mean resistant proportions are similar for most antimicrobials. Resistant proportions of clinical *E. coli* data fluctuate over time, therefore more data is needed to quantify the association. The random sample of commensal *E. coli* from healthy animals seems more suitable to monitor time trends in AMR. The selected sample of clinical isolates results in a higher chance to detect low prevalent resistance: i.e. cefotaxime and colistin. The two surveillance systems have complementary advantages, it is therefore advisable to monitor AMR both in commensal *E. coli* from healthy broilers and in clinical *E. coli* from diseased broilers.

The use of whole-genome sequencing to monitor antimicrobial resistance

By sequencing the whole genome of bacteria, similar information on AMR prevalence can be acquired as by culture-based AST (McDermott et al., 2016; Shelburne et al., 2017; Mahfouz et al., 2020). In addition, WGS reveals

characteristics of resistance genes and their genetic environment (for example location on mobile genetic elements), as well as genetic relatedness of bacteria: crucial information by which the spreading potential of resistance genes is determined. These aspects are valuable to AMR monitoring (Hendriksen et al., 2019), but to make the best use of WGS, some challenges have to be addressed. The first challenge is to determine test validity of WGS without using culture-based AST as the gold standard, Chapter 7. The other challenge is how the benefits of WGS (apart from detection of resistance genes) can be used for AMR monitoring, investigated in Chapter 8.

In Chapter 7, we used Bayesian latent class analysis to evaluate the accuracy of WGS (Illumina sequencing) versus culture-based AST, without assuming one test as the gold standard. The model assessed the two independent tests in three animal populations (N=150, 50 bacterial isolates per population): veal calves, pigs, and broilers from fresh faeces collected at farms in the Netherlands in 2014-2015. Resistance genes were identified with ResFinder 3.0 (Bortolaia et al., 2020) and compared with broth microdilution. This analysis showed that WGS-based AST is just as suitable to monitor AMR in *E. coli* from livestock as culture-based AST: both methods have high sensitivity and specificity. The latent class model estimates the true resistance prevalence by combining the data with prior information, showing how well tests identify this true prevalence (Johnson et. al 2019). Gentamicin and azithromycin resistance are low prevalent in the animal populations and in the prior information, resulting in wide probability intervals for the sensitivity. Therefore, sensitivity for low prevalent resistance should be further investigated with latent class analysis. Furthermore, we compared WGS results for different length and identity settings of gene alignment. Few differences were found between (length/identity %) settings 60/90, 90/90 and 95/95. The default setting of 60/90 of ResFinder seemed suited to monitor AMR in livestock.

In Chapter 8, we analysed the genetic relatedness and presence of plasmids and resistance genes in the same 150 *E. coli* isolates and showed how WGS (Illumina) can be used to monitor AMR. In general, no spread of specific resistant bacterial clones in animal species was found. On some farms, genetically identical strains were found in multiple animals, with resistance to the same antimicrobial classes and plasmids of the same replicon types. This indicates that sampling one animal per farm instead of multiple animals leads to a representative isolate collection, and thereby prevents bias in trends monitored in the whole animal population. As a consequence of the method (short read sequencing), plasmid location could be confirmed for few resistance genes. However, significantly

elevated levels of specific plasmid replicon types warrant further investigation by long-read sequencing: in veal calves IncHI2 was frequent (36%), and in broilers: IncI1 (46%) IncI2 (22%) and p0111 (46%). Col plasmids were more frequent in broilers (44%) and pigs (34%), compared to veal calves (12%). Short read sequencing (by Illumina) should be combined with long read sequencing (by Nanopore or PacBio) for optimal insight in the genetic environment of resistance genes.

Future evaluation and interpretation of AMR monitoring

Effective monitoring of AMR requires standardization and international harmonization, to enable comparison of outcome data. As we show, the randomized sample in commensal *E. coli* from healthy animals is suitable to analyse AMR trends, as we have seen and in Chapter 2, 4 and 6. Part of the findings in the thesis can be extrapolated to AMR monitoring in *Salmonella, Campylobacter,* or other pathogens, but sampling in pathogens is hard to randomize (illustrated in Chapter 6 for clinical *E. coli*) as they cannot be isolated from each animal.

To further enhance interpretation of AMR monitoring data, quantitative analyses should be incorporated in routine monitoring. In this thesis we developed models and the R code necessary to process large amounts of data. Implementing these statistical analyses in AMR monitoring requires dedicated time and means. This is advised, because in future the amount of data and data complexity will further increase, illustrated in Chapter 7 and 8. To further improve AMR monitoring, we promote evaluation of AMR surveillance systems, as discussed in Chapter 3. It would also help to harmonize the interpretation objectives of AMR monitoring over countries, next to harmonization of laboratory methods, and to determine validity of the statistical methods used over countries (i.e. in data with more variation, from different countries and animal sectors).

The random sample used in AMR monitoring in commensal *E. coli* is by design a yearly sample, and not 'real-time' monitoring. To increase the sensitivity for trend changes, to detect for example a sudden increase of resistance, sentinel farms could be followed over time (longitudinally). However, this would introduce bias compared to the current random sample, which as we show generates objective, comparable outcome data. Next to optimal AMR trend analysis, a surveillance system should ideally have early detection of hazards. For that purpose, complementary sampling strategies should be applied, by taking a risk-based sample (for example from treated animals, i.e. diseased, or young) next to a random sample. Risk-based sampling can increase early

detection of emerging resistance and low prevalent resistance. For hazard-specific surveillance (increasing the detection of specific micro-organisms, for example by enriched culture media), the system should be as 'real-time' as possible. To conclude, *E. coli* is a useful indicator to monitor AMR, provided that bias in the sampling is prevented, and that proper statistical methods are used for the evaluation and interpretation.

