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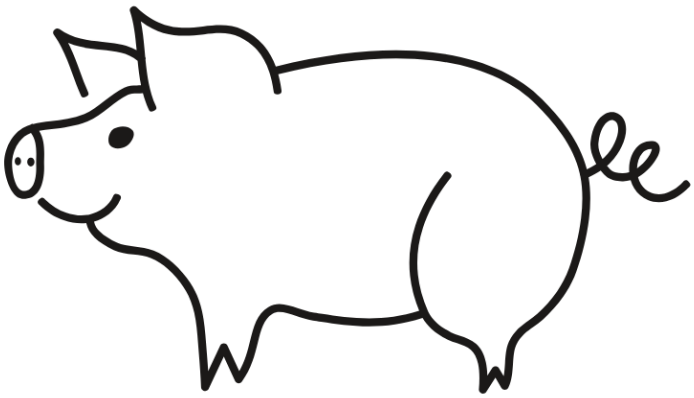
Risk factors associated with the abundance of antimicrobial resistance gene *tet*(W) and class 1 integron (*intI*1) in pigs in the Netherlands

General Research Project



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Plain language summary

In the 21st century, a significant threat to public health is antimicrobial resistance (AMR). This happens when bacteria become resistant to antibiotics, making these drugs less effective in treating infections. AMR leads to more infectious diseases that cannot be easily treated. The use of antibiotics in humans and animals contributes to this problem, making our medicines less effective. Reducing the use of antibiotics in both animals and humans is vital to fight AMR. The amount of antibiotics used in animal farming is growing with the two common types of antibiotics used in pig farming being penicillins and tetracyclines. Tetracyclines are cheap antibiotics that were initially used to treat infections but are now given to animals to help them grow. In the Netherlands, these antibiotics are widely used in pig farming. There's been a big effort to reduce the use of antibiotics in the livestock industry, which has led to a significant decrease in antibiotic use between 2009 and 2021. The link between antimicrobial resistance and antimicrobial use (AMU) has been studied by researchers showing that a decrease in AMU results in lower antimicrobial resistant genes. This research aimed to understand the relationship between antibiotic use, farming practices, and the presence of resistance genes in Dutch pig farms. We specifically looked at two resistance genes: *tet*(W) and class 1 integron (*intI*1). This study was carried out on 36 pig farms in the Netherlands. It took place over two different times, with a year between them. The farms were divided into two main types: "farrowing" farms, which focus on breeding piglets, and "farrow-to-finish" farms, which manage all stages of pig development. Samples were collected from 60 pigs on each farm. These samples were then grouped into sets of six based on the pigs' ages. The study also used questionnaires that were filled out by veterinarians and farmers. These questionnaires collected information about the farms, including their size, the type of pig production, and various farming practices like hygiene and animal management. Data analysis was performed. We looked at factors related to farm characteristics, hygiene, biosecurity, cleaning, planning, feeding and water supply. For each variable, we used a statistical model to check its impact. The study found that the use of tetracycline antibiotics decreased over time, and so did the levels of resistance genes. The results suggested that factors related to how farms are managed play a in the abundance of these resistance genes. For instance, farms that had good cleaning and disinfection practices had lower levels of resistance genes. Using antibiotics in the first week of a piglet's life was linked to higher levels of *tet*(W) genes, while quarantining new animals on farms was associated with more *intI*1 genes. This study gives us a better understanding of how tetracycline resistance and resistance genes are connected to various factors on Dutch pig farms. Lower antibiotic use and resistance genes show that interventions can make a difference. Farm practices, hygiene, and the care of piglets and sows all play important roles in managing antimicrobial resistance. Further research should explore piglet feeding practices and sow management in more detail.

Abstract

Antimicrobial resistance (AMR) poses a substantial threat to public health in the 21st century. This research investigates the relationship between the abundance of antimicrobial resistance gene(*tet*(W) and class 1 integron (*intI*1)) and various risk factors related to tetracycline use, farm characteristics, hygiene, biosecurity, animal care, cleaning, planning, feeding and water supply, on Dutch pig farms. A longitudinal study was conducted on 36 pig farms, with samples collected at two time points, with a 12-month interval between them. On each farm, swab samples were taken from 60 pigs, who were then pooled by 6 pigs within the same age category. At the same time questionnaires were filled by veterinarians and farmers. qPCR was performed to quantify the abundance of *tet*(W) and *intI*1 along with the 16S rRNA gene used for the normalization of ARG copies. Associations between the abundance of antimicrobial resistant genes and different risk factors were assessed using a mixed-effects model. The results highlight the effectiveness of interventions aimed at reducing antimicrobial use in livestock. In the model covering all farms, it was determined that the use of early-life antibiotics, switching the piglets after the third day, and providing water via a nipple were significantly associated with the abundance of *tet*(W). These resistance genes were linked to the annual loss of sows, as well as cleaning and feeding procedures, in farrow-to-finish farms. According to our research, the annual number of piglets per sow and the existence of quarantine procedures were associated to the abundance of class 1 integron genes. While this study provides valuable insights into AMR in pig farming, cautious interpretation is advised due to its data-driven nature.

