
DTU

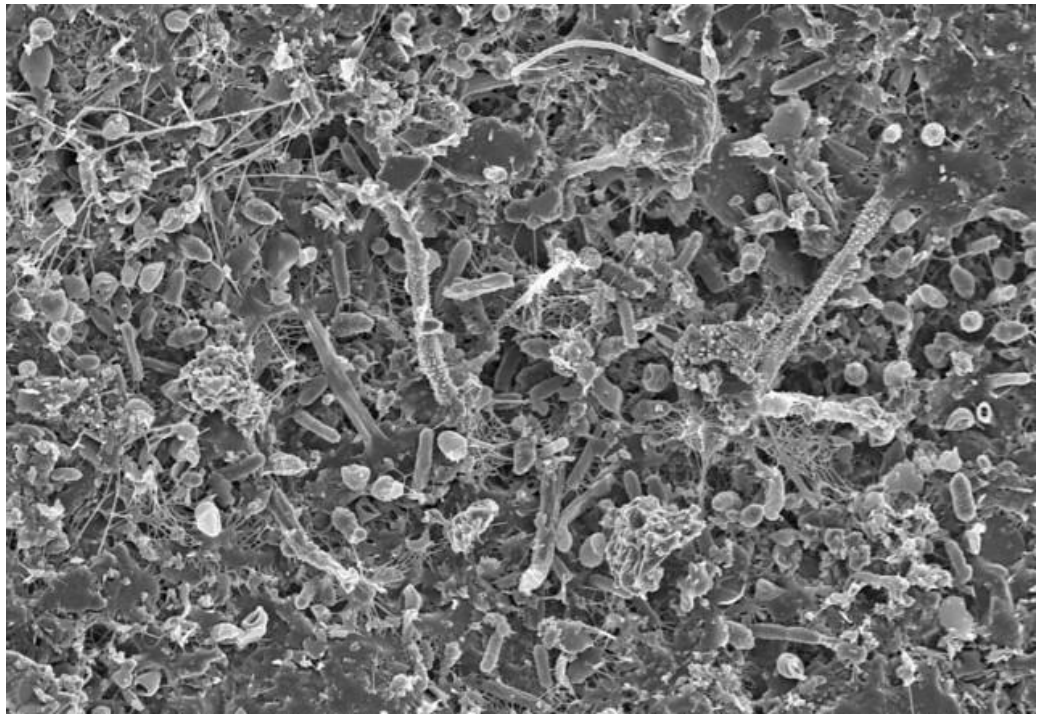
Patrick Munk

Metagenom-baseret overvågning af resistens i dyr

Hvad er metagenomics?

- "'...the application of modern genomics techniques to study the communities of microorganisms directly in their natural environments, by passing the need for isolation and lab cultivation of individual species" – Chen and Patcher (2005)

- **Oversat**
  "Anvendelsen af moderne genomiske metoder til at studere mikrobielle samfund i deres naturlige miljøer og dermed springe dyrkning og isolering over"



Hastigheden vi læser bakteriers DNA

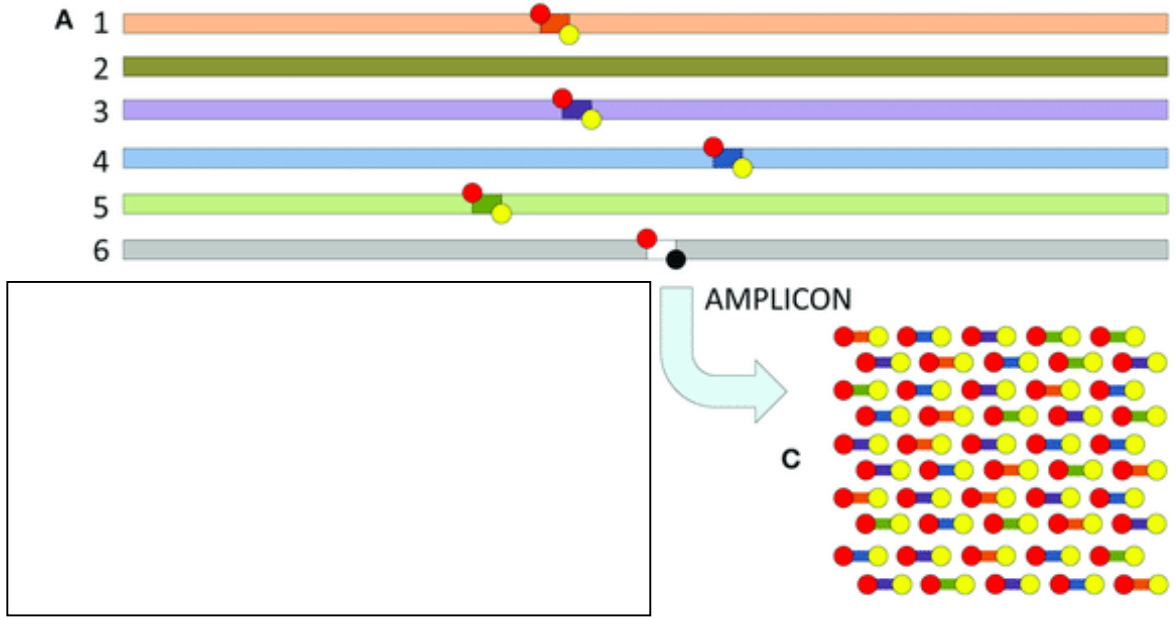
Illumina NovaSeq 6000

- Køretid: ~ 44 timer (2 x S4 flow cells)
- Max kapacitet
  - 20 milliarder DNA-sekvenser
  - 6000 Gbp (6 Tbp)

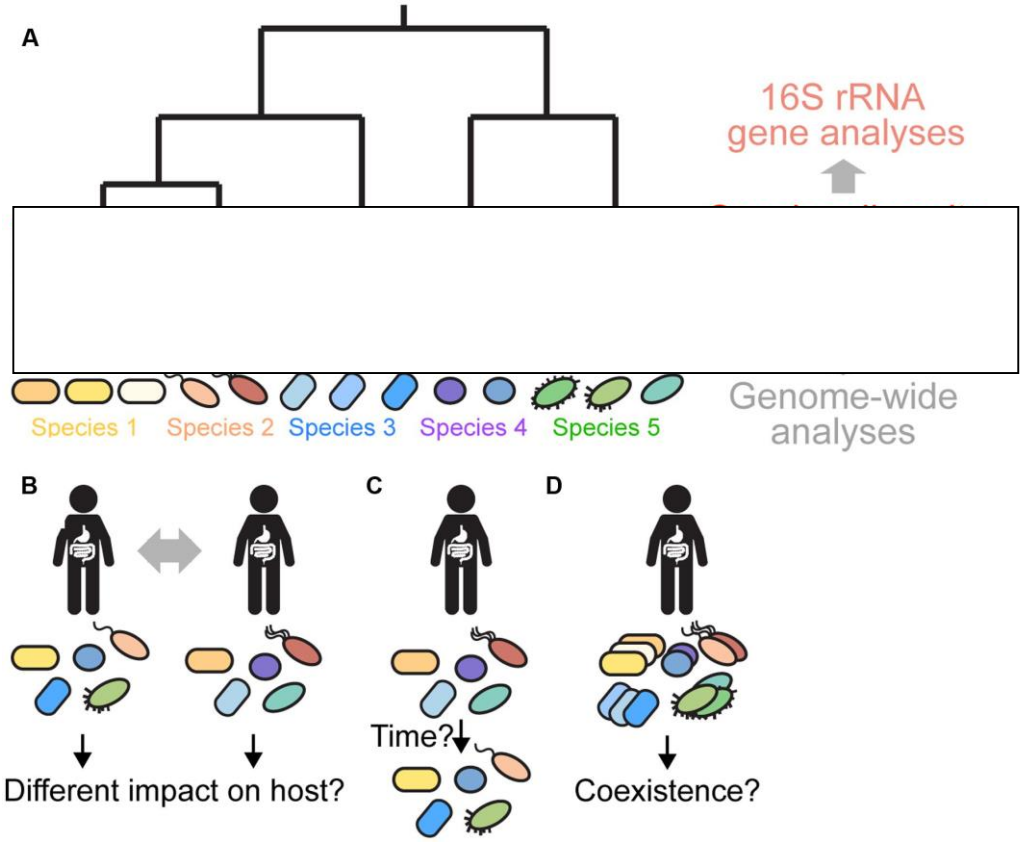
Kan læse 38 millioner baser i sekundet!

Forestil jer at læse disse bøger på 1/4 sekund!