References

Adeolu, M., Alnajar, S., Naushad, S., R, S.G., 2016. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov. divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. nov. Int J Syst Evol Microbiol 66, 5575-5599.

Aenishaenslin, C., Häsler, B., Ravel, A., Parmley, E.J., Mediouni, S., Bennani, H., Stärk, K.D.C., Buckeridge, D.L., 2021. Evaluating the Integration of One Health in Surveillance Systems for Antimicrobial Use and Resistance: A Conceptual Framework. Front Vet Sci 8, 611931-611931.

Aenishaenslin, C., Häsler, B., Ravel, A., Parmley, J., Stärk, K., Buckeridge, D., 2019. Evidence needed for antimicrobial resistance surveillance systems. Bull World Health Organ 97, 283-289.

Bennani, H., Mateus, A., Mays, N., Eastmure, E., Stärk, K.D.C., Häsler, B., 2020. Overview of Evidence of Antimicrobial Use and Antimicrobial Resistance in the Food Chain. Antibiotics (Basel) 9, 49.

Bortolaia, V., Kaas, R.S., Ruppe, E., Roberts, M.C., Schwarz, S., Cattoir, V., Philippon, A., Allesoe, R.L., Rebelo, A.R., Florensa, A.F., Fagelhauer, L., Chakraborty, T., Neumann, B., Werner, G., Bender, J.K., Stingl, K., Nguyen, M., Coppens, J., Xavier, B.B., Malhotra-Kumar, S., Westh, H., Pinholt, M., Anjum, M.F., Duggett, N.A., Kempf, I., Nykäsenoja, S., Olkkola, S., Wieczorek, K., Amaro, A., Clemente, L., Mossong, J., Losch, S., Ragimbeau, C., Lund, O., Aarestrup, F.M., 2020. ResFinder 4.0 for predictions of phenotypes from genotypes. J Antimicrob Chemother.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., Hanage, W.P., 2015. Antibiotics in agriculture and the risk to human health: how worried should we be? Evol Appl 8, 240-247.

Chantziaras, I., Boyen, F., Callens, B., Dewulf, J., 2014. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J Antimicrob Chemother 69, 827-834.

Chantziaras, I., Smet, A., Filippitzi, M.E., Damiaans, B., Haesebrouck, F., Boyen, F., Dewulf, J., 2018. The effect of a commercial competitive exclusion product on the selection of enrofloxacin resistance in commensal E. coli in broilers. Avian Pathology 47, 443-454.

Dorado-Garcia, A., Mevius, D.J., Jacobs, J.J., Van Geijlswijk, I.M., Mouton, J.W., Wagenaar, J.A., Heederik, D.J., 2016. Quantitative assessment of antimicrobial resistance in livestock during the course of a nationwide antimicrobial use reduction in the Netherlands. J Antimicrob Chemother 71, 3607-3619.

EFSA, 2017. 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 – Joint Interagency Antimicrobial Consumption and Resistance Analysis (JIACRA) Report. *EFSA J* 2017;15:4872.

EURL-AR, 2021. EQAS Reports. Retrieved from https://www.eurl-ar.eu/reports.aspx

Frimodt-Moller, N., 2004. Microbial Threat--The Copenhagen Recommendations initiative of the EU. J Vet Med B Infect Dis Vet Public Health 51, 400-402.

Hendriksen, R.S., Bortolaia, V., Tate, H., Tyson, G.H., Aarestrup, F.M., McDermott, P.F., 2019. Using Genomics to Track Global Antimicrobial Resistance. Front Public Health 7, 242.

Hoelzer, K., Wong, N., Thomas, J., Talkington, K., Jungman, E., Coukell, A., 2017. Antimicrobial drug use in food-producing animals and associated human health risks: what, and how strong, is the evidence? BMC Vet Res 13, 211.

Johnson, I., Hansen, A., Bi, P., 2018. The challenges of implementing an integrated One Health surveillance system in Australia. Zoonoses Public Health 65, e229-e236.

Machuca, J., Briales, A., Labrador, G., Díaz-de-Alba, P., López-Rojas, R., Docobo-Pérez, F., Martínez-Martínez, L., Rodríguez-Baño, J., Pachón, M.E., Pascual, Á., Rodríguez-Martínez, J.-M., 2014. Interplay between plasmid-mediated and chromosomal-mediated fluoroquinolone resistance and bacterial fitness in Escherichia coli. Journal of Antimicrobial Chemotherapy 69, 3203-3215.

Mahfouz, N., Ferreira, I., Beisken, S., von Haeseler, A., Posch, A.E., 2020. Large-scale assessment of antimicrobial resistance marker databases for genetic phenotype prediction: a systematic review. Journal of Antimicrobial Chemotherapy 75, 3099-3108.

McDermott, P.F., Tyson, G.H., Kabera, C., Chen, Y., Li, C., Folster, J.P., Ayers, S.L., Lam, C., Tate, H.P., Zhao, S., 2016. Whole-Genome Sequencing for Detecting Antimicrobial Resistance in Nontyphoidal Salmonella. Antimicrob Agents Chemother 60, 5515-5520.