Introduction

One of the significant dangers to public health in the twenty-first century is antimicrobial resistance (AMR), which happens when bacteria change in response to the use of antibiotics result in antibiotics' reduced efficacy in treating infections (Murray, 2022). The AMR problem is the increased incidence of infectious diseases that impact people on a worldwide scale but cannot be treated with any antimicrobial medication currently on the market (Michael et al., 2014). Antimicrobial usage (AMU) has paved the way for the development of AMR in bacterial populations, leading to an increase in the failure of antimicrobial therapies in humans and animals (Murray, 2022). Antimicrobial-resistant infections result in a minimum of 50,000 annual fatalities in Europe and the United States, along with approximately 700,000 worldwide, with projected estimates suggesting a potential increase to 10 million annual deaths globally attributable to antimicrobial resistance by 2050 (O'Neill, 2014). Between the years of 2000 and 2015, the total amount of antibiotics utilized worldwide increased by 65%, from 21.1 to 34.8 billion defined daily doses (DDDs), while the daily consumption rate of antibiotics increased by 39%, from 11.3 to 15.7 DDDs per 1,000 individuals (Klein et al., 2019). The contact between humans and farm animals is challenging, and there are various potential channels via which resistant bacteria could spread. The complexity is increased by the ability of resistance genes to spread between several commensal bacterial species (Laxminarayan et al., 2013).

Reduced prevalence of antibiotic-resistant microorganisms in animals and humans is associated with treatments that limit the use of antibiotics in livestock (Tang et al., 2017). Antimicrobial use is important to combat antimicrobial resistance because excessive or inappropriate use of antimicrobials can contribute to the development and spread of resistant bacteria (Sanjeet Bagcchi, 2023). Animals are frequently given antibiotics for non-therapeutic objectives, including promoting growth, in addition to therapeutic or prophylactic purposes. The total amount of antimicrobials used in animal food was predicted to be 131,109 tons in 2013, and the amount is expected to increase to 200,235 tons by 2030(Van Boeckel et al., 2017).

Two types of antibiotics that are most often used in pig farming worldwide are penicillins and tetracyclines (Lekagul et al., 2019). Tetracyclines are extremely affordable antibiotics that were first used to treat and prevent infections in both humans and animals in the 1940s. They are also utilized as growth promoters in animal feed at subtherapeutic doses (Chopra & Roberts, 2001). In the Netherlands, in the pig farming sector of the year 2017, first-choice antibiotics, including amphenicols, macrolides/lincosamides, penicillins, pluromutilins, and tetracyclines, collectively accounted for a total usage of 57,716 kilograms, with tetracyclines contributing 30,598 kilograms to this figure (SDA, 2018). In 2011, The Netherlands Veterinary Medicines Authority (SDa) settled on benchmark criteria for veterinary antimicrobial usage on specific livestock farms with the goal of returning antibiotic sales to 1999 levels when growth promoters were banned (Bos et al., 2013, SDa, 2011). Between 2009 and 2021, there was a significant reduction of 70.8% in the overall amount of antibiotics sold in the livestock sector, reflecting a substantial decline in antibiotic usage in this industry. In 2021 the number of kilograms of antibiotics sold was exceeded by 14% by the number of kilograms used (SDa, 2022).

The interaction between AMR in pig farming and a variety of factors has been studied in academic research, which consistently reveals a link between AMU and AMR and emphasizes that ceasing AMU results in decreased antimicrobial resistance (Andersen et al., 2023). Research has also demonstrated that modifying husbandry practices (Soundararajan et al., 2022), considering farm type and size (Lekagul et al., 2019), and accounting for the presence of resistance genes in sows as well as the AMU in sows and piglets (Callens et al., 2015) are associated with the levels of AMR observed in pigs.

The aim of this research was to assess the association between antimicrobial usage, as well as farm management practices, and the abundance of antimicrobial resistance genes (i.e., *tet*(W)) and class 1 integron (*intI*1) on Dutch conventional swine farms.

Materials and methods

Study design

The design of the study has been elaborated on in a different paper (Dohmen et al., 2015). This longitudinal study focused on 36 Dutch pig farms and was conducted over two distinct time points, with a 12-month interval between them. The participating farms were divided into two main categories: "farrowing" farms, which focus on breeding and providing piglets to finishing farms, and "farrow-to-finish" farms, which oversee all phases of pig development. Between March 2011 and September 2011, researchers conducted preliminary site visits to farms and veterinarians. Swab samples were collected from 60 individual pigs on each farm and subsequently grouped into 10 pools of 6 animals. Each pool consisted of an age group in the same pen. These pools have been tailored to include every age category present on the farm, namely suckling piglets, weaning piglets, gilts, sows, and finishing pigs. In the case of farrowing farms that lacked finishing pigs, samples were taken from weaning piglets instead.

Structured questionnaires were submitted by veterinarians and farmers affiliated with each participating farm. The questionnaire was designed to collect data on farm characteristics, encompassing factors such as farm size, production type, and a range of farm practices, including biosecurity measures such as quarantine and pest control, hygiene protocols including the type of cleaning agents applied, and the use of gloves, as well as animal management aspects such as the presence of other animals on the farm, vaccination practices, contact structure, feeding methods, and water supply management. The farm questionnaire was filled out again at the second sampling time to document any shifts or improvements over time.

Laboratory analysis

The laboratory analysis steps were described elsewhere (Dohmen et al., 2015). Briefly, the farm veterinarian used sterile cotton-wool swabs (Cultiplast1) to obtain rectal samples, which were then delivered refrigerated to the laboratory through courier. DNA was isolated using UltraClean1 Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) or DNeasy 96 Blood & Tissue Kit (Qiagen). qPCR was performed to quantify the abundance of *tet*(W) and *intI*1 along with the 16S rRNA gene used for the normalization of ARG copies.