Ikke shotgun metagenomics



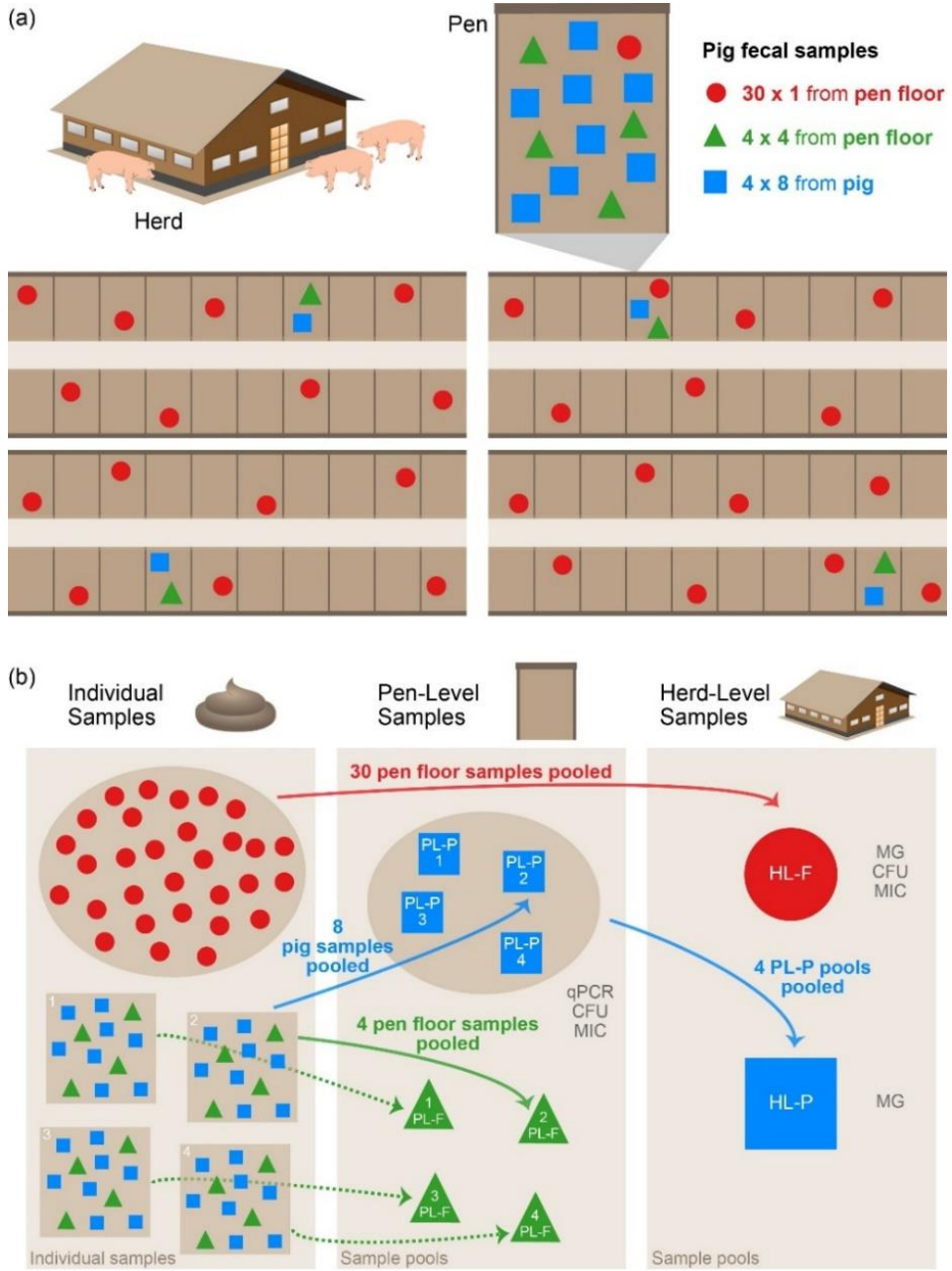
C Sekse (2017) Front Microbio



KM Ellegaard (2016) Front Microbio

Dansk pilotprojekt

- Sammenligne geno- og fænotypiske resistensovervågning i danske svin
- Sammenlign forskellige prøvetagnings-strategier
- Vurder muligheden for at bruge metagenomics til overvågning af resistens på svinegårde
- Vurder muligheden for at måle resistens-ændringer som følge af antibiotika-forbrug



P Munk et al (2017) J. Antimicro Therapy

Det “våde” laboratorie

Prøver der skal analyseres. Fx spildevand, svine- eller mennekse-lort

DNA ekstraktion fra prøve
BE Knudsen et al. (2016) mSystems

DNA revet til 3-400 bp

(PCR-free) DNA library prep
PCR: fugl og fisk
PCR-free: pattedyr og spildevand

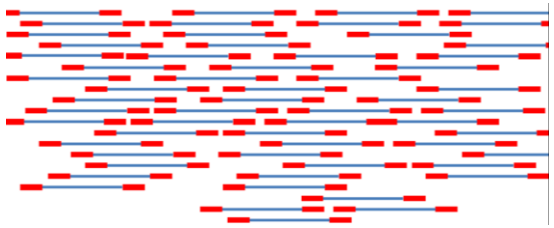
NovaSeq6000

2 x 35M 150bp fragmenter = 10 Gbp

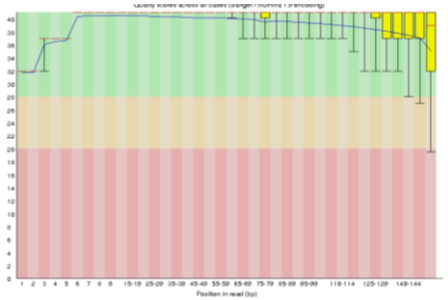
~1500 DKK

efter ekstraktion

Det "tørre" laboratorie



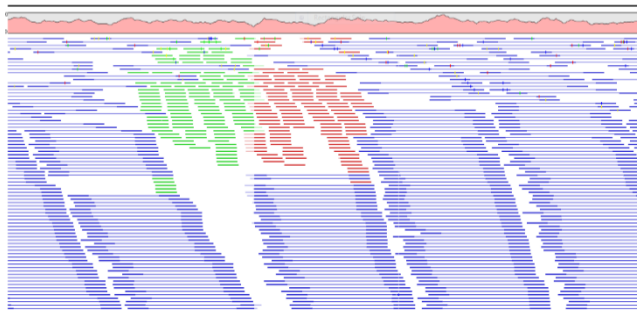
PE DNA reads
>35M/ sample



QC, kvalitet- og

adapter-trimming

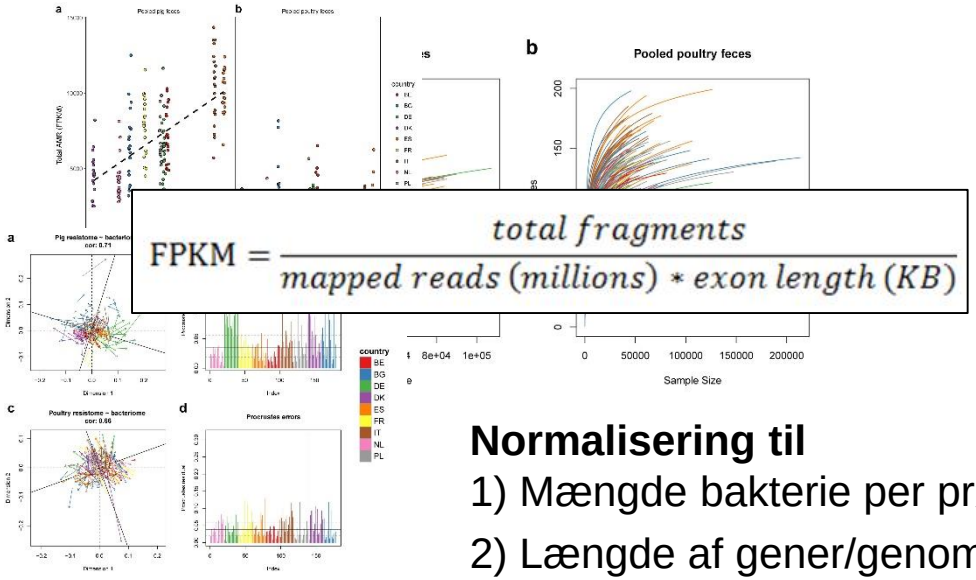
bbduk2



KMA
Reads mapped til **ResFinder**, Bakterie-genomer etc.