Mevius, D., Heederik, D., 2014. Reduction of antibiotic use in animals "let's go Dutch". Journal für Verbraucherschutz und Lebensmittelsicherheit 9, 177-181.

Michael, C.A., Dominey-Howes, D., Labbate, M., 2014. The antimicrobial resistance crisis: causes, consequences, and management. Front Public Health 2, 145.

Munk, P., Andersen, V.D., de Knegt, L., Jensen, M.S., Knudsen, B.E., Lukjancenko, O., Mordhorst, H., Clasen, J., Agerso, Y., Folkesson, A., Pamp, S.J., Vigre, H., Aarestrup, F.M., 2017. A sampling and metagenomic sequencing-based methodology for monitoring antimicrobial resistance in swine herds. J Antimicrob Chemother 72, 385-392.

Netherlands Veterinary Medicines Institute (SDa). 2018. Usage of Antibiotics in Agricultural Livestock in the Netherlands in 2018. Retrieved from https://cdn.i-pulse.nl/autoriteitdiergeneesmiddelen/userfiles/Publications/2018-def-rapport1.pdf

O'Neill, 2016. Tackling drug-resistant infections globally: Final report and recommendations. The review on antimicrobial resistance. Chaired by Jim O'Neill.

Roth, N., Käsbohrer, A., Mayrhofer, S., Zitz, U., Hofacre, C., Domig, K.J., 2019. The application of antibiotics in broiler production and the resulting antibiotic resistance in Escherichia coli: A global overview. Poult Sci 98, 1791-1804.

Shelburne, S.A., Kim, J., Munita, J.M., Sahasrabhojane, P., Shields, R.K., Press, E.G., Li, X., Arias, C.A., Cantarel, B., Jiang, Y., Kim, M.S., Aitken, S.L., Greenberg, D.E., 2017. Whole-Genome Sequencing Accurately Identifies Resistance to Extended-Spectrum β-Lactams for Major Gram-Negative Bacterial Pathogens. Clin Infect Dis 65, 738-745.

Taylor, N.M., Wales, A.D., Ridley, A.M., Davies, R.H., 2016. Farm level risk factors for fluoroquinolone resistance in E. coli and thermophilic Campylobacter spp. on poultry farms. Avian Pathology 45, 559-568.

Ventola, C.L., 2015. The antibiotic resistance crisis: part 2: management strategies and new agents. P t 40, 344-352.

Vieira, A.R., Collignon, P., Aarestrup, F.M., McEwen, S.A., Hendriksen, R.S., Hald, T., Wegener, H.C., 2011. Association Between Antimicrobial Resistance in Escherichia coli Isolates from Food Animals and Blood Stream Isolates from Humans in Europe: An Ecological Study. Foodborne Pathogens and Disease 8, 1295-1301.

Walker, B., Barrett, S., Polasky, S., Galaz, V., Folke, C., Engström, G., Ackerman, F., Arrow, K., Carpenter, S., Chopra, K., Daily, G., Ehrlich, P., Hughes, T., Kautsky, N., Levin, S., Mäler, K.-G., Shogren, J., Vincent, J., Xepapadeas, T., de Zeeuw, A., 2009. Looming Global-Scale Failures and Missing Institutions. Science 325, 1345-1346.

WHO, 2015. Global action plan on antimicrobial resistance. Retrieved from https://www.who.int/antimicrobial-resistance/publications/global-action-plan/en/

WHO, 2019. Critically important antimicrobials for human medicine, 5th revision. World Health Organization Advisory Group on Integrated Surveillance of Antimicrobial Resistance (WHO AGISAR). Critically important antimicrobials for human medicine, 6th Revision, (ed.) World Health Organization. (Geneva), 2019.

Summary

Summary

Effective antimicrobials are essential for adequate healthcare, but unfortunately, worldwide antimicrobial resistance (AMR) threatens this effectiveness, caused by antimicrobial use (AMU). The possibilities for development of antimicrobials are limited, and new antimicrobials will not become widely available. This leaves prudent AMU and other interventions to limit existing AMR as an important strategy and therefore, AMR must be monitored. Production animals are a relevant reservoir to monitor, because AMR may be transmitted to humans directly, or indirectly via food or the environment. This thesis is about monitoring of AMR in livestock as public health hazard in indicator organism *Escherichia coli*.

In the European Union, monitoring of AMR in animals as public health hazard is performed by European legislation in commensal *E. coli* and food-borne pathogens *Salmonella* and *Campylobacter*. The international legislation has led to harmonisation and standardisation of the sampling and the microbiological methods. Elements not prescribed create room for improvement. The evaluation and interpretation by statistical analysis of AMR monitoring results is not prescribed, but is challenging and will be more complex when the amount of data increases. The updated EU legislation in 2020 has allowed whole-genome sequencing (WGS) as alternative method to culture-based antimicrobial susceptibility testing in AMR monitoring. So far, no statistical approaches were described to evaluate WGS versus culture-based methods. Analyses can be improved for optimal evaluation and interpretation of AMR monitoring data. Therefore, the first aim of this thesis is to evaluate AMR monitoring results with statistical methods. The second aim is to improve the interpretation of AMR monitoring in commensal *E. coli*. The third aim is to assess WGS versus culture-based methods to monitor AMR.

