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# Continuing occurrence of vancomycin resistance determinants in Danish pig farms 20 years after removing exposure to avoparcin

Authors

Anna Camilla Birkegård[1], Kaare Græsbøll[1], Julie Clasen[3], Tariq Halasa[2], Nils Toft[2], Anders Folkesson[3*]

*Corresponding author: Anders Folkesson, Department of Biotechnology and Biomedicine, Technical University of Denmark, Kemitorvet Building 204, 2800 Kgs. Lyngby, Denmark, afol@dtu.dk +4520120657

[1]Technical University of Denmark, Department of Applied Mathematics and Computer Science, Asmussens Allé Building 303B, 2800 Kgs. Lyngby, Denmark

[2]Technical University of Denmark, National Veterinary Institute, Kemitorvet Building 204, 2800 Kgs. Lyngby, Denmark

[3], Technical University of Denmark, Department of Biotechnology and Biomedicine,, Kemitorvet Building 204, 2800 Kgs. Lyngby, Denmark

## Highlights

- Vancomycin resistance determinants were present in all screened Danish pig farms (665 finisher farms and 78 sow farms)
- Vancomycin resistance determinants belonging to *vanG* cluster were found in lower levels in sow farms that in finisher farms
- Vancomycin resistance determinants belonging to *vanB* cluster were found in lower levels in finisher farms that in sow farms
- The present of vancomycin resistance determinants in pig faeces constitute a reservoir for transfer of vancomycin resistance to human

## Abstract

Vancomycin-resistant *Enterococcus* spp. is a major health problem worldwide and livestock have been implicated in constituting a reservoir for the transmission of vancomycin resistance to zoonotic pathogens. Vancomycin resistance determinants can be situated on mobile genetic elements and transferred between bacterial species The livestock reservoir must therefore be included in a risk assessment of the vancomycin resistance burden. Avoparcin, a vancomycin analogue, has not been used in Danish pig production for over 20 years and vancomycin has never been used. The objective of this study was to screen faecal samples from Danish pig farms for nine selected vancomycin resistance determinants. We found at least four different vancomycin resistance determinants in all screened Danish pig farms (665 finisher farms and 78 sow farms). The vancomycin resistance determinants present in *vanB* or *vanG* clusters were found at significantly different levels in sow and finisher farms. However, *vanA* was not detected in any of the farms. In conclusion, vancomycin resistance determinants are still present in Danish pig production 25 years after the ban on avoparcin use.

## Keywords

Avoparcin; glycopeptides; swine; antimicrobial resistance

## Introduction

Avoparcin (a vancomycin analogue) has not been used in Danish pig production since 1995 when it was banned from use in pig farms in an attempt to end the use of growth promoters (DANMAP, 2016). This was followed by a total ban on the use of avoparcin as a growth promoter in the EU in 1997, and a ban on the use of all antibiotics as growth promoters in livestock within the EU in 2006 (European Union, 2006).

Vancomycin-resistant *Enterococci* spp. (VRE) is a major nosocomial health problem (Puchter et al., 2018). Vancomycin resistance determinants (VRDs) can be carried on mobile genetic elements (MGEs)(Ahmed and Baptiste, 2017), and animal reservoirs might therefore be relevant in the risk assessment of the vancomycin resistance burden (Freitas et al., 2009; Gouliouris et al., 2018; Hammerum, 2012; Olsen et al., 2012). Following the ban on vancomycin analogues being used as growth promoters, the percentage of *Enterococcus faecium* isolates resistant to vancomycin in Danish livestock animals decreased from 20% in 1995 to 6% in 2000 (Aarestrup et al., 2001). Furthermore, VRE has not been observed in Danish pigs since 2010 (DANMAP, 2011), but it was recently observed in Danish poultry (Leinweber et al., 2018).

VRDs confer resistance towards vancomycin by different mechanisms (Table 1). Some genes activate other VRDs, others hinder the binding of vancomycin to the bacterial targets, while other VRDs has unknown resistance mechanisms. The determinants included in this study are all been found to be present on mobile elements and thus may be transferred to pathogenic bacteria that pose a danger to human health.The objective of this study was to screen faecal samples from Danish pig farms using total community DNA for selected VRDs and to quantify the levels of these using qPCR.