	Farm1	Farm2	Farm3
Ref1	17	19	14
Ref2	2	4	0
Ref3	31	0	2

Gen x sample
Tælle-matrix



$$FPKM = \frac{total\ fragments}{mapped\ reads\ (millions) * exon\ length\ (KB)}$$

Normalisering til
1) Mængde bakterie per prøve
2) Længde af gener/genomer

EFFORT Projektet

- EFFORT = Ecology from Farm to Fork Of microbial drug Resistance and Transmission
- EU FP7 projekt som sluttede i vinteren 2018
- Tungt brug af metagenom-sekvensering til måling af resistens

AMR overvågning i stor skala

- Overvåg AMR I svin og kyllinger I 9 europæiske lande
- Prøver fra over 9000 dyr
- 181 svine- og 178 kyllinge-gårde
- >5,000,000,000,000 bp sekvenseret

P Munk et al (2018) - rdcu.be/3ora

ARTICLES
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nature microbiology
Corrected: Author Correction

Abundance and diversity of the faecal resistome in slaughter pigs and broilers in nine European countries

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Antimicrobial resistance (AMR) in bacteria and associated human morbidity and mortality is increasing. The use of antimicrobials in livestock selects for AMR that can subsequently be transferred to humans. This flow of AMR between reservoirs demands surveillance in livestock and in humans. We quantified and characterized the acquired resistance gene pools (resistomes) of 181 pig and 178 poultry farms from nine European countries, sequencing more than 5,000 Gb of DNA using shotgun metagenomics. We quantified acquired AMR using the ResFinder database and a second database constructed for this study, consisting of AMR genes identified through screening environmental DNA. The pig and poultry resistomes were very different in abundance and composition. There was a significant country effect on the resistomes, more so in pigs than in poultry. We found higher AMR loads in pigs, whereas poultry resistomes were more diverse. We detected several recently described, critical AMR genes, including *mcr-1* and *optrA*, the abundance of which differed both between host species and between countries. We found that the total acquired AMR level was associated with the overall country-specific antimicrobial usage in livestock and that countries with comparable usage patterns had similar resistomes. However, functionally determined AMR genes were not associated with total drug use.

Antimicrobial resistance (AMR) is considered one of the largest threats to human health[1]. In addition to the use of antimicrobial agents in humans, livestock is considered an important source of AMR, potentially compromising human health[2]. Besides AMR in zoonotic pathogens, AMR in commensal bacteria is worrisome because of its ability to spread horizontally to pathogens.

Multiple studies have shown that the use of antimicrobials in livestock will lead to an increased occurrence of AMR and that the reduction of usage will eventually lead to reduced resistance[3–8]. Several national surveillance programmes have been implemented to monitor the occurrence of AMR in different reservoirs and follow trends over time[1,9–11]. There are major differences in antimicrobial consumption patterns between different countries globally and also within Europe[12]. Major differences in the occurrence of AMR have also been observed among indicator organisms (for example, *Escherichia coli*) isolated from different European countries[3,13]. Current monitoring efforts are mainly based on culturing indicator bacteria followed by phenotypic AMR determination[3,14]. This procedure only targets a limited number of species present in the gut microbiota and, therefore, probably represents only a fraction of its resistome (the collective pool of AMR genes). Metagenomic approaches have been used in several recent studies and have shown that metagenomic read mapping describes AMR abundance in bacterial communities more accurately than commonly used technologies on selected indicator organisms[15–17]. A recent study focused on sampling a diverse group of individual pigs from 11 farms in 3 countries and showed that genetics, age, diet and geography all probably influence the pig microbiota, but little information is available for the poultry microbiota[16].

As part of the European Union-funded EFFORT project (www.effort-against-amr.eu), we sampled >9,000 animals in 181 pig and 178 poultry herds in 9 European countries, generating herd-level composite samples as previously described[17]. Metagenomic sequencing of these samples gives us a unique insight into the abundance, diversity and structure of the acquired pig and broiler resistomes in Europe. An association between AMR gene abundance and national veterinary antimicrobial usage (AMU) was also analysed. The results and raw data presented here can be used as a baseline for future metagenomic AMR monitoring. To our knowledge, this study represents the single largest metagenomic AMR monitoring effort of livestock: both in terms of countries (9), herds included (359), individual animals sampled (>9,000) and sequencing effort (>5,000 Gb)[16].

Results

Acquired resistome characterization. The total AMR load varied significantly across samples, depending on both the host animal

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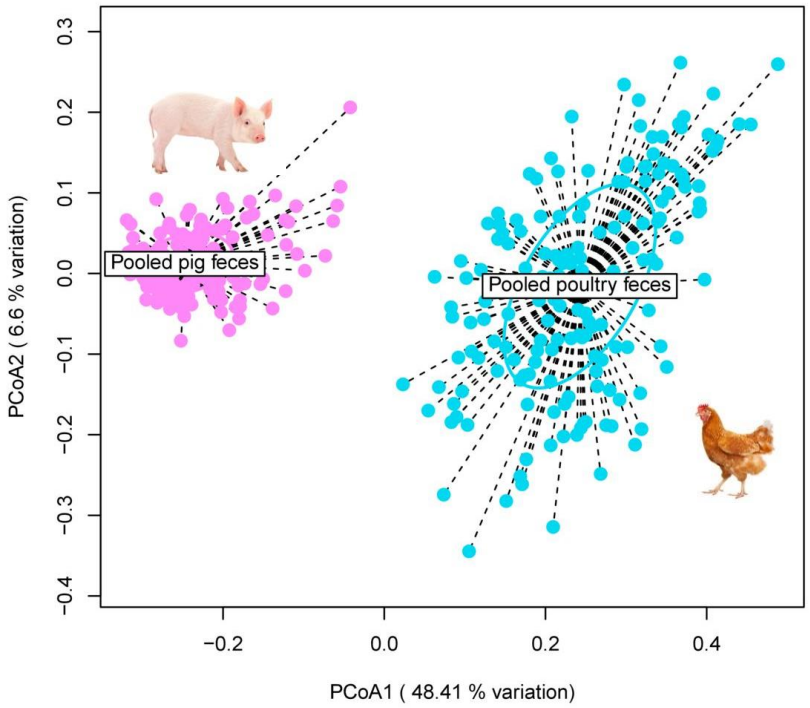
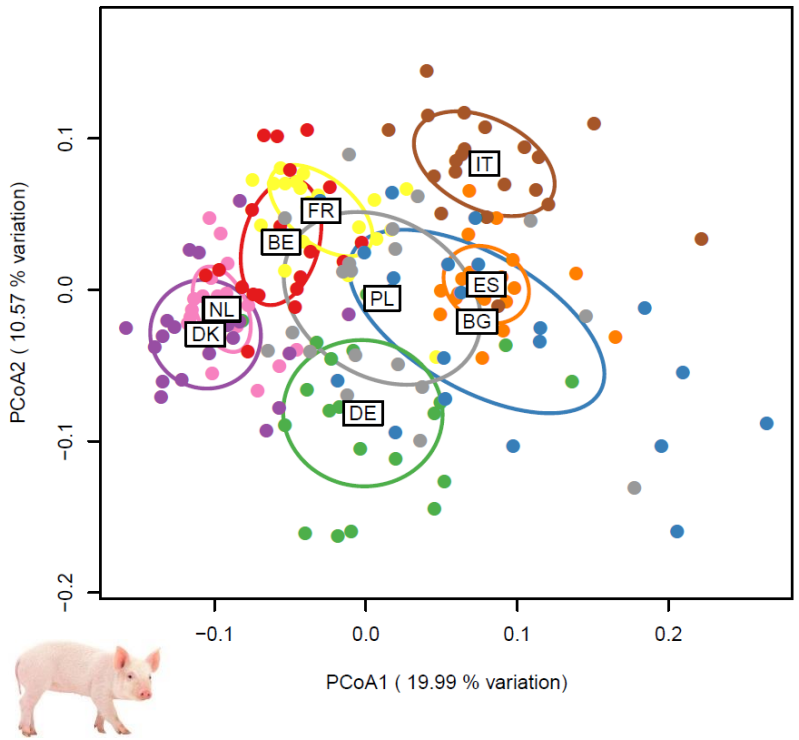
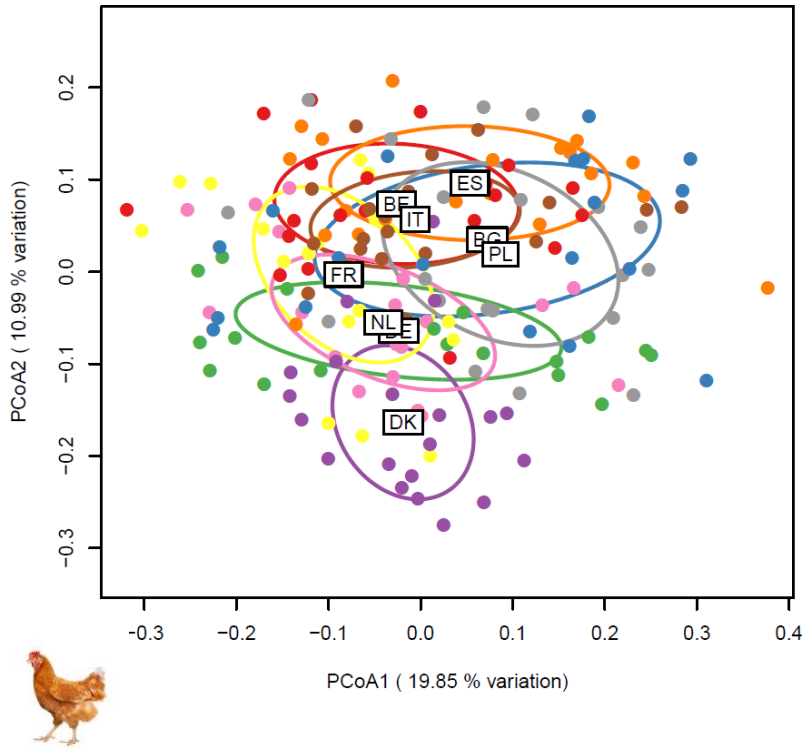
Norway
Finland
Baltic Sea
Estonia
Latvia
North Sea
Lithuania
Denmark
United Kingdom
Ireland
Belarus
Poland
Netherlands
Germany
Belgium
Czechia
Slovakia
Ukraine
Austria
Moldova
Hungary
France
Romania
Croatia
Serbia
Black Sea
Italy
Bulgaria
Portugal
Spain
Tyrrhenian Sea
Greece
Turkey