Data on antimicrobial use

In the Netherlands, national databases compile information on all antimicrobial medications shipped to individual farms. The owners of the research farms submitted their written approval for the 2-year period of data retrieval on antimicrobial use (Dorado-García et al., 2015). The sector quality system's national databases were used to retrieve all antibiotic prescriptions written for each farm. Tetracycline use was expressed as defined daily dosages per animal per year (DDDA/Y) per farm. A DDDA/Y of 1 indicates that the average animal in the population received one day's worth of antimicrobial exposure annually (Bos et al., 2013).

Data analysis

Statistical analyses were performed using R version 4.2.2. The initial dataset consisted of 497 variables and 412 observations. Before conducting any analyses, a comprehensive data cleaning process was performed to ensure the integrity and quality of the dataset.

Additionally, the high number of NAs was handled by systematically eliminating variables containing more than 10% missing values. We performed variable selection in our dataset using a threshold-based approach to ensure that all categorical variables retained in the analysis met a minimum level variation criterion of 10%. Specifically, for each categorical variable, we examined the distribution of its levels and assessed whether any of them had a proportion below the predefined threshold. If any category within a variable fell below the 10% proportion threshold, the entire variable was removed from the dataset to maintain consistency and prevent potential issues arising from imbalanced variables.

In a further effort to reduce the number of variables, univariable analysis was performed on blocks of variables selected by categories such as farm description, hygiene, divisions, vaccine materials, animal care, planning, and water supply, among others. This process involved categorical and continuous independent variables. For each of these variables, a linear mixed-effects model was used. Depending on the point of interest, either the number of gene copies of *tet*(W) or *intI*1 was considered as the outcome variable. To account for variations between farms, a unique farm identification number was assigned as a random effect with a random intercept. Each regression model incorporated five covariates: the total number of sows as an indicator of farm size, the use of tetracyclines (DDDA/Y) as a continuous variable, time to account for the two different time points, age representing five distinct age categories of pigs, and farm type categorized into four levels: closed farrow to finish, closed farrow, open farrow to finish, and open farrow(a closed farm is described as a farm that doesn't get external supply of gilts and an open farm is one that receives external supply of gilts at least once a year from at least one supplier). Coefficients (including estimates, standard errors, degrees of freedom, p-values, and confidence intervals) are calculated for the independent variables. The selection was based on a data-driven methodology, with the inclusion criteria of a p-value equal or lower than 0.1.

The variance inflation factor (VIF) was calculated for the model to identify any multicollinearity issues. These variables measure how inflated the estimated regression coefficient variances are in comparison to instances where the predictor variables are not linearly connected (Copas et al., 1987). Variables

were removed from the selected variables list based on a threshold of 5 for the VIF score. These steps were systematically performed for every model based on each block of variables.

After we conducted univariable analyses to identify potential predictor variables, we included them in our final linear mixed-effects regression model. We computed the VIF scores and we iteratively removed variables with VIF scores exceeding 5, ensuring that all retained factors had values below this threshold. Subsequently, we employed the "step" function from the "lmerTest" package in R to perform an automatic backward analysis. This procedure systematically eliminated predictor variables that did not significantly contribute to the model's explanatory power. Backward elimination begins by first including all predictors in the model and then checking to see if the AIC decreases as each variable is eliminated. When a variable has been removed, the impact of the remaining predictors is reevaluated, and the procedure is repeated until the AIC increases as a result of the elimination of all variables (Field et al., 2012). This concluded with a final mixed-effects model (Figure 1). The same analysis was performed on a separate model including only farrow-to-finish farms. Maximum likelihood (ML) estimation was used.

Given the multiple testing procedures carried out in the preceding steps, it was imperative to employ familywise error rate control measures. Pairwise comparisons are employed to manage the familywise error rate by adjusting the significance level for each individual test in a manner that ensures the overall probability of committing a Type I error (denoted as α) across all comparisons remains at 0.05. There exist various methods for controlling the familywise error rate, but the most widely used and straightforward approach, known as the Bonferroni correction, involves dividing α by the number of comparisons, denoted as "k." This division guarantees that the cumulative risk of Type I errors does not exceed 0.05 (Field et al., 2012).