## Material and methods

### Collection of samples

The faecal samples that were collected to represent finisher farms have previously been described (Birkegård et al., 2017). Briefly, samples were collected at five abattoirs in Denmark. Samples from five pigs from each farm were collected at the slaughter line after the removal of the gut from the carcass, by squeezing faecal material into an empty sampling vial. Samples from 687 finisher farms were collected.

The collection of samples from sow farms was described in Birkegård et al. (2018). Briefly, samples from 17 sow farms were collected at the farm (from sows at different production cycles, at the choice of the farmer) and samples from 65 sow farms were collected at an abattoir that only slaughtered sows. For both sample sites, samples from five pigs from each farm were collected by digital manipulation of the rectum.

In the sample were included sow farms that delivered pigs to the finisher farms (52 pairs) (Birkegård et al. 2018).

## Quantification of vancomycin resistance

The samples from sow and finisher farms were handled in the same way. The five samples were pooled into one aliquot per farm, and DNA was extracted as previously described (Clasen et al., 2016). The levels of nine VRDs were then quantified using qPCR primers sets previously reported by Johnson et.al (2016) (Table 1). For qPCR amplification, samples (40ng/µl) were investigated using a Fluidigm HD Biomark System and Gene expression 48 x 48' arrays according to the manufacturer's instructions. The fluorescent dye EvaGreen was added to the reaction mixture to enable real time quantification of amplification. The following amplification protocol was used: 10 min at 95°C, followed by 35 cycles of 15 s at 95°C and 60 s at 60°C for extension and annealing, where the fluorescence was measured after each cycle. A melting curve analysis was performed at the end of the qPCR in order to detect unspecific amplicons. Primers were synthesized at DNA Technology A/S (Aarhus Denmark). VRD levels were measured as Relative quantification (RQ) values that were determined for each of the samples as follows:

$RQ = C_{\text{Refence gene}} - C_{\text{qGene of interest}}$ (Equation 1)

Where 16S was used as a reference gene presumed to be representative of 100% of the microbiota in the faecal samples at all times. Therefore, RQ values were calculated from all samples normalized against their respective 16S rDNA.

### Difference between age groups

The difference between the number of VRD, prevalence of the different VRD, and the VRD levels in sow and finisher farm populations was tested using a t-test, Fisher's exact test and Wilcoxon rank sum test respectively.

### Software

Data management, statistical analyses and graphical presentation was done in R version 3.4.0 (R Core Team, 2017) using R studio (RStudio team, 2016).

## Results

### Exclusion of farms

Samples from 26 farms were excluded because the quality of the run on the qPCR was not good enough to quantify eight out of nine VRDs.

The final study population consisted of 665 finisher farms and 78 sow farms.

### Vancomycin resistance

Eight VRDs were found in 284 (43%) of the finisher farms and 31 (40%) sow farms (Fig. 1). A t-test showed that there was no significant different in the number of genes between sow and finisher farms.

The prevalence of the VRD varied. It was highest for *vanTG* in finishers and *vanXB* in sows; and the lowest for *vanWB* in finishers and *vanTG* in sows (Table 2). There was a significant difference in the prevalence between sow and finisher farms for *vanTG*, *vanWG*, and *vanRB*.

There was a significantly lower level of the following vancomycin resistance genes in sows than in finishers: *vanTG* and *vanWG* (p-value < 0.0001). In contrast, there was a significantly higher level of the following vancomycin resistance genes in sows than in finishers: *vanHB* (p-value = 0.0009),,

*vanRB* (p-value < 0.0001), *vanSB* (p-value = 0.0007), *vanWB* (p-value = 0.005), , *vanXB* (p-value < 0.0001) and *vanYB* (p-value = 0.004; Fig. 2).

## Discussion

Vancomycin analogues have not been used in the Danish pig industry for 20 years at the time of this study (DANMAP, 2016). However, we still found determinants conferring vancomycin resistance in all study farms. The prevalence of the determinants varied (Table 2), and different levels were found in sow and finisher farms (Fig. 2). The factors driving the prevalence of VRDs in the absence of glycopeptide selections are unknown. The VRDs may have an unknown ecological function besides conferring resistance that keep the genes prevalent in the intestine of Danish pigs. Co-selection with other resistance genes on the same mobile genetics elements could also be a explanation. Macrolide resistance determinants have been genetically linked to *vanA* in Enterococci from poultry (Borgen et al., 2002).