Chapter 2 aims to model the time trends in AMR monitoring data in commensal *E. coli* from the Netherlands, 1998 to 2016, in broilers, slaughter pigs, veal calves, to evaluate if trends and trend changes as a result of interventions were observed. The rates of increase or decrease of AMR over time are captured in a model (Poisson regression). Since 2009, as a likely effect of AMU interventions, a decrease over time for most antimicrobials is found in broilers and pigs, for some antimicrobials this decrease is faster than in others. From this evaluation, we conclude that monitoring data from *E. coli* is suitable to quantify trends over time, to follow AMR in animal populations and measure the effects of interventions.

In Chapter 3 tools are assessed which can be used to evaluate AMR monitoring. The applicability of six different evaluation tools for integrated surveillance was assessed by case studies in eight countries. Results show that although some tools cover relevant aspects better than others, there is not one best tool for evaluation of integrated AMR surveillance: the suitability of the tool depends on the evaluation objective. In general, more scientific expertise on evaluation of AMR monitoring from an integrated perspective is needed. An online platform was created in this consortium to further develop and share evaluators experience.

In Chapter 4, a need of policy makers is addressed for a clear overview of AMR monitoring outcome, to develop and adjust policy timely. This chapter aims to summarise AMR over multiple antimicrobial classes, to develop AMR monitoring outcome indicators. A multivariate cluster analysis was applied to AMR monitoring data from the Netherlands, 2007 to 2018, in broilers, slaughter pigs, veal calves, and dairy cows. This resulted in four clusters containing combinations of resistance to multiple antimicrobial classes. These clusters are useful as monitoring outcome indicators, because they distinguish different levels of multidrug resistance (i.e. resistant to three or more antimicrobial classes) and indicate development of AMR over time and in the different animal sectors. The clusters were compared with outcome indicators reported by the European Food Safety Authority (EFSA), and were found more specific and potentially more practical. In order to apply them for benchmarking of AMR, we recommend to verify this cluster methodology with data from different countries.

In Chapter 5, AMR is described in commensal *E. coli* from livestock in several European countries, and the correlation between AMR and AMU in several European countries. The relationship with AMU and the outcome indicators reported by EFSA was evaluated. From this analysis, it could be concluded that there was a large variation of AMR and AMU between different countries. Based on the correlations, AMR for some antimicrobial classes was prevalent independent of AMU. The strength of correlations differed per antimicrobial class. The indicators used by EFSA did not correlate to overall AMU, indicating they are not specific.

Chapter 6 compares AMR monitoring in commensal *E. coli* isolated from healthy animals with clinical resistant *E. coli* from diseased broilers in the Netherlands, 2014 to 2019. Differences and similarities in the two types of AMR monitoring are described. Monitoring methodology in the two programs is different as they

have different aims. The sample is different, the test panels of antibiotics are different (focused on human versus animal health) and the criteria (breakpoints) to determine resistance differ. Despite these differences, resistant percentages are similar for most antimicrobials. The random sample of commensal *E. coli* from healthy broilers seems more suitable to monitor time trends in AMR. The selected sample from diseased broilers results in a higher chance to detect low prevalent resistance. The two surveillance systems are complementary, so it is advisable to monitor AMR both in commensal *E. coli* from healthy broilers and in clinical *E. coli* from diseased broilers.

In Chapter 7, we used Bayesian latent class analysis to evaluate the accuracy of WGS (Illumina sequencing) versus culture-based AST to monitor AMR, without assuming one test as the gold standard. This was analysed in three animal populations (N=150, 50 bacterial isolates per population): veal calves, pigs, and broilers, from fresh faeces collected at farms in the Netherlands in 2014-2015. Resistance genes (identified with the ResFinder 3.0 database) were compared with broth microdilution. This showed that WGS is just as suitable to monitor AMR in *E. coli* from livestock as culture-based AST: both methods have high sensitivity and specificity.

Chapter 8 describes the additional benefits of WGS, in the same 150 commensal *E. coli* isolates from livestock. WGS reveals characteristics of resistance genes and their genetic environment (for example location on mobile genetic elements: plasmids), and relatedness of bacteria: crucial information determining the spread of resistance. In this data, no spread of genetically related bacteria was found in animal species. On some farms, identical strains were found in multiple animals, with resistance to the same antimicrobial classes and plasmids of the same replicon types. Consequently, sampling one animal per farm instead of multiple animals leads to a representative bacterial isolate collection. This indicates that the current methodology in AMR monitoring prevents bias in monitored AMR trends. As a consequence of the sequencing method (short read sequencing), plasmid location could be confirmed for few resistance genes. To improve the quality of WGS as tool for AMR monitoring, short read sequencing should be combined with long read sequencing for optimal AMR monitoring.

The conclusions from this thesis are that *E. coli* is a useful indicator to monitor AMR in livestock, provided that bias in the sampling is prevented, and that proper statistical methods are used for the evaluation and interpretation. As we show, the randomized sample from healthy animals is well suited to analyse AMR trends over time. Other types of samples such as risk-based sampling (for

example from diseased animals) are useful to detect rare or emerging resistance, but should be used next to a random sample to ensure representativeness for the whole animal population. To improve interpretation of AMR monitoring data, quantitative analyses should be incorporated in routine monitoring, because in the future the amount and complexity of data will further increase. The validity of the statistical approaches in this thesis should be further investigated in data with more variation, from different countries. We promote further evaluation of AMR surveillance systems, and the analysis of AMR monitoring outcomes should be harmonized, next to already existing harmonization of laboratory methods.