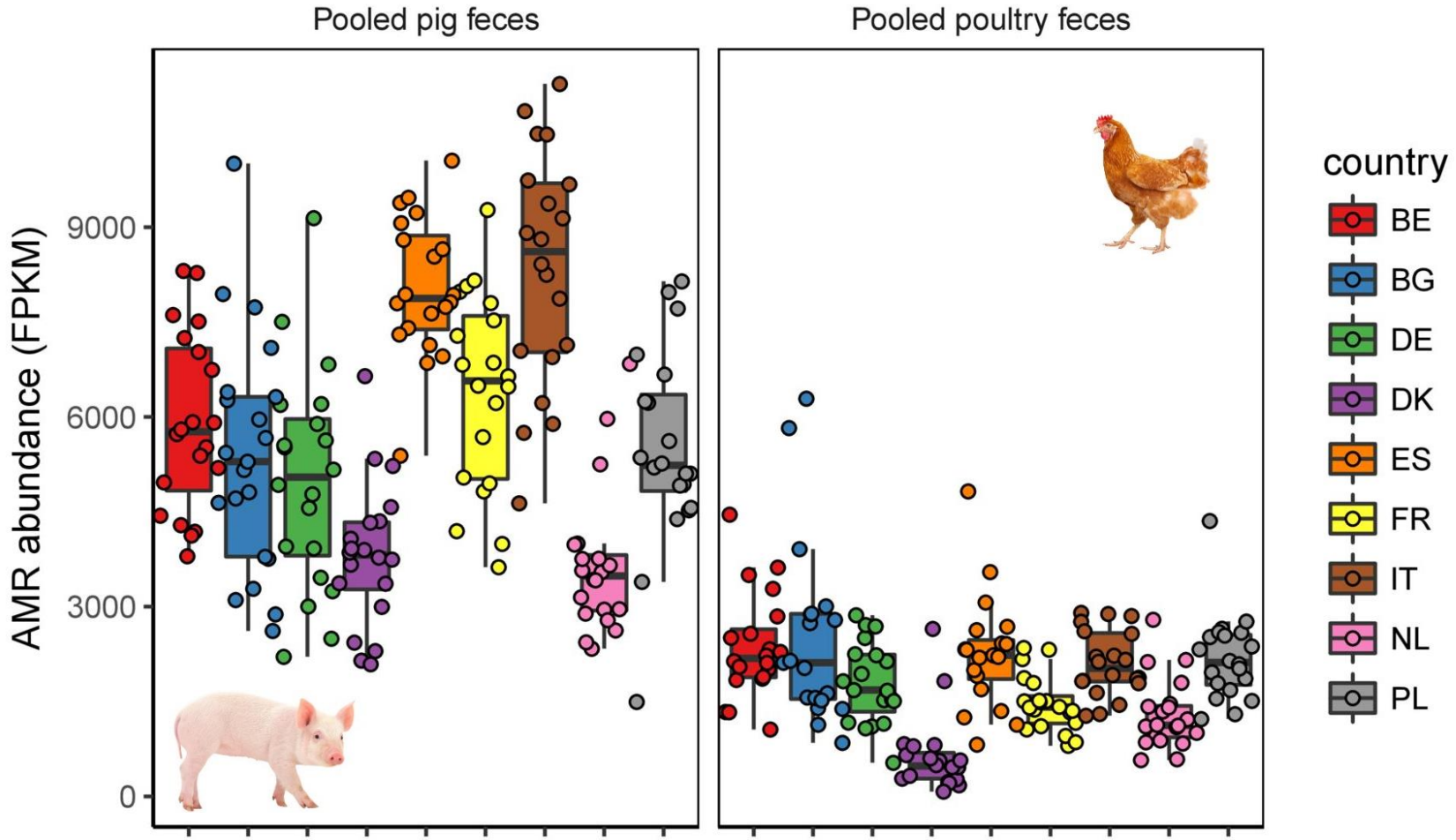
Ordination af resistomet



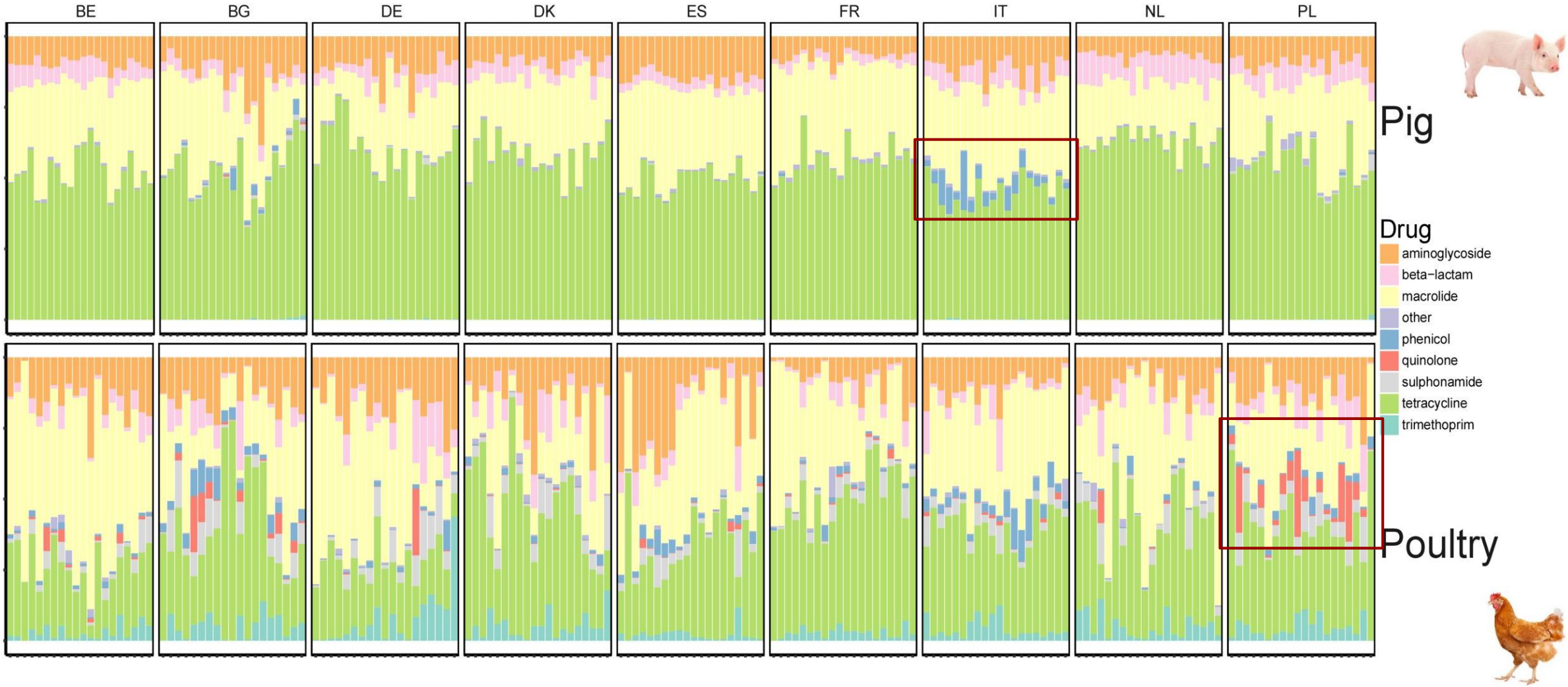