Bonferroni-corrected p-value = $\frac{\alpha}{k}$

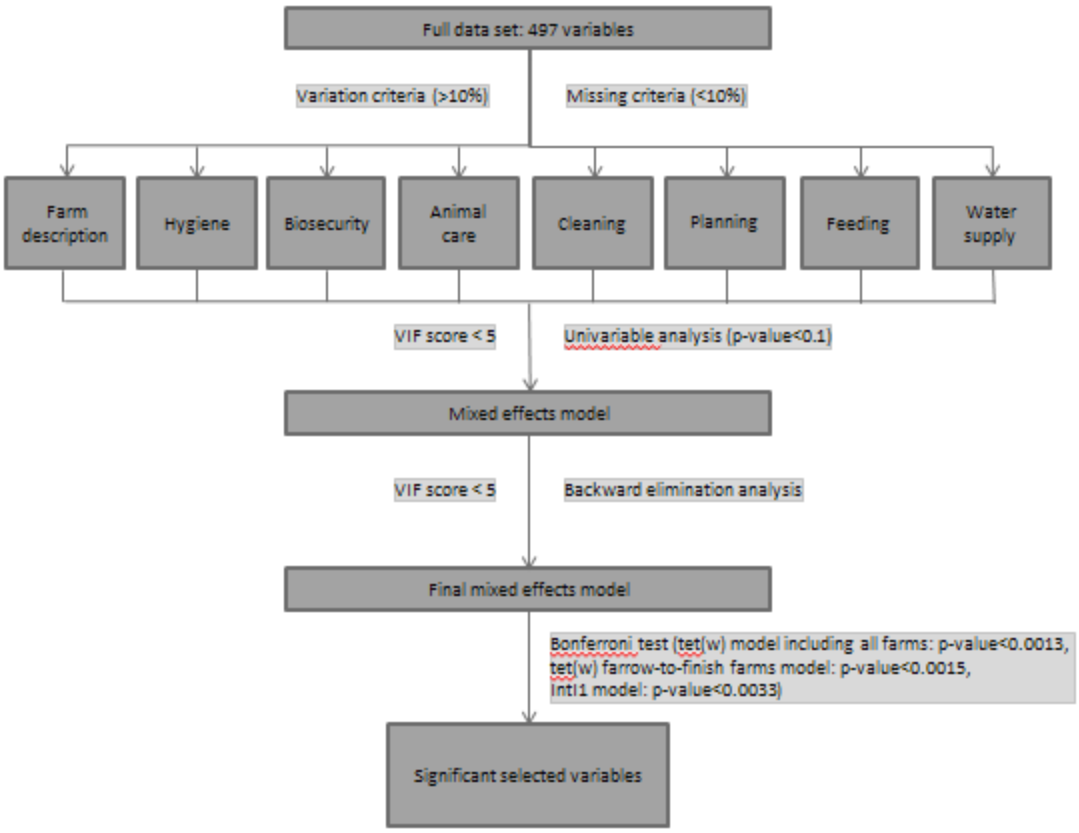

Figure 1 Visual scheme of the model building process

Results

Descriptive results

Table 1 outlines the characteristics of the 36 farms in this study. The overall use of tetracycline demonstrated a significant decrease over a 12-month span, although this reduction was not uniform across all farm types (Figure 2). During the initial sampling period, the mean relative abundance of *tet*(W) was 0.57 across all farms, with a slightly higher value of 0.58 observed for farrow-to-finish farms (Figure 3). At the second time sampling moment, there was a substantial reduction in these values. The mean relative abundance of *tet*(W) decreased to 0.14 for all farms and 0.16 for farrow-to-finish farms. In the case of *intI*1 (Figure 4), the initial mean relative abundance was -2.34. However, at the second time sampling moment, the reduction in *intI*1 abundance was even more pronounced, dropping to -5.22. An analysis of Table 2 reveals a noteworthy decline in the prevalence of both *tet*(W) and *intI*1 over the course of the two distinct time points across all age categories.

Table 1 Farm characteristics

Farm characteristics	n = 36 farms
Farm type	
Open	14
Closed	22
Production type	
Farrow-to-finish	24
Farrowing	12
	Median (min - max)
Number of sows	365 (110-1018)

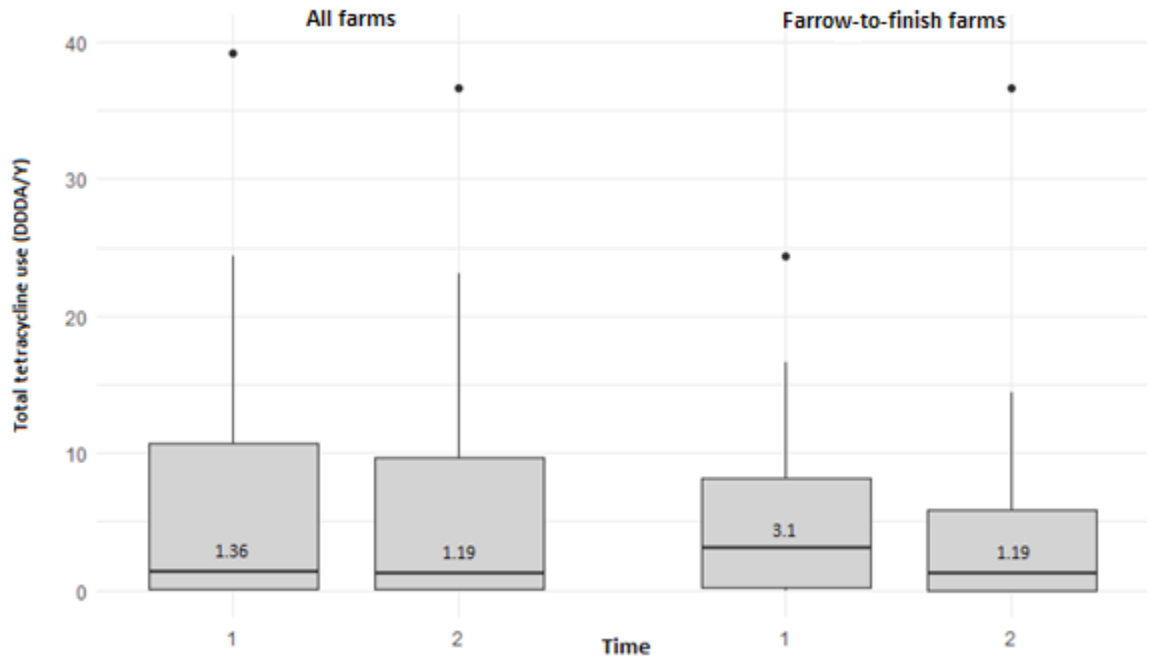

Figure 2 Total use of tetracyclines (DDDA/Y) at the 2 sampling points for all farms and farrow-to-finish farms