Universiteit Utrecht

Samenvatting in het Nederlands

Samenvatting in het Nederlands

Effectieve antibiotica zijn essentieel voor een goede gezondheidszorg. Helaas wordt de effectiviteit van antibiotica bedreigd door antimicrobiële resistentie (AMR), een urgent wereldwijd probleem. De mogelijkheden om nieuwe antibiotica te ontwikkelen zijn beperkt. Als ze al ontwikkeld worden, zullen deze nieuwe middelen spaarzaam worden ingezet, om nog meer resistentievorming te voorkomen. Het behouden van de effectiviteit van bestaande antibiotica is dan ook een belangrijke strategie om AMR tegen te gaan. Om die reden dient AMR te worden gemonitord in reservoirs waarin resistentie zich kan ontwikkelen. Voedselproducerende dieren zijn zo'n relevant reservoir, omdat het gebruik van antibiotica bacteriën selecteert die resistent zijn (oftewel selectiedruk). Vervolgens kan AMR worden overgedragen van dier op mens, door direct contact, of indirect via voedsel of via het milieu. Dit proefschrift gaat over de monitoring van AMR in voedselproducerende dieren in de indicatorbacterie *Escherichia coli.*

In de Europese Unie wordt AMR als volksgezondheidrisico gemonitord in *E. coli*, *Salmonella* en *Campylobacter*. Dit is verplicht volgens Europese regelgeving. De internationale regelgeving heeft geleid tot harmonisering en standaardisatie van protocollen voor onder andere het nemen van steekproeven en de gebruikte laboratoriummethoden. De analysemethode voor evaluatie en interpretatie van de AMR monitoring uitkomsten is echter niet voorgeschreven, maar wordt steeds complexer doordat de hoeveelheid uitkomstdata toeneemt. In 2020 is de Europese regelgeving vernieuwd en wordt nu 'Whole Genome Sequencing' (WGS) ook toegestaan als monitoringsmethode, in plaats van het bepalen van de gevoeligheid door middel van kweekplaten met antibiotica erin. Tot zover waren er geen methoden gepubliceerd of voorgeschreven om WGS statistisch te evalueren ten opzichte van de huidige gouden standaard: de traditionele kweek met gevoeligheidsbepaling. Ook in meer algemene zin kunnen analysemethoden van AMR monitoringsdata worden verbeterd. Het eerste doel van dit proefschrift is daarom: het evalueren van AMR monitoring door middel van statistische analyses van AMR monitoring data. Het tweede doel is het verbeteren van de interpretatie van AMR monitoring met behulp van analyses. De derde doelstelling is om de accuraatheid van WGS statistisch te evalueren ten opzichte van de traditionele gevoeligheidsbepaling.

In Hoofdstuk 2 worden AMR monitoring data uit Nederlandse landbouwhuisdieren van 1998-2016 gemodelleerd om trends in de tijd of een trendbreuk te detecteren, die mogelijk een gevolg zijn van veranderingen

of interventies in antibioticumgebruik. De mate van toename of afname van AMR over de tijd wordt gekwantificeerd met een statistisch regressiemodel (Poisson regressie). Sinds 2009 werd een afname van resistentie over de tijd waargenomen voor vrijwel alle antibiotica in vleeskuikendata en varkens, volgend op interventies in het antibioticumgebruik in deze dieren. Voor sommige antibiotica is deze afname van resistentie in de tijd sneller dan voor anderen. De conclusie uit deze evaluatie is dat de AMR monitoring data uit *E. coli* geschikt zijn om trends over de tijd te kwantificeren, om daarmee in dierpopulaties AMR te kunnen volgen evenals mogelijke effecten van interventies.

In Hoofdstuk 3 worden verschillende tools getest om AMR monitoring te evalueren. De toepasbaarheid van zes verschillende tools (bedoeld om geïntegreerde monitoring te evalueren) werden bestudeerd door middel van casestudies in acht verschillende landen. De resultaten laten zien dat sommige tools de relevante aspecten beter behandelen dan andere, maar dat er niet één tool het beste is om geïntegreerd AMR monitoring te evalueren. De bruikbaarheid van de tools hangt af van het specifieke evaluatiedoel. In algemene zin is er veel meer wetenschappelijke kennis nodig over de evaluatie van AMR monitoring vanuit een geïntegreerd perspectief. Er is een online platform gestart om evaluatie-ervaringen te delen en verder te ontwikkelen.

In Hoofdstuk 4 wordt ingegaan op een behoefte van beleidsmakers om een duidelijk overzicht te hebben van AMR monitoringuitkomsten, om beleid tijdig te ontwikkelen en aan te passen. In deze studie wordt AMR van verschillende antibioticaklassen samengevat, om daarmee AMR uitkomstindicatoren te ontwikkelen. Daartoe is een multivariate clusteranalyse toegepast op AMR monitoring data uit Nederland van 2007-2018 in vleeskuikens, varkens, vleeskalveren en melkkoeien. Dit leverde vier clusters op met daarin 201 combinaties van resistentie tegen verschillende antibioticaklassen. Deze clusters zijn bruikbaar als uitkomstindicatoren van AMR monitoring, omdat ze onderscheid kunnen maken tussen verschillende niveaus van multiresistentie (dat wil zeggen: resistent tegen drie of meer antibioticaklassen). Ook geven de clusters een indicatie van hoe AMR zich ontwikkelt over de tijd in de verschillende dierpopulaties. Deze clusters zijn vervolgens vergeleken met uitkomstindicatoren opgesteld door de European Food Safety Authority (EFSA). De indicatoren uit deze studie werden specifieker en potentieel beter toepasbaar bevonden dan die van EFSA. Om ze echt toe te passen als benchmark voor AMR raden we aan deze clusterindeling eerst te verifiëren met data uit verschillende landen.