Vancomycin resistance genes of the *vanG* cluster included in this study (*vanTG* and *vanWG*) were found at higher levels in sows compared to finishers, whereas VRD of the *vanB* cluster (*vanHB*, *vanRB*, *vanSB*, *vanWB*, *vanXB* and *vanYB*) were found at lower levels in finishers. To the best of our knowledge, this is the first time that this has been observed.

The most prevalent type of VRD reported in *Enterococcus* spp. worldwide is *vanA* (Cattoir and Leclercq, 2013), but this was not found in any of the farms screened in this study, either because it was not detectable due to the dilution effect, or because *vanA* was truly absent from the Danish pig population. However, the *vanB* cluster is reported to be highly prevalent in human outbreaks of VRE (Cattoir and Leclercq, 2013). Determinants belonging to this cluster were present in almost all farms (Table 2), and genes of the *vanG* cluster were also detected in *Enterococcus* spp. (Table 1).

Pig faeces constitute a reservoir for vancomycin determinants via faecal contamination of the

carcass, which can potentially be transferred from pigs to humans via food consumption. Even though no VRE was found in pigs in 2015 in Denmark (DANMAP, 2016), porcine faeces could still be a reservoir of VRDs. This have been shown to occur between intestinal bacteria both *in vitro* and in the intestine of both humans and animals (Bourgeois-Nicolaos et al., 2006; Dahl et al., 2007; Hammerum 2012, Lester et al., 2006; Lester and Hammerum, 2010; Lim et al., 2006; Moubareck et al., 2003; Sletvold et al., 2007). It would be relevant for future studies to investigate the factors associated with the presence of VRDs in Danish pig farms and the organisms harbouring these VRDs. This information can be used to propose measures to minimise the VRDs and therefore reduce the risk of this resistance transferring to humans.

Furthermore, it is of great concern that vancomycin resistance has been observed in *Staphylococcus aureus*, as vancomycin is a last-resort therapy option for the treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) (Van Tyne and Gilmore, 2014).

## Conclusion

VRDs are still present in Danish pig production more than 20 years after the ban on vancomycin use. The presence of VRDs in pig faeces represents a reservoir for the transfer of vancomycin resistance to humans.