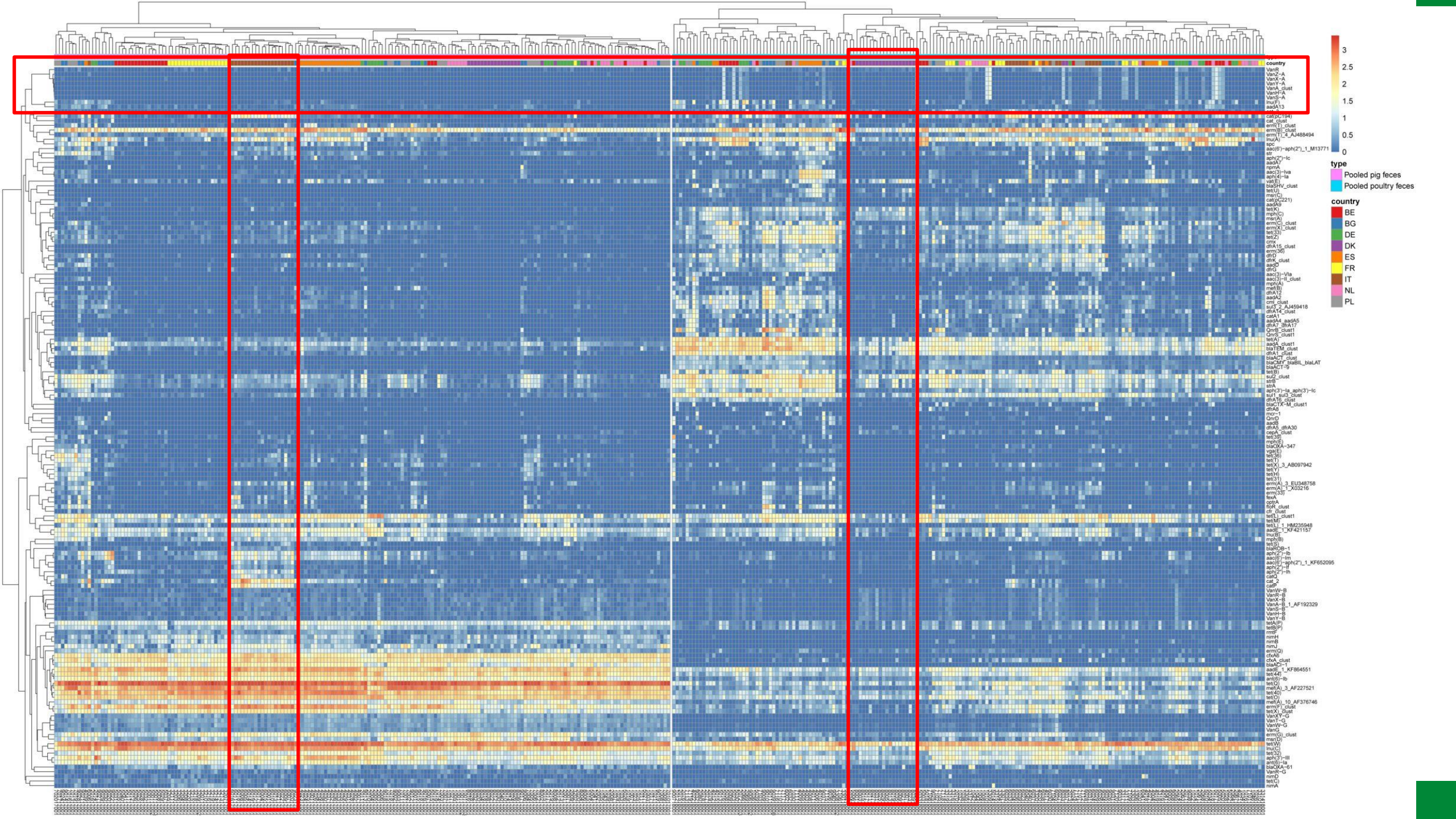


Total resistens



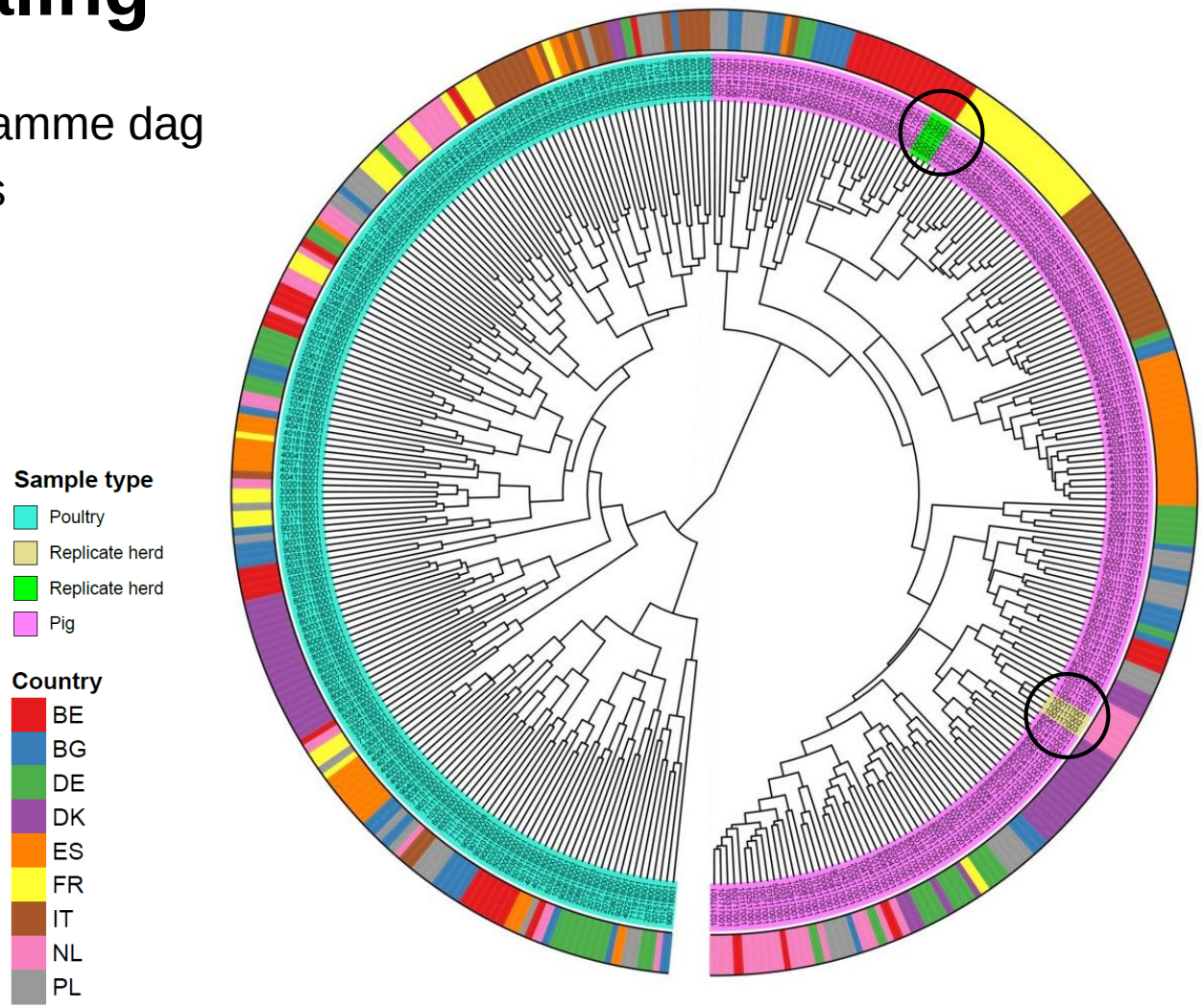
Resistens fordelt på antibiotika-klasser