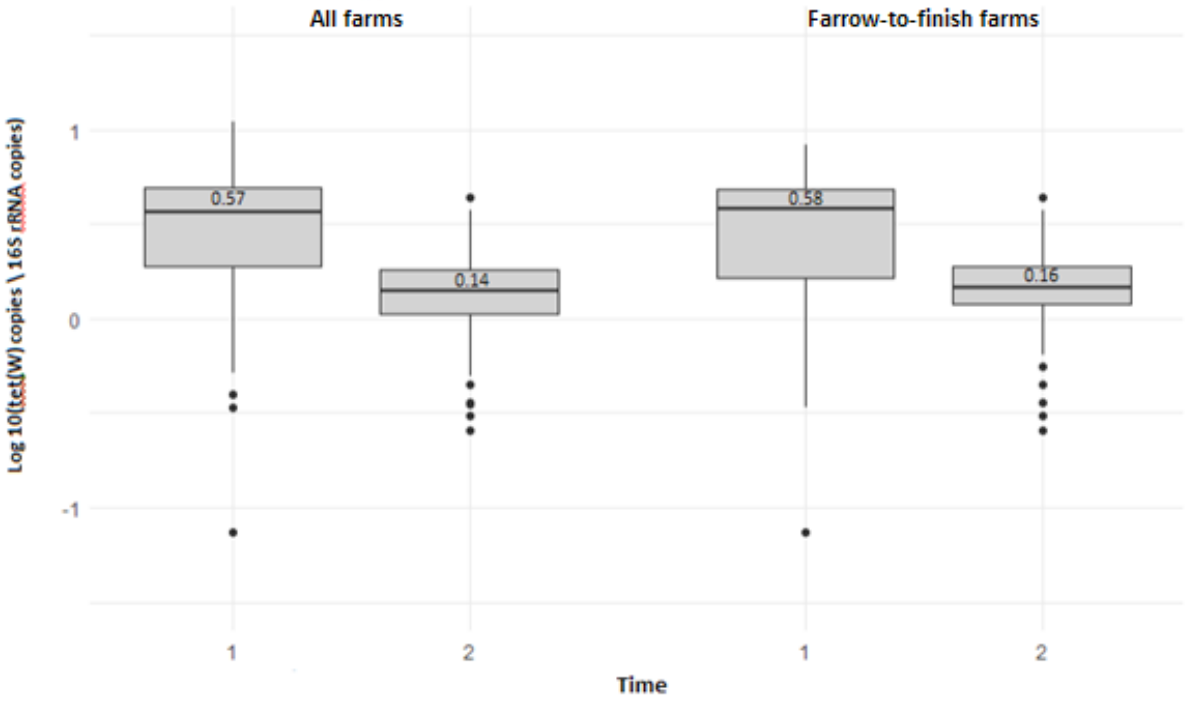

Figure 3 Relative abundance of tet(W) at the 2 sampling points for all farms and farrow-to-finish farms

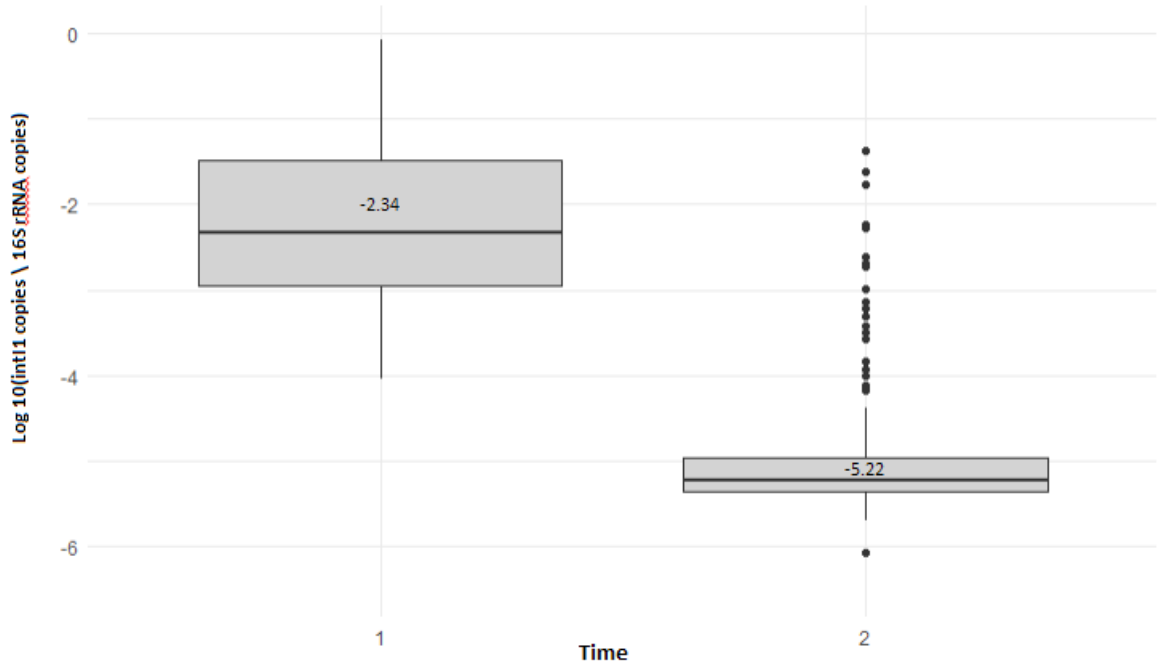

Figure 4 Relative abundance of intl1 at the 2 sampling points

Table 2 Relative abundance of tet(W) and intl1 at the 2 sampling points for all age categories

