9

RESISTANCE IN ANIMAL PATHOGENS

9. Resistance in animal pathogens

Highlights

Surveillance of antimicrobial resistance in 2023 focused on pathogenic bacteria from pigs and included results obtained through antimicrobial susceptibility testing (AST) and/or whole genome sequencing (WGS) of isolates belonging to *Actinobacillus pleuropneumoniae* (AST and WGS), *Bordetella bronchiseptica* (AST and WGS), *Clostridium perfringens* (WGS), *Erysipelothrix rhusiopathiae* (WGS), haemolytic and non-haemolytic *Escherichia coli* (AST and WGS), *Glaesserella parasuis* (WGS), *Klebsiella pneumoniae* (AST and WGS), *Salmonella enterica* (AST and WGS), *Staphylococcus hyicus* (AST and WGS) and *Streptococcus suis* (AST and WGS).

Most pathogenic bacteria isolated from pigs in 2023 displayed similar frequencies of phenotypic resistance as in 2022 (1-year period) and 2018 (5-year period). However, nine pathogen-drug combinations were associated with significantly increased resistance, whereas one was associated with a significantly decreased frequency.

The increased frequency of neomycin resistance in haemolytic *E. coli* (52.3%) is concerning because it is one of only few drugs recommended in Denmark as first choice for treating *E. coli*-associated post-weaning diarrhea. The rapid increase in neomycin resistance coincided with increased use of neomycin in weaners.

The increased frequency of gentamicin resistance in haemolytic *E. coli* (35.2%) is also concerning because it is considered critically important for human medicine by the World Health Organization.

WGS-based detection of resistance mechanisms (genes and point mutations) in pathogenic bacteria isolated from pigs in 2023 showed that 22 pathogen-resistance mechanism combinations were associated with significantly increased frequencies when compared to 2022 (1-year period) and 2021 (2-year period), whereas one was associated with a significantly decreased frequency.

Resistance towards carbapenems, 3rd, 4th and 5th generation cephalosporins, oxazolidinones and polymyxins remained at a low level.

The observed concordance between AST results and WGS-based detection of resistance mechanisms was 99.6% for *A. pleuropneumoniae*, 79.6% for *B. bronchiseptica*, 94.2% for haemolytic *E. coli*, 94.3% for non-haemolytic *E. coli*, 76.0% for *K. pneumoniae*, 97.3% for *S. enterica*, 91.5% for *S. hyicus* and 94.5% for *S. suis*.

9.1 Introduction

Antimicrobial susceptibility testing (AST) and surveillance of antimicrobial resistance (AMR) in pathogenic bacteria from pigs, including *Actinobacillus pleuropneumoniae*, haemolytic *Escherichia coli* and *Streptococcus suis*, have been part of the DANMAP programme since 2015. In 2020, the Danish Veterinary and Food Administration (DVFA) asked the Danish Veterinary Consortium (DK-VET) to investigate whether it would be possible to implement whole genome sequencing (WGS) in the surveillance of AMR in pathogenic bacteria from food-producing animals as a basis to detect resistance mechanisms (genes and point mutations). WGS-based AMR surveillance in pathogenic bacteria from pigs commenced in January 2021 and included AST and/or WGS of isolates belonging to *A. pleuropneumoniae* (AST and WGS) *Bordetella bronchiseptica* (AST and WGS), *Clostridium perfringens* (WGS), *Erysipelothrix rhusiopathiae* (WGS), haemolytic and non-haemolytic *E. coli* (AST and WGS), *Glaesserella parasuis* (WGS), *Klebsiella pneumoniae* (AST and WGS), *Salmonella enterica* (AST and WGS), *Staphylococcus hyicus* (AST and WGS) and *S. suis* (AST and WGS), which were identified in clinical samples submitted by veterinarians to the Veterinary Laboratory, The Danish Agriculture and Food Council.

9.2 Temporal trends of AMR in pathogenic bacteria from pigs

The Veterinary Laboratory performed AST of isolates belonging to *A. pleuropneumoniae*, *B. bronchiseptic*a, haemolytic and non-haemolytic *E. coli*, *K. pneumoniae*, *S. enterica*, *S. hyicus* and *S. suis*. Table 9.1 shows the frequencies of resistant isolates in 2023, while all results from 2016-2023 can be found on DK-VET's homepage (https://www.vetssi.dk/). Data are based on epidemiological cut-offs (ECOFFs) and clinical breakpoints when ECOFFs are unavailable (https://www.vetssi.dk/).