In Hoofdstuk 5 beschrijven we AMR in commensale *E. coli* uit landbouwhuisdieren in verschillende Europese landen en wordt de correlatie met antibioticumgebruik in deze landen berekend. Daarnaast is de relatie met de uitkomstindicatoren door EFSA geëvalueerd. De conclusie was dat er grote variatie is van AMR en antibioticumgebruik in verschillende landen. De correlaties van AMR met het antibioticumgebruik gaven aan dat AMR voor sommige antibioticaklassen onafhankelijk van het antibioticumgebruik aanwezig is (d.w.z. de correlatie met antibioticumgebruik verschilde sterk per antibioticumklasse). De indicatoren door EFSA correleerden niet met het algehele antibioticumgebruik, dit benadrukte dat die indicatoren inderdaad niet specifiek zijn.

In Hoofdstuk 6 wordt AMR monitoring in commensale *E. coli* uit gezonde vleeskuikens vergeleken met AMR monitoring in klinische *E. coli*-isolaten, verzameld uit zieke vleeskuikens uit Nederland, 2014-2019. Deze twee monitoringssystemen hebben verschillende doelstellingen en zijn daarom ook verschillend ingericht. De beoogde steekproef verschilt en de testpanels van antibiotica zijn verschillend. De AMR monitoring in gezonde dieren richt zich meer op antibiotica die voor de volksgezondheid belangrijk zijn, terwijl het testpanel voor zieke dieren vooral bestaat uit antibiotica die voor gebruik in de veterinaire praktijk belangrijk zijn. Ook verschillen de criteria om resistentie te definiëren, oftewel de gebruikte breekpunten. Ondanks deze verschillen zijn overeenkomstige resistentiepercentages gevonden voor de meeste antibiotica in deze studie. De gerandomiseerde steekproef in commensale *E. coli* van gezonde dieren lijkt beter geschikt om AMR trends in de tijd waar te nemen. Het geselecteerde sample in zieke dieren leidt tot een hogere kans om laagprevalente resistenties te detecteren. De twee monitoringssystemen zijn dus complementair aan elkaar. Voor een volledig overzicht van AMR raden we aan zowel in commensale als in klinische *E. coli*-bacteriën te blijven monitoren.

In Hoofdstuk 7 is Bayesiaanse statistiek gebruikt om de accuraatheid van WGS als testmethode te vergelijken met gevoeligheidsbepalingen door middel van kweek, zonder dat één van beide tests als de gouden standaard wordt beschouwd. Dit is gedaan door een 'latent class' analyse in drie dierpopulaties (N=150, 50 bacteriële isolaten per populatie): vleeskalveren, vleesvarkens en vleeskuikens, waarvan verse faeces verzameld is op bedrijven in Nederland in 2014-2015. De uitkomst voor resistentiegenen in deze isolaten (aangetoond met de ResFinder 3.0 database voor resistentiegenen) werd vergeleken met uitkomst van de kweek met gevoeligheidsbepaling. Dit liet zien dat WGS net zo geschikt is om AMR te monitoren in *E. coli* uit landbouwhuisdieren als de kweek

met gevoeligheidsbepalingen: beide methoden hebben een hoge sensitiviteit en specificiteit.

In Hoofdstuk 8 worden de additionele voordelen van WGS als monitoringsmethode beschreven aan de hand van de data uit dezelfde 150 *E. coli*-isolaten. WGS levert allerlei informatie over de resistentiegenen en hun genetische achtergrond. Bijvoorbeeld waar ze gelokaliseerd zijn in het bacteriële genoom, of dat ze op bepaalde mobiele genetische elementen liggen, plasmiden genoemd. Ook laat WGS zien of en hoe sterk bacteriën aan elkaar verwant zijn, dit is cruciale informatie om de verspreiding van resistentiegenen in kaart te kunnen brengen. In deze studie bleek binnen de diersoorten geen verwantschap van de geteste stammen voor te komen, maar op sommige bedrijven werden wél genetisch identieke bacteriestammen gevonden uit meerdere dieren. Deze waren resistent tegen dezelfde antibiotica en droegen dezelfde typen plasmiden bij zich. Dit betekent dat het raadzaam is om per bedrijf maar één dier te bemonsteren in plaats van meerdere dieren, om te voorkomen dat er oververtegenwoordiging optreedt in de steekproef van de AMR monitoring, die de waargenomen trends zou kunnen beïnvloeden. Als gevolg van de gebruikte methode (WGS met korte genetische fragmenten, oftewel 'short reads') werd maar voor enkele resistentiegenen een verband aangetoond tussen resistentiegenen en hun specifieke genetische locatie op plasmiden. Bij toepassen van WGS voor AMR monitoring bevelen we daarom aan 'short read' sequencing te combineren met 'long read' sequencing, zodat dat verband vaker kan worden aangetoond.