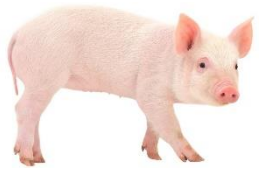

country
VanR
VanZ_A
VanX_A
VanY_A
VanA_clust
VanH_A
VanS_A
lnu(F)
aadA13
cat(pC194)
cat_clust
ermT(L)_clust
ermB(C)_clust
tet(T)_1_4_AJ488494
lnu(A)
spc
aac(6')−aph(2")_1_M13771
str
aph(2")−Ic
aadA7
nprA
aac(3)−Iva
aph(4)−Ia
vat(E)
blaSHV_clust
tet(U)
msr(C)
cat(pC221)
aadA9
tet(K)
mph(C)
msr(A)
ermC)_clust
ermX)_clust
tet(33)
tet(Z)
cmx
dfrA15_clust
erm(36)
dfrD
dfrK_clust
aadD
dfrG
aac(3)−VIa
aac(3)−II_clust
mph(A)
mef(B)
dfrA12
aadA2
cml_clust
sul3_2_AJ459418
dfrA14_clust
catA1
aadA4_aadA5
dfrA7_dfrA17
QnrB_clust1
QnrS_clust1
tet(A)
aadA_clust1
blaTEM_clust
dfrA1_clust
blaACT_clust
blaCMY_blaBIL_blaLAT
blaACT−9
tet(B)
sul2_clust
strB
strA
aph(3')−Ia_aph(3')−Ic
sul1_sul3_clust
dfrA16_clust
blaCTX−M_clust1
dfrA8
mcr−1
QnrD
aadB
dfrA5_dfrA30
cepA_clust
tet(39)
mph(E)
blaOXA−347
vga(E)
tet(36)
tet(T)
tet(X)_3_AB097942
tet(Y)
tet(H)
tet(31)
erm(A)_3_EU348758
erm(A)_1_X03216
erm(33)
flexA
optA
floR_clust
cfr_clust
tet(L)_clust1
tet(M)
tet(L)_1_HM235948
aadE−1_KF421157
lnu(B)
mph(B)
tet(S)
blaROB−1
aph(2")−Ib
aac(6')−Im
aac(6')−aph(2")_1_KF652095
aph(2")−If
aph(2")−Ih
catQ
cat_2
catP
VanW−B
VanR−B
VanX−B
VanA−B_1_AF192329
VanS−B
VanH−B
VanY−B
tet(A/P)
tet(B/P)
rmtF
nimH
nimB
nimJ
erm(Q)
cfxA6
cfxA_clust
blaACI−1
aadE_1_KF864551
tet(44)
ant(6)−Ib
tet(Q)
mef(A)_3_AF227521
tet(40)
tet(O)
mef(A)_10_AF376746
ermF)_clust
tet(X)_clust
VanXY−G
VanT−G
VanW−G
VanG
ermG)_clust
msr(D)
tet(W)
lnu(C)
tet(32)
aph(3')−III
ant(6)−Ia
blaOXA−61
VanR−G
nimD
tet(C)
nimA
3
2.5
2
1.5
1
0.5
0
type
Pooled pig feces
Pooled poultry feces
country
BE
BG
DE
DK
ES
FR
IT
NL
PL

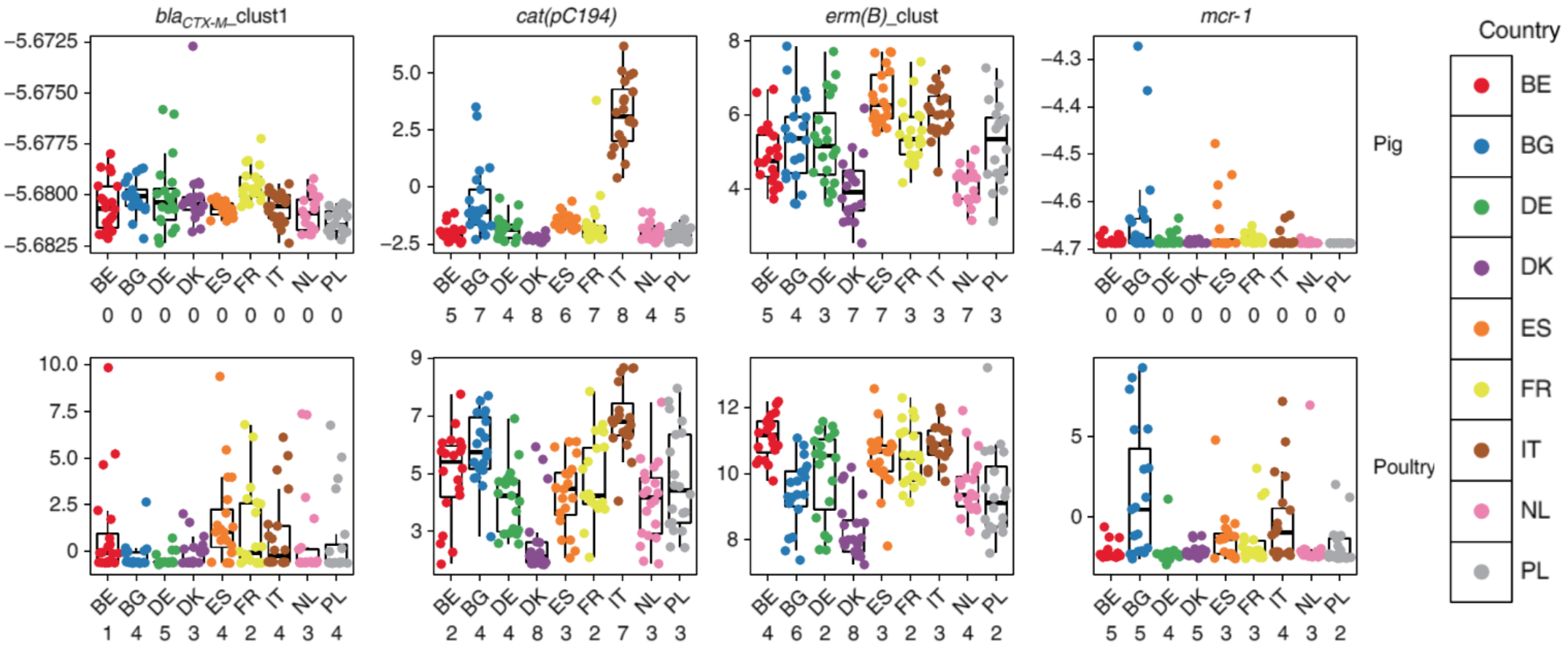
Reproducerbarhed af måling

- To svine-gårde fik taget prøver 3 gange samme dag
- 3 runder a 25 individuelle prøver = 3 pools
- 91.5 – 93.3% Bray Curtis similarity (BE)
- 93.6 – 93.7% Bray Curtis similarity (NE)