	All farms tet(W)			Farrow-to-finish farms tet(W)			All farms intI1		
Age category	Pool samples (n = 369)	No. of tet(W) gene copies - median no. (interquartile range) for t0	No. of tet(W) gene copies - median no. (interquartile range) for t12	Pool samples (n = 355)	No. of intl1 copies - median no. (interquartile range) for t0	No. of intl1 copies - median no. (interquartile range) for t12	Pool samples (n = 240)	No. of tet(W) gene copies - median no. (interquartile range) for t0	No. of tet(W) gene copies - median no. (interquartile range) for t12
Suckling piglets (Kraam)	106	0.53 (0.27-0.66)	0.13(0.06-0.26)	103	-2.02(-2.55-(-1.10))	-5.01(-5.23-(-4.83))	70	0.58(0.27-0.69)	0.13(-0.05-0.26)
Weaned piglets (Gespeend)	104	0.63(0.28-0.73)	0.10(0.01-0.23)	95	-2.86(-3.45-(-1.95))	-5.27(-5.44-(-5.04))	59	0.58(0.15-0.67)	0.13(0.03-0.23)
Rearing gilts (Gelten)	60	0.67(0.30-0.77)	0.14(0.08-0.26)	60	-2.56(-2.84-(-2.00))	-5.32(-5.41-(-5.19))	39	0.66(0.30-0.74)	0.16(0.10-0.29)
Fatteners (Vlees)	25	0.32(0.16-0.48)	0.22(0.17-0.32)	24	-2.23(-2.23-(-2.23))	-5.12(-5.35-(-4.66))	25	0.32(0.16-0.48)	0.22(0.17-0.32)
Sows (Zeugen)	74	0.54(0.28-0.69)	0.10(-0.04-0.22)	73	-2.25(-2.65-(-1.46))	-5.22(-5.28-(-5.03))	47	0.54(0.16-0.68)	0.14(0.018-0.21)

Analysis of the *tet*(W) model including all farms

In the context of univariable analysis, 38 variables related to farm characteristics, hygiene, biosecurity, animal care, cleaning, planning, feeding and water supply were identified with p-values falling below 0.1. The details of these variables are outlined in Table 6. After computing the VIF score, 27 variables were included in the backward elimination analysis. The multivariable analysis has shown the fixed factor accounting for the 2 sampling times to be significant with p-values less than 0.001 (Table 3). When considering age categories, fatteners exhibited a notably higher *tet*(W) gene abundance (Estimate = 0.15, p = 0.004) compared to sows, while other age categories did not show significant differences. Farm type, farm size and tetracycline usage were explored as well, but did not reach statistical significance. Having the only entrance through a hygiene lock (p = 0.004), cleaning the piglet passage with soaking agent (p=0.006), cleaning the gilt passage with disinfection agent (p=0.03), allowing a 24 hours dry period after cleaning the mating passage (p=0.03), feeding the piglets with sows with milk (p=0.009), were additional independent variables that were included in the multivariable analysis, although they did not attain statistical significance. After performing the Bonferroni correction (p<0.0013) the establishments that mainly supply water via a nipple in the farrowing section (p<0.001), and in which piglets can still be switched after the third day (p=0.0011) presented significantly lower *tet*(W) gene abundance. Using antibiotics during the first week of life (p<0.001) was associated with higher gene abundance.

Table 3 Multivariable analysis for the tet(W) model including all farms

Variable	Category	N	Estimate	95%CI	P-value
Age	Sows	74	Ref	Ref	-
	Suckling piglets	106	0.003	-0.06-0.07	0.917
	Weaned piglets	104	0.05	-0.02-0.11	0.182
	Gilts	60	0.07	-0.008-0.14	0.081
	Fatteners	25	0.15	0.05-0.26	0.004
Sampling time	0	180	Ref	Ref	-
	12 months	192	-0.36	-0.42-(-0.31)	<0.001
Farm type	Open farrow-to-finish	130	Ref	Ref	-
	Closed farrow-to-finish	113	0.03	-0.09-0.15	0.63
	Open farrowing	100	0.04	-0.07-0.17	0.47
	Closed farrowing	29	-0.01	-0.17-0.17	0.91
Farm size(number of sows)		372	0.0002	-0.00003-0.0004	0.09
Tetracycline use		372	0.002	-0.002-0.006	0.30
The only entrance to the farm is the hygiene lock*	No	163	Ref	Ref	-
	Yes	209	-0.12	-0.20-(-0.04)	0.004
All piglets are given an injection of antibiotics in their first week of life	No	133	Ref	Ref	-
	Yes	229	0.16	0.08-0.25	<0.001
After the third day, piglets can still be switched	No	228	Ref	Ref	-
	Yes	144	-0.14	-0.22-(-0.06)	0.0011
Piglet passage is cleaned with soaking agent*	No	313	Ref	Ref	-
	Yes	59	-0.16	-0.28-(-0.05)	0.006
Gilt passage is cleaned with disinfection agent*	No	313	Ref	Ref	-
	Yes	59	0.12	0.01-0.24	0.03
After cleaning the mating passage there is a dry period of at least 24 hours*	No	332	Ref	Ref	-
	Yes	40	0.13	0.02-0.25	0.03
Piglets with sow are fed with milk*	No	205	Ref	Ref	-
	Yes	167	0.09	0.02-0.16	0.009
In the farrowing section drinking water is mainly supplied via a nipple	No	44	Ref	Ref	-
	Yes	328	-0.25	-0.4-(-0.13)	<0.001

* Not significant variable because it didn't meet the Bonferroni correction criteria