Table 9.1 Phenotypic antimicrobial resistance among pathogenic bacteria from pigs, Denmark, 2023 DANMAP 2023

Antimicrobial agent	Ap	Bb	H-Ec	NH-Ec	Kp	Se	Sh	Ss
	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)	R (%)
Amoxicillin	0.0%	ND	73.8%	77.4%	ND	78.0%	100.0%*	ND
Amoxicillin-clavulanic acid	ND	ND	14.1%	12.6%	0.0%	4.9%	ND	ND
Cefpodoxime	ND	ND	4.7%	0.5%	ND	ND	ND	ND
Cefquinome	ND	ND	ND	ND	3.8%	ND	ND	ND
Ceftiofur	1.3%	ND	ND	ND	ND	ND	ND	ND
Colistin	ND	ND	0.3%	0.5%	0.0%	ND	ND	ND
Doxycyclin	0.0%	ND	53.0%	58.4%	34.6%	85.4%	ND	40.4%
Enrofloxacin	2.6%	ND	12.8%	4.7%	ND	ND	0.0%*	0.0%
Florfenicol	0.0%	3.1%	20.8%	28.9%	26.9%	39.0%	25.0%*	0.0%
Gentamicin	ND	ND	35.2%	18.4%	11.5%	43.9%	ND	ND
Lincomycin	ND	ND	ND	ND	ND	ND	100.0%*	ND
Neomycin	ND	ND	52.3%	18.9%	15.4%	ND	ND	ND
Penicillin	0.0%	ND	ND	ND	ND	ND	100.0%*	2.1%
Spectinomycin	ND	ND	68.8%	41.6%	ND	65.9%	ND	ND
Streptomycin	ND	ND	82.9%	76.3%	ND	82.9%	ND	ND
Tetracycline	ND	ND	71.1%	71.6%	34.6%	85.4%	ND	ND
Tiamulin	0.0%	ND	ND	ND	ND	ND	100.0%*	ND
Tildipirosin	0.0%	0.0%	ND	ND	ND	ND	ND	ND
Tilmicosin	0.0%	ND	ND	ND	ND	ND	37.5%*	ND
Trimethoprim-sulfamethoxazole	0.0%	ND	58.4%	72.6%	50.0%	46.3%	62.5%*	15.6%
Tulathromycin	0.0%	6.3%	ND	ND	ND	ND	ND	ND
Tylosin	ND	ND	ND	ND	ND	ND	37.5%*	ND

Data are based on epidemiological cut-offs (ECOFFs) and clinical breakpoints when ECOFFs are unavailable (https://www.vetssi.dk/)
Percentages based on small sample sizes (n<20) are indicated by asterisks and should be interpreted with caution.
Abbreviations: Ap, *Actinobacillus pleuropneumoniae*; Bb, *Bordetella bronchiseptica*; H-Ec, haemolytic *Escherichia coli*; NH-Ec, non-haemolytic *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Se, *Salmonella enterica*; Sh, *Staphylococcus hyicus*; Ss, *Streptococcus suis,* R, resistant; ND, not determined

Most pathogenic bacteria isolated from pigs in 2023 displayed similar frequencies of phenotypic resistance as in 2022 (1-year period) and 2018 (5-year period). However, nine pathogen-drug combinations were associated with significantly increased resistance, whereas one was associated with significantly decreased resistance. Table 9.2 and Figure 9.1 show all significant changes in phenotypic resistance over a 1-year period (2023 vs. 2022) and a 5-year period (2023 vs. 2018).

Haemolytic *E. coli* displayed significantly increased resistance to florfenicol, gentamicin, neomycin, spectinomycin, streptomycin and tetracycline (Figure 9.1).

The increased frequency of neomycin resistance in haemolytic *E. coli* (52.3%) is worrisome because it is one of only a few drugs recommended in Denmark as first choice for treating *E. coli*-associated post-weaning diarrhea. Furthermore, haemolytic *E. coli* also displayed medium to high frequencies of resistance to the other first-choice drugs, including amoxicillin-clavulanic acid (14.1%), spectinomycin (68.8%), trimethoprim-sulfamethoxazole (58.4%) and streptomycin (82.9%). *E. coli* isolates from 2023 were not tested for susceptibility to the remaining first-choice drug ampicillin, but it should be noted that we observed a high frequency of ampicillin resistance in haemolytic *E. coli* from 2022 (60.9%). The rapid increase in neomycin resistance coincided with increased use of neomycin in weaners (Figure 9.2) following two recent decisions to restrict the use of alternative drugs in pigs: 1) the Danish Yellow Card initiative to reduce the use of colistin in 2016 and 2) the European Union-wide ban of medicinal zinc in 2022.