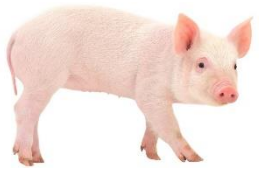


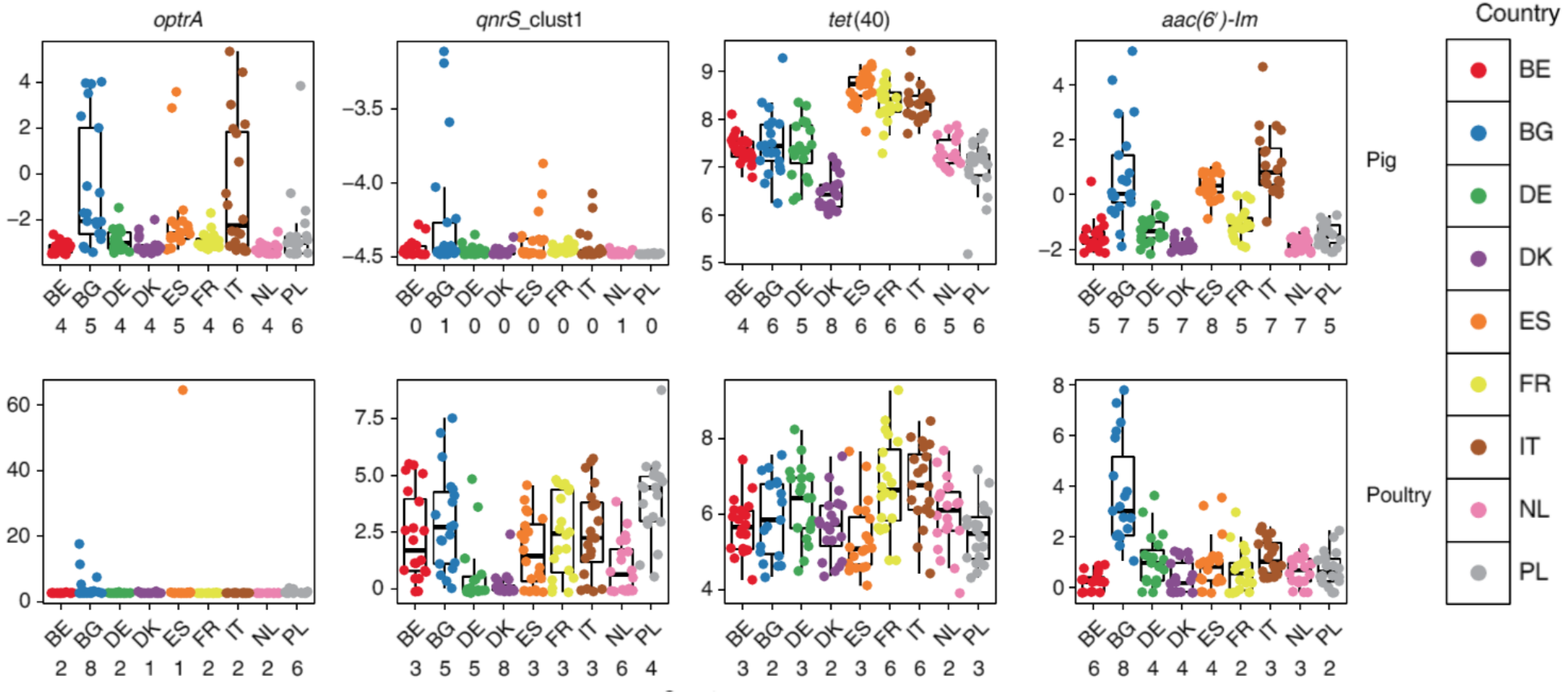
Gener med forskellig abundans



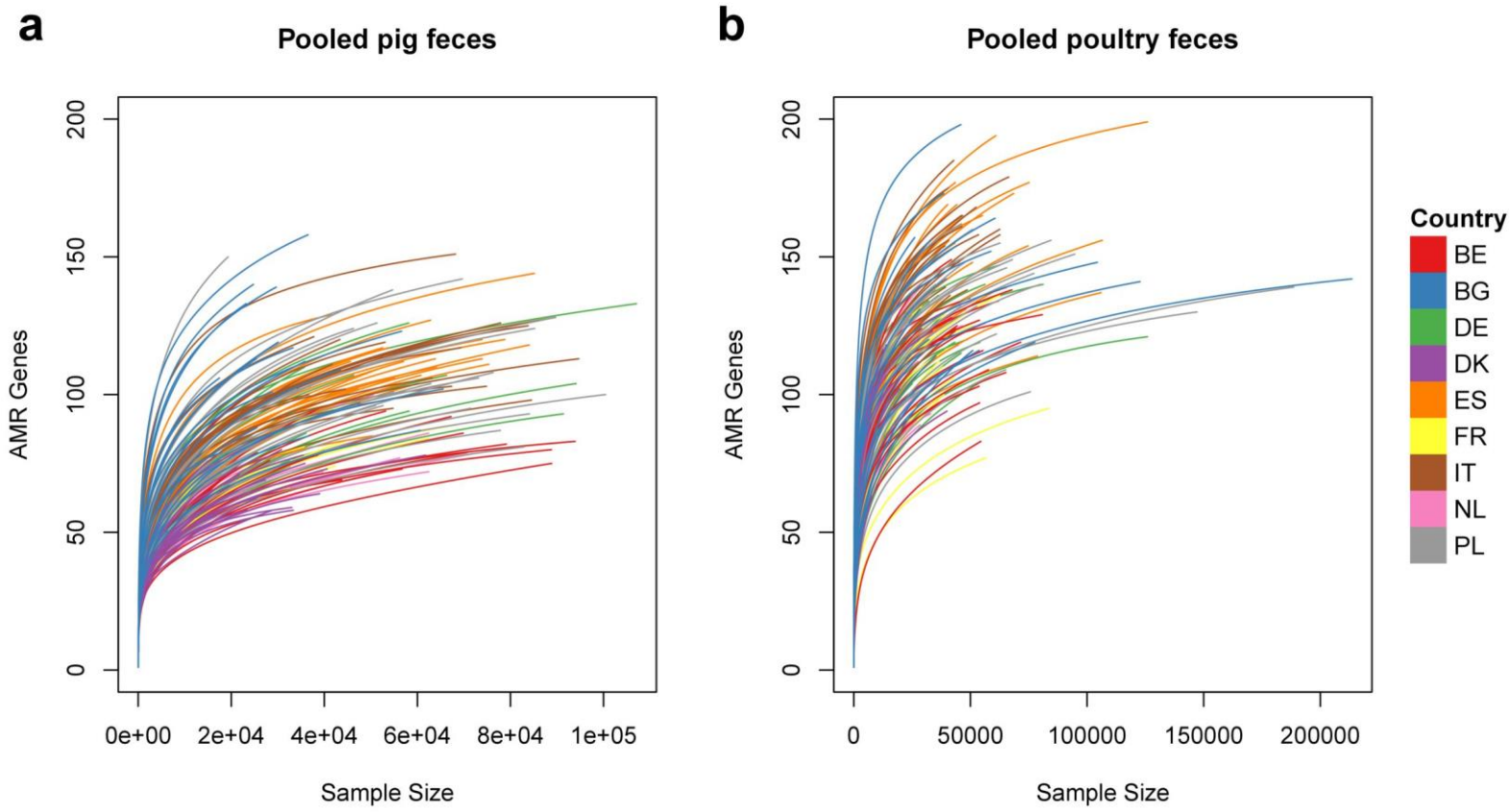


Gener med forskellig abundans II



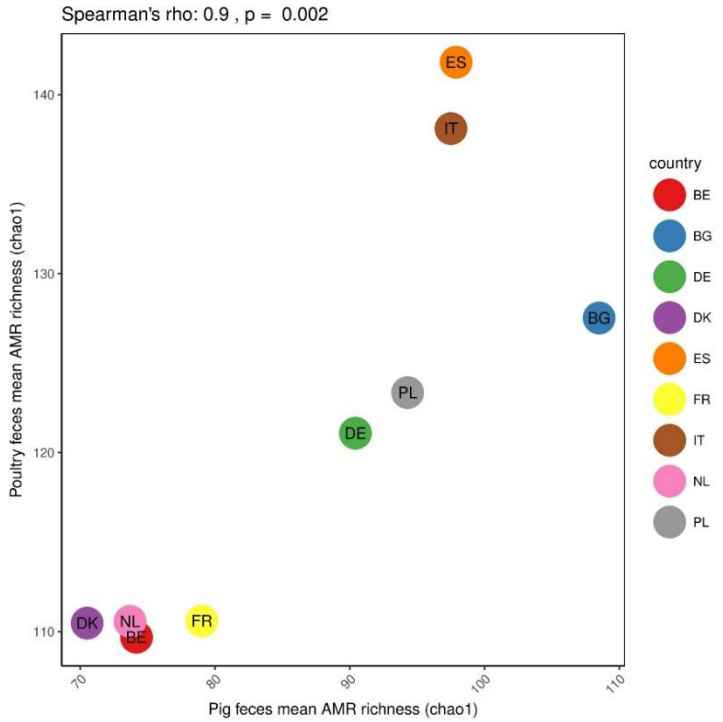


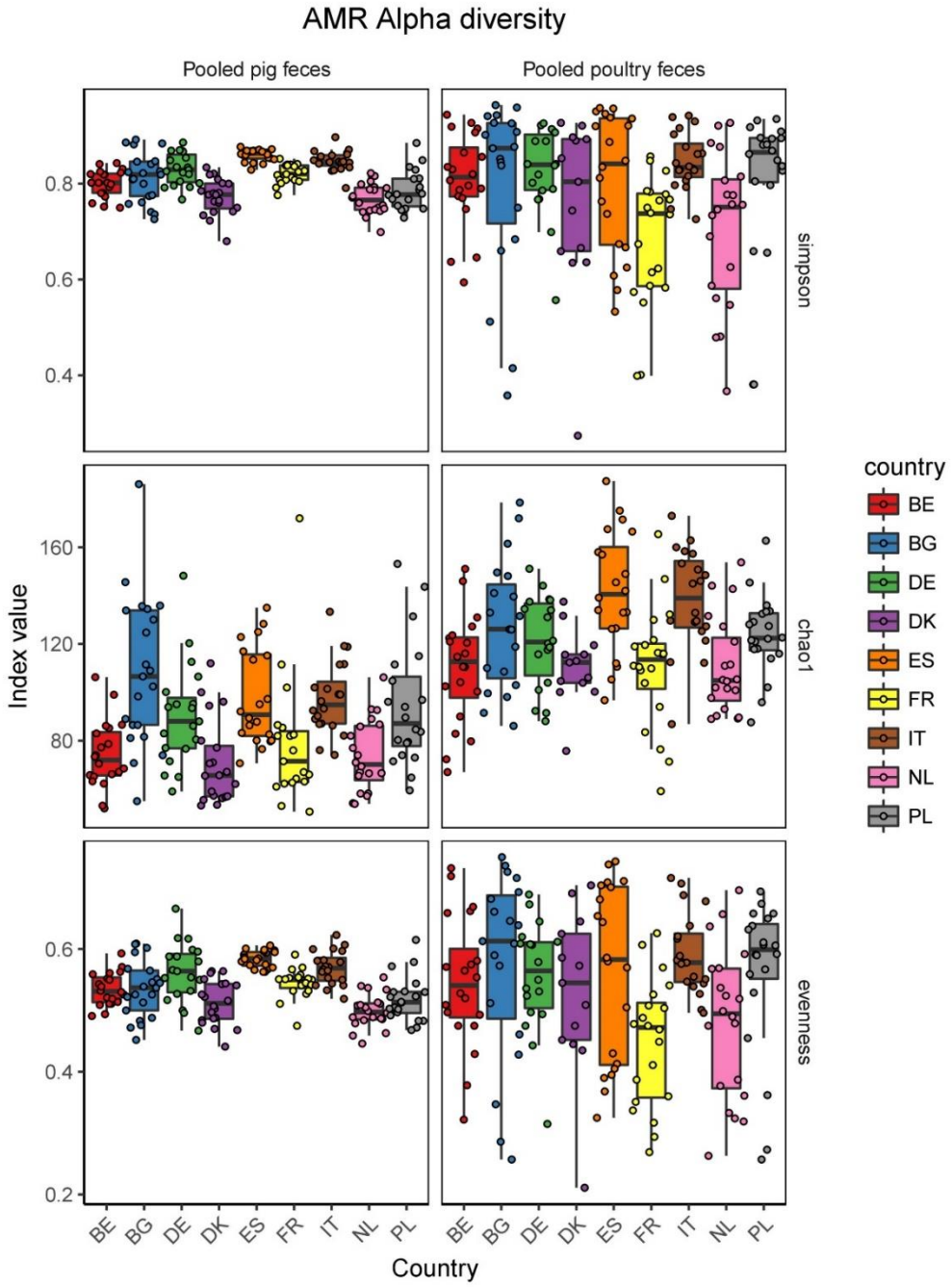
Diversitet af resistensgener



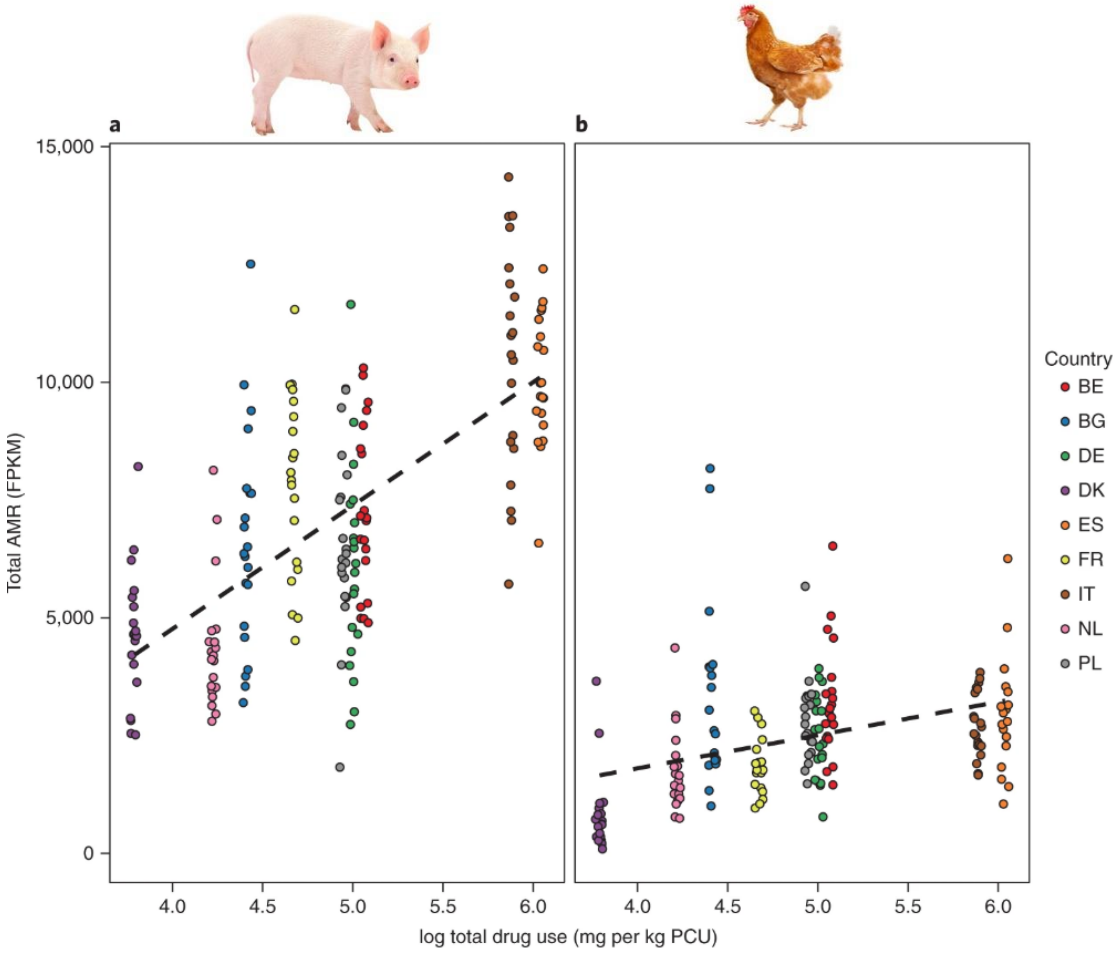
AMR gene diversity

- Alpha diversity in sample resistomes:
  - Evenness (Pielou)
  - Richness (Chao1)
  - Diversity (Simpson)





Faktorer der påvirker resistens-niveauet



JAC

Determinants of the pig antimicrobial resistome in Europe

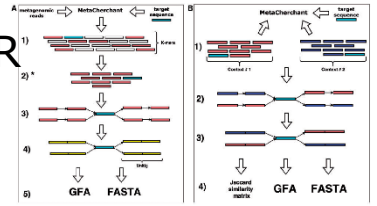
Table 3. Results random-effects meta-analysis: lower aggregation resistance gene clusters (90% identity, $q<0.1$, $p_h>0.05$)

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

LV Gompel et al. (2019) J Antimicro Therapy

Hvad nu?

Bestemmelse af AMR genomiske kontekst

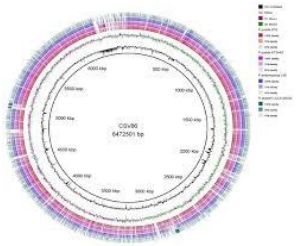


EI Olekhnovich (2018) Bioinformatics