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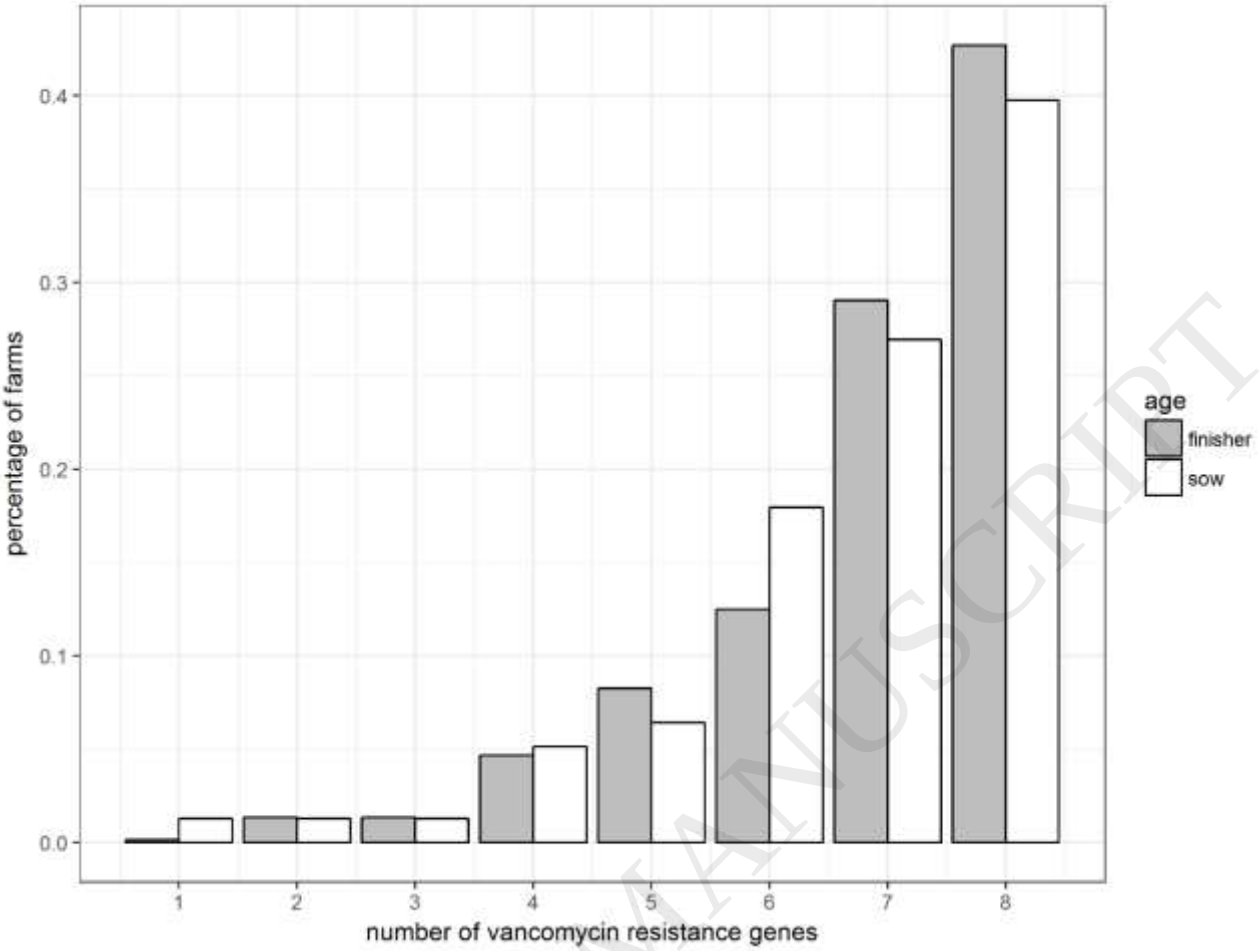