The increasing frequency of gentamicin resistance in haemolytic *E. coli* (35.2%) is also worrisome because it is considered critically important for human medicine by the World Health Organization.

Non-haemolytic *E. coli* displayed significantly increased resistance to florfenicol and significantly decreased resistance to amoxicillin-clavulanic acid, while *S. enterica* and *S. hyicus* displayed significantly increased resistance to gentamicin and tiamulin, respectively. However, the results for *S. hyicus* are based on <20 isolates and should be interpreted with caution (Figure 9.1).

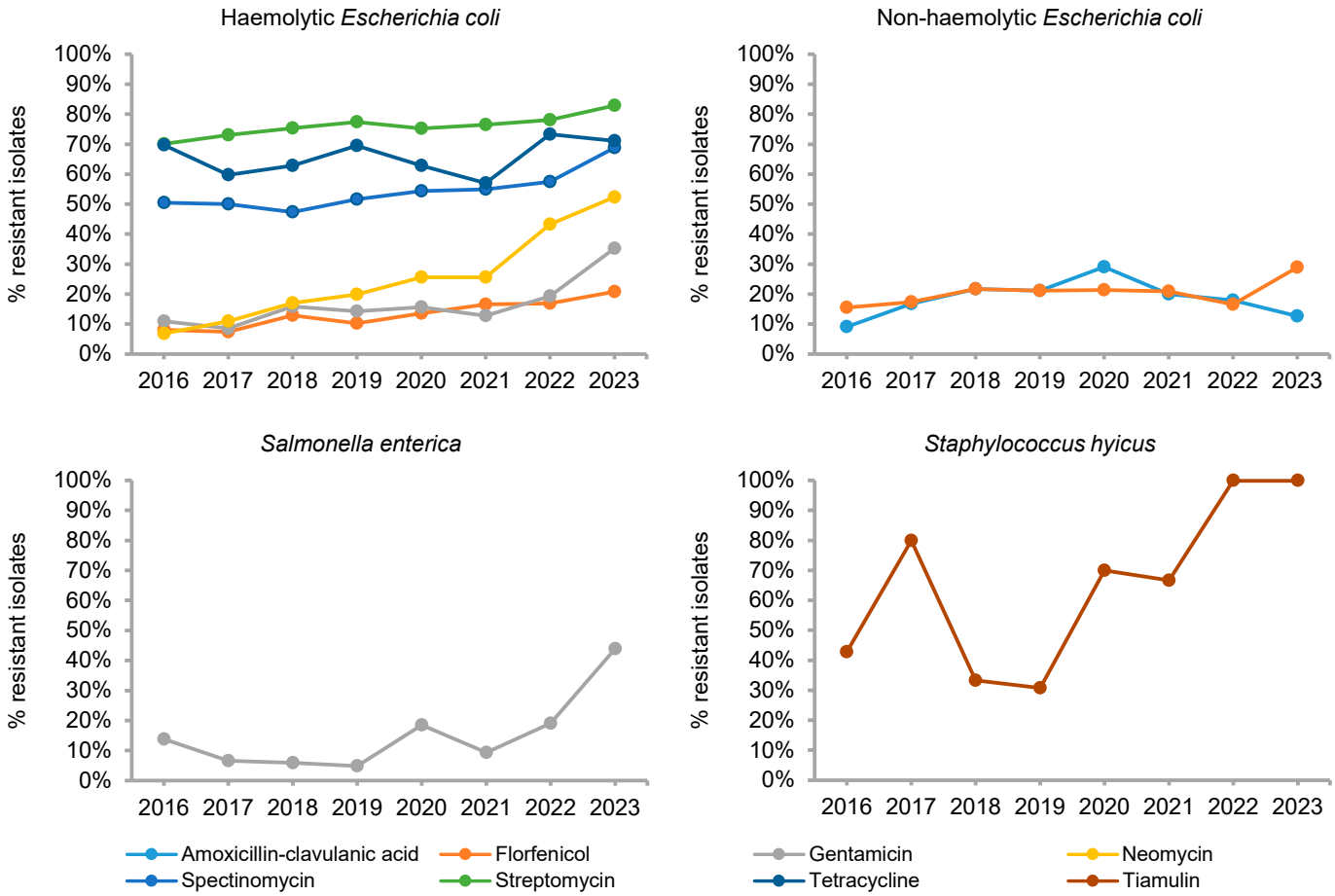
Table 9.2 Statistically significant temporal changes in antimicrobial resistance phenotypes among pathogenic bacteria from pigs, Denmark, 2023 vs. 2022 and 2023 vs. 2018