In dit proefschrift concluderen we dat het monitoren in *E. coli* een bruikbare indicatie oplevert van AMR in een dierpopulatie, vooropgesteld dat de steekproeven representatief zijn voor de gemonitorde populatie en dat de juiste statistische methoden worden gebruikt voor de evaluatie en interpretatie. Zoals we laten zien helpt het randomiseren van een steekproef om trends te kunnen monitoren over de tijd. Andere steekproeven, zoals een risico-gebaseerde steekproef (bijvoorbeeld van zieke dieren), zijn nuttig om zeldzame of opkomende resistentievormen te monitoren. Deze dienen wel naast een gerandomiseerde steekproef te worden gebruikt om te zorgen dat gemonitorde trends in AMR representatief zijn voor de gehele dierpopulatie. Om de interpretatie van AMR monitoring data verder te verbeteren moeten de kwantitatieve analyses zoals hier toegepast, worden ingebed in routinemonitoring. Des te meer omdat in de toekomst de hoeveelheid data en de complexiteit ervan nog verder toe zal nemen. De statistische benaderingen in dit proefschrift kunnen worden gevalideerd door ze te testen op AMR data van andere landen. Verder bevelen we aan om in

de toekomst AMR monitoring meer te evalueren en om analysemethoden voor uitkomsten van AMR monitoring ook te harmoniseren, zoals dat al gedaan is voor de gebruikte laboratoriummethoden.

Dankwoord

Dankwoord

Heel veel dank gaat uit naar mijn begeleiders Dik Mevius, Gerdien van Schaik en Kees Veldman, die bijgedragen hebben met hun uitgebreide kennis, maar minstens evenveel toewijding en betrokkenheid. Dank jullie wel voor het veelvuldig lezen en helpen verbeteren van al mijn stukken, de inhoudelijke discussies die we hadden waren stimulerend en werden steeds leuker naarmate het proefschrift (en ikzelf) vorderde. Het laatste jaar door corona waren we volledig aangewezen op online meetings. Het heeft ons er niet van weerhouden samen een goed resultaat neer te zetten en daar ben ik heel trots op en dankbaar voor!

Speciale dank gaat ook uit naar Mike Brouwer, Jeanet van der Goot en voormalig collega Daniela Ceccarelli die eveneens betrokken waren tijdens mijn PhD. Ik heb van jullie ook veel geleerd, niet alleen inhoudelijk maar ook in bredere zin in de ontwikkeling tot wetenschapper.

Naast deze collega's zijn er nog vele anderen die zich hebben ingespannen om bij te dragen aan deze promotie. Natuurlijk allereerst onze analisten op het antibioticalab: Joop Testerink, Marga Japink, Alieda van Essen, Yvon Geurts, Arie Kant en bij Bioinformatica Albert de Boer en Frank Harders. Ook Teresita Bello Gonzalez uit de AMR groep bij WBVR wil ik bedanken als gewaardeerde collega. Daarnaast zijn er nog vele andere medewerkers van Wageningen Bioveterinary Research (WBVR) die direct of indirect hebben bijgedragen. Ronald Petie dank ik voor het meedenken in R en alles wat ik daardoor geleerd heb. De mensen in de epidemiologiegroep voor het sparren over modellen. Ik wil vele collega's bij WBVR bedanken niet alleen voor de inhoudelijke bijdrage of het werk op het lab, maar ook voor koffiemomenten, een praatje tussendoor, wandelingetje etc. Dat geldt ook voor de mensen van de kunstcommissie (Monique, Saskia, Jan) sport- en carpoolmaatjes (o.a. Conny, Manouk, Marloes), collega PhD students en de jonge projectleidersgroep. Last but not least bij WBVR wil ik graag mijn leidinggevenden bedanken: Hetty, Hendrik-Jan en Ludo.

Naast de mensen bij WBVR zijn er natuurlijk ook mensen bij de Universiteit Utrecht die hier in dank genoemd moeten worden. Met name de veterinaire epidemiologiegroep bij de afdeling Population Health Sciences, Faculteit Diergeneeskunde UU (voorheen Hoofdafdeling Landbouwhuisdieren) en in het bijzonder Hans Vernooij voor het altijd willen meedenken over statistiek. Voor de bijdrage aan de statistische modellen dank ik ook Bas Engel van Biometris, Wageningen Universiteit.

Alle co-auteurs van de hoofdstukken wil ik graag bedanken, onder andere Cajo ter Braak, met wie ik samen heb gewerkt aan Hoofdstuk 4 en van wie ik heel veel heb geleerd over het toepassen van multivariate analyses. Maar ook de mensen binnen het Co-Eval-AMR netwerk (o.a. Lis, Marianne) en binnen EFFORT (Roosmarijn, Liese, Haitske en Jaap). Voor het inzamelen van de monsters en coördineren van de monitoring dank ik ook Ben Wit en Petra Dop. De mensen bij Royal GD met wie we hebben samengewerkt ben ik dankbaar: Jeanine Wiegel en Annet Heuvelink.

De mensen van ProefschriftMaken wil ik graag bedanken, in het bijzonder Vera van Beek voor het vormgeven van dit boekje.