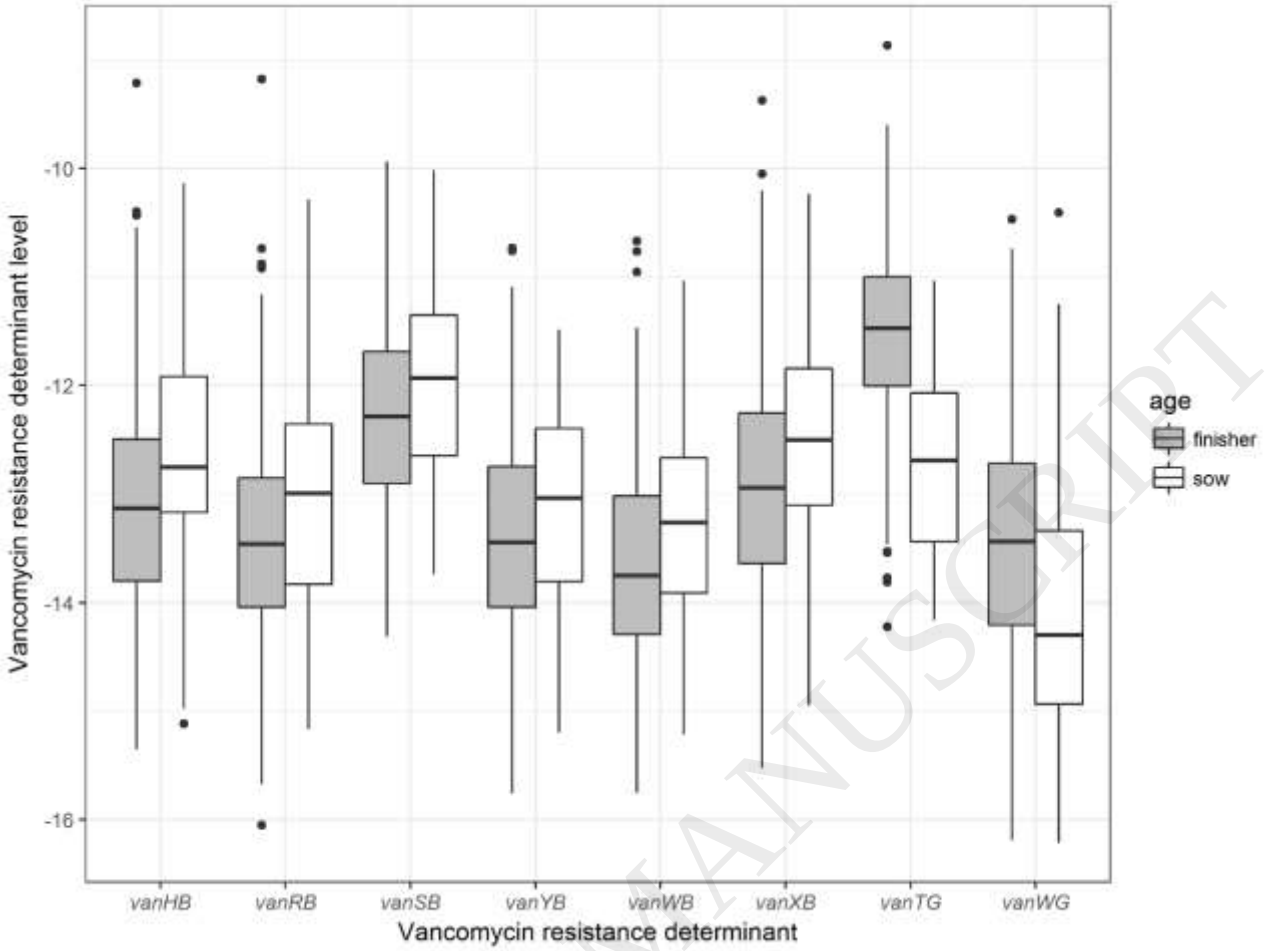
## Figure legends