Analysis of the *tet*(W) model including only farrow-to-finish farms

From the univariable analysis 33 variables related to farm characteristics, hygiene, biosecurity, animal care, cleaning, planning, feeding and water supply were selected based on a p-value threshold of 0.1. Table 7 highlights the characteristics of these variables. After checking the VIF score, 16 variables were included in the backward elimination analysis. Similarly to the past model, the variable accounting for the 2 sampling time points was found significant ($p<0.001$) through the multivariable analysis (Table 4). Among age categories, gilts (Estimate = 0.11, $p = 0.03$) and fatteners (Estimate = 0.14, $p = 0.02$) showed a substantial increase in *tet*(W) gene abundance compared to sows, while other age categories did not show significant differences. Farm size and tetracycline use were explored as covariates as well. However, neither of these variables showed significant associations with *tet*(W) gene abundance within

farrow-to-finish farms, as evidenced by p-values of 0.50 and 0.24, respectively. Other variables included in the model but without any statistical significance were the mean growth per piglet per day (p=0.007) and cleaning the fattener passage with a soaking agent (p=0.04). Following the application of the Bonferroni correction feeding piglets with sow milk (p=0.001) was associated with increased gene abundance. On the other hand, the percentage of annual sow loss (p<0.001) and cleaning the fattener passage with a disinfection agent (p<0.001) were negatively associated with the abundance of *tet*(W) genes.

Table 4 Multivariable analysis for the tet(W) model including farrow-to-finish farms

Variable	Category	N	Estimate	95%CI	P-value
Age	Sows	47	Ref	Ref	-
	Suckling piglets	70	0.03	-0.06-0.12	0.55
	Weaned piglets	59	0.05	-0.04-0.15	0.28
	Gilts	39	0.11	0.008-0.21	0.03
	Fatteners	25	0.14	0.02-0.26	0.02
Sampling time	0	120	Ref	Ref	-
	12 months	123	-0.28	-0.34-(-0.20)	<0.001
Farm size(number of sows)		243	0.00006	-0.0002-0.0002	0.50
Tetracycline use		243	0.003	-0.002-0.008	0.24
Mean growth per piglet per day*		243	0.0008	0.0002-0.0014	0.007
Percentage of loss of sows per year		223	-0.003	-0.004-(-0.001)	<0.001
Piglets with sow are fed with milk	No	130	Ref	Ref	-
	Yes	113	0.12	0.05-0.20	0.0011
Fattener passage is cleaned with disinfection agent	No	194	Ref	Ref	-
	Yes	49	-0.20	-0.31-(-0.09)	<0.001
Fattener passage is cleaned with soaking agent*	No	194	Ref	Ref	-
	Yes	49	-0.11	-0.22-0.001	0.04

* Not significant variable because it didn't meet the Bonferroni correction criteria

Analysis of the *intI*1 model

15 variables related to farm characteristics, biosecurity, animal care, cleaning, feeding and water supply were selected through the univariable analysis based on a p-value lower than 0.1 (Table 8). 14 variables were then included in the backward elimination analysis due to selection based on a VIF score threshold of 5. The multivariable analysis indicates the significance of the time variable (p<0.001) (Table 5). In contrast to the previous models that had identified fatteners as substantial contributors, the analysis concerning *intI*1 genes showed that suckling piglets (p = 0.07) displayed an increase in gene abundance relative to sows. The type of farm also emerged as a notable determinant. Closed farrow-to-finish farms (estimate: 0.23, p = 0.09) and closed farrowing farms (estimate: 0.3, p = 0.16) were both associated with increased *intI*1 gene abundance, relative to open farrow-to-finish farms, serving as the reference category. The farm size did not show a significant association with *intI*1 gene abundance (p = 0.74). Feeding piglets with sows with mush/pulp (p=0.03) was a variable included in the multivariable analysis but didn't have any statistical significance. After implementing the Bonferroni correction method, the quarantine of newly delivered animals (p=0.0027) has been shown to play a significant role being associated with higher levels of class 1 integron. The mean number of weaned piglets per sow per year (p<0.001) was linked to lower abundance of *intI*1 genes.

Table 5 Multivariable analysis for the intl1 model

Variable	Category	N	Estimate	95%CI	P-value
Age	Sows	73	Ref	Ref	-
	Suckling piglets	103	0.23	-0.02-0.47	0.07
	Weaned piglets	95	-0.21	-0.46-0.04	0.10
	Gilts	60	-0.15	-0.43-0.13	0.30
	Fatteners	24	0.07	-0.32-0.46	0.72
Sampling time	0	167	Ref	Ref	-
	12 months	191	-2.7	-2.9-(-2.55)	<0.001
Farm type	Open farrow-to-finish	125	Ref	Ref	-
	Closed farrow-to-finish	108	0.23	-0.02-0.48	0.09
	Open farrowing	97	-0.04	-0.34-0.25	0.78
	Closed farrowing	28	0.3	-0.09-0.65	0.16
Farm size(number of sows)		358	-0.00008	-0.0005-0.0004	0.74
Mean number of weaned piglets per sow per year		358	-0.05	-0.07-(-0.03)	<0.001
Newly delivered animals are placed in quarantine	No	226	Ref	Ref	-
	Yes	132	0.46	0.18-0.73	0.0027
Piglets with sow are fed with mush/pulp*	No	198	Ref	Ref	-
	Yes	160	-0.25	-0.45-(-0.04)	0.03

* Not significant variable because it didn't meet the Bonferroni correction criteria

Discussion

The longitudinal study, conducted on 36 Dutch pig farms, aimed to investigate the associations between the abundance of *tet*(W) and *intl*1 genes, and tetracycline use and different farm characteristics and practices over two time points separated by 12 months using a linear mixed-effects regression model. The results suggested that the decrease in ARGs was driven by risk factors related to the litter size per sow, cleaning, feeding and other farm management practices.