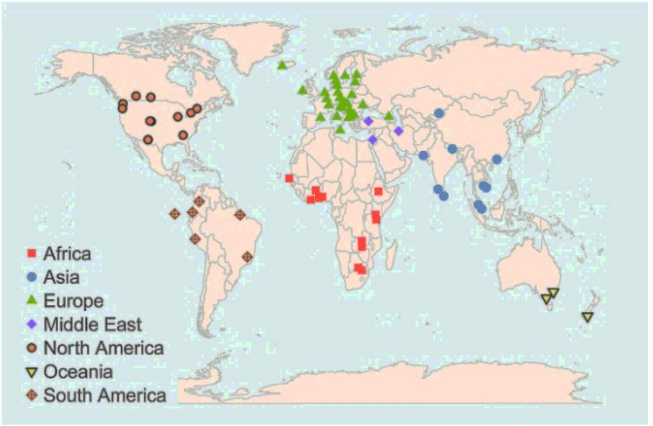
Supplement til dansk AMR overvågning i dyr

Bestemmelse af resistens i flere dyrearter I Europa

Rekonstruktion af genomer fra metagenomer



Overvågning af AMR I spildevand i 100+ lande



R Hendriksen et al (2019) Nature Comm

Konklusioner omkring metagenomisk AMR-måling

Cons

- Svært at optimere en enkelt DNA-ekstraktions-protokol til mange prøvetyper
- Sensitivitet er stadigvæk lavere end ved fx selektiv opdyrkning
- Det er ikke trivielt at bestemme i hvilken kontekst hvert AMR-gen sidder

Pros

- Hundredvis af resistensgener i alle bakterier kan kvantificeres separat vha. ét assay
- Metoden er præcis nok til at måle effekter af fx antibiotikaforbrug og biosecurity
- Metagenomisk data har ekstremt stort genbrugspotentiale
  - Kan søge efter nyopfundne resistensgener
  - Kan også detektere og kvantificere fx bakterielle arter
  - Kan bruges til at opdage helt nye bakterie- og virus-arter

Tak for jeres opmærksomhed!