Tijdens mijn promotie mocht ik ook studenten begeleiden, Ingrid Cardenas (WUR) en Ursula Bergwerff (UU). Ik vond het leuk om te zien hoe gemotiveerd jullie waren en heb er zelf ook van geleerd jullie als Masterstudenten te begeleiden.

Cindy Dierikx (bij wie ik zelf ooit stage liep) verdient hier ook speciale dank, omdat zij mij mede (samen met Dik natuurlijk) heeft geïnspireerd om dit onderzoek te gaan doen en me altijd is blijven aanmoedigen mijn ambitie na te streven om aan AMR te werken. Dankjewel!

Mijn coach Marjanska en anderen, zoals Sara mijn fysiotherapeut, wil ik bedanken voor hun hulp om (wanneer nodig) fysieke en mentale barrières weg te nemen om gezond tot deze prestatie te komen.

Mijn vader Cees Hesp heeft in de eindfase mijn stukken nagelezen als plaatsvervangend 'native speaker' van de Engelse taal. Hij staat altijd klaar met raad en daad, dankjewel daarvoor lieve Pap! Ook mijn lieve moeder Liane Hesp wil ik bedanken voor de 'mental support'. Het was fijn dat ik altijd bij jullie kon komen eten op de terugweg uit Lelystad. Jullie zijn me ontzettend dierbaar.

Mijn lieve en toegewijde zusje Kylie Hesp wil ik bedanken voor haar letterlijk altijd klaarstaan voor een gesprek. Wat hebben wij veel gebeld afgelopen jaren, het moet wel een recordtijd aan zussen-gesprekken zijn! Natuurlijk ook dank aan mijn andere lieve broertjes en zusje Casper, James en Myriam Hesp voor alle wandelingen, samen tekenen en Sinterklaas surprises. Jullie zijn altijd in mijn gedachten. Ook mijn dierbare nichtje en neefje Tara en Auke wil ik hier noemen. Mijn nichtje en neef Stephanie en Alexander Meulenbelt.

Opa Hesp en wijlen Oma Hesp wil ik bedanken, het is niet in woorden uit te drukken hoe jullie me hebben gesteund en mede grootbrachten. Net zoals mijn lieve tante Désirée, oom André en Opa Piet. Jullie hebben mij gebracht waar ik nu ben. Ook Tante Henny wil ik graag bedanken voor de mooie momenten die we samen hadden, jij bent voor mij ook familie.

Mijn schoonfamilie, de familie De Boer wil ik graag bedanken omdat die in de laatste fase ook heeft meegeleefd. Carly en René, Menno, Hans en Opa en Oma de Boer: dankjewel voor de gezelligheid en etentjes die me fijne afleiding in drukke tijden hebben gegeven.

Sommige mensen zijn familie, zonder bloedband: mijn lieve en gezellige vrienden. Ik hoop dat ik niemand vergeet te noemen maar wil in ieder geval mijn oud-bestuursgenootjes (de pinguïns) bedanken: Jorien, Gert-Jan, Pieter, en Nienke! Daarnaast natuurlijk mijn lieve vriendinnen Marije, Anne, Diana, Judith, Tjarda, Dorien, Sylvia, Floor en Franca. Natuurlijk ook in het bijzonder nog eens de paranimfen: Jorien en Anne. Dat jullie maar veel (nieuw) leven in de brouwerij mogen brengen op de feestelijke promotieplechtigheid.

Tot slot kom ik bij mijn lieve Jelle. Hoe moet ik dit nu in woorden uitdrukken? Je was en bent mijn grootste steun, mijn liefste maatje. Naast je lieve toewijding als partner voor alle mentale ondersteuning heb je je zelfs ook nog ingezet met Excel-skills en om me te helpen de cover te ontwerpen. Je bent mijn held! Naast mijn promotie waren wij afgelopen tijd ook veel aan het bouwen aan onze toekomst. Ik hoop dat nog lang (wat zeg ik, altijd!) met jou samen te doen. Ik ben zo ontzettend dankbaar dat ik jou ken!

En aan iedereen die heeft bijgedragen: ontzettend veel DANK!

About the author

About the author

Graduated as a veterinarian at Utrecht University in 2013, Ayla Hesp worked in veterinary practice as companion animal veterinarian and as locum veterinarian in The Netherlands. Before, she worked as a consultant in Animal Science at the University for Applied Sciences (HAS Hogeschool Den Bosch, NL). Ayla started a PhD at the Faculty of Veterinary Medicine, Utrecht University, as an employee of Wageningen Bioveterinary Research (WBVR), Lelystad, the Netherlands in October 2016. Since then she worked at WBVR in research projects on antimicrobial resistance, bacteriology and epidemiology. Ayla is passionate about improving public health by the 'One Health, One Medicine' principle, her special interest is the interaction between research, policy and practice.

Effective antimicrobials are essential for adequate healthcare, but unfortunately, worldwide antimicrobial resistance (AMR) threatens this effectiveness. The possibilities for development of new antimicrobials are limited. This leaves prudent antimicrobial use and other interventions to limit existing AMR as an important strategy. Therefore, AMR must be monitored in the relevant reservoirs, such as livestock. The interpretation by statistical analysis and evaluation of AMR monitoring is not prescribed by legislation, but is challenging and will be even more complex when the amount of data increases. This PhD thesis provides insight in how to optimize evaluation and interpretation of AMR monitoring in livestock in the indicator organism *Escherichia coli*.