Figure 1
The proportion of farms where increasing number of different vancomycin resistance determinants were found. Vancomycin resistance determinant levels below the primer specific limit of detection were counted as not present at the farm.

Figure 2
Distribution of vancomycin resistance determinant levels. Levels below the primer specific limit of detection were counted as not present at the farm and were excluded from this figure.






## Tables

**Table 1: Overview of the vancomycin resistance determinants included in the study**

| VRD cluster | VRD | Primer sequence | VRD function[a] | Found in the following bacterial species[a] |
|---|---|---|---|---|
| *vanB* | *vanHB*[g] | Forward: GAGGTTTCCGAGGCGACAA<br><br>Reverse: CTCTCGGCGGCAGTCGTAT | Prevents vancomycin binding | *Enterococcus*[c]*,, Eggerthella, Clostridium* |

| | | | | |
|---|---|---|---|---|
| | *vanRB*[b] | Forward: GCCCTGTCGGATGACGAA<br>Reverse: TTACATAGTCGTCTGCCTCTGCAT | Activates the transcription of *vanH*, *vanA*, and *vanX* in response to vancomycin. | *Enterococcus*[c], *Bacillus, Paenibacillus, Clostridium, Eggerthella,Carnobacterium, Hungatella, Clostridiales, Lysinibacillus, Listeria, Viridibacillus, Geomicrobium, Firmicures, Candidatus* |
| | *vanSB* | Forward: GCGCGGCAAATGACAAC<br>Reverse: TTTGCCATTTTATTCGCACTGT | Activates *vanR* by phosphorylation | *Enterococcus*[c], *Bacillus, Peptoclostridium, Clostridium, Paenibacillus, Streptococcus*[d], *Eggerthella, Hungatella, Lysinibacillus* |
| | *vanWB* | Forward: CGGACAAAGATACCCCCTATAAAG<br>Reverse: AAATAGTAAATTGCTCATCTGGCACAT | accessory gene with unknown function[e] | *Enterococcus*[b], *Clostridium* |
| | *vanXB* | Forward :AGGCACAAAATCGAAGATGCTT<br>Reverse : GGGTATGGCTCATCAATCAACTT | Alters the vancomycin binding site | *Enterococcus*[c], *Streptococcus, Clostridium, Desulfotignum, Eggerthella, and Desulfovibrio* |
| | *vanYB*[f] | Forward: GGCTAAAGCGGAAGCAGAAA<br>Reverse: GATATCCACAGCAAGACCAAGCT | Alters vancomycin binding site | *Acetobacterium, Aerococcus, Alphaproteobacteria, Anaerotignum, Aneurinibacillus Anoxybacillus, Arenibacter, Arthrobacter, Bacillus, Bacteroidetes, Betaproteobacterium, Bifidobacterium, Brevibacterium, Butyribacterium, Clostridioides, Clostridium, Eggerthella, Eisenbergiella, Enterococcus*[c], *Erysipelothrix, Eubacteriaceae, Firmicutes, Fructobacillus, Geobacillus, Halobacillus, Isoptericola, Jeotgalibaca, Lactococcus, Lacunisphaera, Leptolyngbya, Lysinibacillus, Macrococcus, Microbacterium, Mycobacterium, Nocardia, Oceanobacillus, Oerskovia, Oxobacter, Paenibaciulls, Paraliobacillus, Parcubateria, Pelotomaculum, Piscirickettsiaceae, Prochlorococcus, Pseudoalteromonas, Rathayibacter, Rhodococcus, Shewanella, Sphingomonas, Spirochaetes, Streptococcus, Synechoccous, Tenericutes, Verrucomicrobia, Vibrio, Weissella,* |
| *vanG* | *vanTG* | Forward: CGTGTAGCCGTTCCGTTCTT | Decrease bacteria binding affinity to vancomycin[e] | *Enterococcu*[c], *Clostridioides, Clostridium, Herbinix, Paenibacillus, Peptoclostridium, Ruminococccus,* |

| | | | | |
|---|---|---|---|---|
| | *vanWG* | Reverse: CGGCATTACAGGTATATCTGGAAA<br>Forward: ACATTTTCATTTTGGCAGCTTGTAC<br>Reverse: CCGCCATAAGAGCCTACAATCT | Accessory gene with unknown function[e] | Enteroccous, Bacillus |
| *vanA*[g] | *vanA* | Forward:AAAAGGCTCTGAAAACGCAGTTAT<br>Reverse: CGGCCGTTATCTTGTAAAAACAT | Preventing vancomycin binding | *Amycolatopsis, Clostridiales, Clostridioides, Clostridium, Comamonas, Desulfitobacterium, Enterococcus*[c]*, Eubacterium, Firmicutes, Flavonifractor, Microbacterium, Oerskovia, Oxobacter, Paenibacillus, Paenibacillus, Rhodococcus, Staphylococcus, Streptomycetes* |

[a] according to www.uniprot.org(The UniProt Consortium, 2017), [b]Member of the two-component regulatory system VanS/VanR(The UniProt Consortium, 2017), [c]reviewed entry, *Enterococcus faecalis*. [d]reviewed entry, *Streptococcus gallolyticus* [e]Information from CARD(Jia et al., 2017), [f] Vancomycin-inducible, penicillin-resistant & Insensitive to beta-lactams.(The UniProt Consortium, 2017), [g]required for high-level resistance to glycopeptide antibiotics(The UniProt Consortium, 2017). [g]required for high-level resistance to glycopeptide antibiotics(The UniProt Consortium, 2017). VRD: vancomycin resistance determinant

**Table 2: Prevalence of the selected vancomycin resistance determinants from faecal samples obtained from 665 finisher and 78 sow farms**

| | *vanHB* | *vanRB* | *vanSB* | *vanWB* | *vanXB* | *vanYB* | *vanTG* | *vanWG* |
|---|---|---|---|---|---|---|---|---|
| Number of finisher farms found in | 593 (90%) | 523 (79%) | 559 (84%) | 459 (69%) | 613 (92%) | 557 (84%) | 654 (98%) | 608 (91%) |
| Number of sow farms found in | 69 (88%) | 71 (91%) | 71 (91%) | 58 (74%) | 74 (95%) | 68 (87%) | 57 (73%) | 58 (74%) |
| Significant difference | No | Yes ($p = 0.01$) | No | No | No | No | Yes ($p < 0.0001$) | Yes ($p < 0.0001$) |