Over the course of the study, a notable reduction in both tetracycline usage and the abundance of *tet*(W) and *intl*1 genes was observed. It is well documented that antibiotic exposure is not the sole key variable influencing the abundance of AMR genes (Birkegård et al., 2017, Vieira et al., 2009). Other studies have shown that there is a negative association between antimicrobial resistance and protocols related to cleaning and disinfection (Mencía-Ares et al., 2021, Burow & Käsbohrer, 2017, Davies & Wales, 2019). Similarly, our study described the cleaning of the fatteners passage with disinfectant as a protective measure against the abundance of *tet*(W) genes in farrow-to-finish farms. The results also emphasize the significance of biosecurity and hygiene practices in AMR dynamics.

The practice of using antibiotics during the piglets first week of life was associated with higher *tet*(W) gene abundance for the model including all farms, indicating a potential link between early-life exposure to antimicrobials and the development of resistance. As reported by Callens et al. in 2015, piglet antimicrobial resistance levels are significantly influenced by the use of antimicrobials in piglets, as well

as by sow resistance levels. Another study suggested that early life antimicrobial intervention in piglets can lead to changes in the abundance of antimicrobial resistance genes (ARGs) in the fecal microbiota (Zeineldin et al., 2019).

There was a positive association observed between the quarantine of newly delivered animals and increased levels of class 1 integrons. It is important to note that the positive association between quarantine practices and higher *intI*1 gene abundance may appear counterintuitive at first glance. However, this observation can be attributed to several underlying factors within the context of our study. Farms implementing quarantine measures often have external suppliers providing them with animals, including newly delivered stock. These external suppliers introduce a new cohort of animals to the farm, potentially carrying their own antimicrobial resistance genes (Yang et al., 2020).

In accordance with the findings of this study, the distribution of drinking water via nipple systems in the farrowing section appeared as a significant factor. The results of the multivariable model including all farms showed that there was a strong negative association between this water route of administration and the abundance of *tet*(W) genes, suggesting a potential effective approach for reducing antibiotic resistance. Nipple-based water supply systems are known for their precision and hygiene, allowing them to prevent contamination and improve water quality (Rauch et al., 2016).

The age category of pigs appeared to be a significant covariate of AMR gene abundance. Fatteners had the lowest *tet*(W) gene abundance among all the age categories at the beginning of the study and the highest abundance at the second sampling time, while suckling piglets displayed an increase in *intI*1 gene abundance. This dynamic between age categories and AMR gene abundance highlights the importance of considering age as a covariate in the assessment of AMR in swine farming practices. The significance of age has been demonstrated in other studies (Dohmen et al., 2017, Yang et al., 2022).

Additionally, feeding practices, specifically the provision of milk to piglets with sow, displayed an association with an elevated abundance of the *tet*(W) gene in farrow-to-finish farms. Previous studies have indicated a similar result, where feeding calves pasteurized or unpasteurized waste milk has led to an increased detection of antimicrobial resistant genes in their fecal samples (Ricci et al., 2017, Maynou et al., 2017). The lack of specific information regarding the type of milk provided to the piglets in our study prevents us from giving a definitive explanation.

The finding that piglets can still be switched between groups after the third day had a significant association with lower *tet*(W) gene abundance regarding the model including all farms. This practice may reflect a strategy to mitigate the spread of antimicrobial resistance genes. While the effect size for the annual loss of sows appeared modest (estimate=-0.003), our study results still proved it significant, suggesting that maintaining a healthier and more stable sow population could contribute to reducing the tetracycline resistant genes in farrow-to-finish farms. On the same note, our study indicates that an increase in weaned piglets per sow per year is associated with a decrease in class 1 integron carriage. This result could suggest that optimizing piglet production may contribute to reducing the abundance of *intI*1 genes. Further research is needed due to the presence of certain findings that raise doubts and could potentially be attributed to incidental outcomes.

It is essential to acknowledge certain limitations of this study. During the data analysis process, a singularity issue was encountered, which prevented the fitting of a model for *intI*1 within farrow-to-finish farms. Additionally, it's important to recognize that this research is inherently data-driven, and while it provides valuable insights into the associations between various factors and antimicrobial resistance, the results should be interpreted cautiously because they could potentially be attributed to incidental outcomes. Another significant limitation of this study is the small sample size relative to the number of variables investigated, potentially impacting the study's statistical power.

Conclusion

In conclusion, our in-depth investigation into Dutch pig farms provides more understanding of of the risk factors associated with the abundance of antimicrobial resistance gene tet(W) and class 1 integron (intl1). The decrease of tetracycline usage and resistance genes indicate the potential impact of interventions, while the dependent variables related to farm characteristics, biosecurity, and management of pigs highlight the complexity of AMR dynamics. Early-life antibiotic usage, switching the piglets after the third day and supplying water via a nipple were identified as influential factors for the abundance of *tet*(W) in the model including all farms. In farrow-to-finish farms these resistant genes were associated with the annual loss of sows and cleaning and feeding practices. Our study suggested that the abundance of class 1 integron genes was correlated to the annual number of piglets per sow and the presence of quarantine protocols. Further studies should focus on feeding practices of piglets and management of sows.

Supplementary data

Tables 5 to 8 are available in a separate document.

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