DANMAP 2023

Pathogen	Antimicrobial agent	2016 R (%)	2017 R (%)	2018 R (%)	2019 R (%)	2020 R (%)	2021 R (%)	2022 R (%)	2023 R (%)	2023 vs. 2022 P value	2023 vs. 2018 P value
H-Ec	Florfenicol	8.1%	7.4%	12.9%	10.3%	13.6%	16.5%	16.9%	20.8%	0.2489	0.0136
	Gentamicin	10.9%	8.6%	15.9%	14.3%	15.6%	12.8%	19.3%	35.2%	0.0000	0.0000
	Neomycin	6.9%	10.9%	17.0%	19.8%	25.6%	25.6%	43.2%	52.3%	0.0270	0.0000
	Spectinomycin	50.5%	50.0%	47.3%	51.6%	54.4%	55.0%	57.4%	68.8%	0.0050	0.0000
	Streptomycin	70.1%	73.0%	75.4%	77.4%	75.2%	76.4%	78.0%	82.9%	0.1479	0.0365
	Tetracycline	69.8%	59.8%	62.9%	69.4%	62.8%	57.0%	73.3%	71.1%	0.5831	0.0388
NH-Ec	Amoxicillin-clavulanic acid	9.1%	16.8%	21.7%	21.1%	29.1%	20.0%	18.0%	12.6%	0.2102	0.0264
	Florfenicol	15.5%	17.3%	21.7%	21.1%	21.3%	20.9%	16.5%	28.9%	0.0090	0.1209
Se	Gentamicin	13.8%	6.6%	5.9%	4.8%	18.5%	9.4%	19.0%	43.9%	0.0186	0.0000
Sh	Tiamulin	42.9%*	80.0%*	33.3%*	30.8*%	70.0%*	66.7%*	100.0%*	100.0%*	1.0000	0.0090

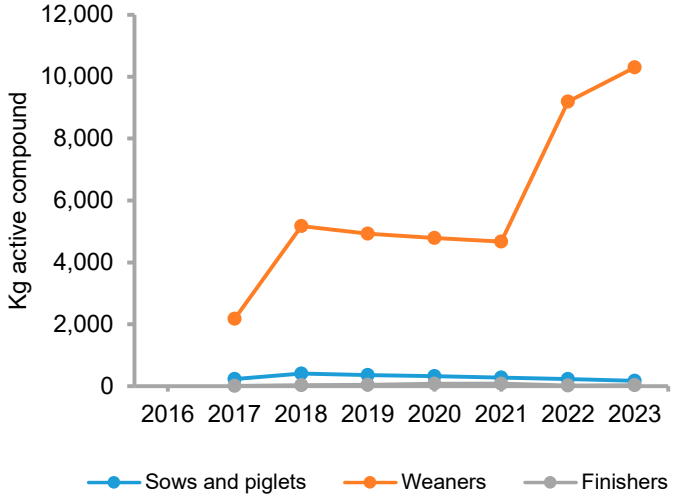
Antimicrobial resistance phenotypes that remained at the same level during 2022-2023 and 2018-2023 were excluded (https://www.vetssi.dk/)
Percentages based on small sample sizes (n<20) are indicated by asterisks and should be interpreted with caution
Abbreviations: H-Ec, haemolytic *Escherichia coli*; NH-Ec, non-haemolytic *Escherichia coli*; Se, *Salmonella enterica*; Sh, *Staphylococcus hyicus;* R, resistant

Figure 9.1 Statistically significant temporal changes in antimicrobial resistance phenotypes among pathogenic bacteria from pigs, Denmark, 2023 vs. 2022 and 2023 vs. 2018
DANMAP 2023



The percentages of tiamulin-resistant *Staphylococcus hyicus* isolates are based on small sample sizes (n<20) and should therefore be interpreted with caution

Figure 9.2 Neomycin use in pigs by age group, Denmark, 2016-2023
DANMAP 2023



9.3 WGS-based detection of resistance mechanisms

A randomly selected subset of *A. pleuropneumoniae* (n=284), *B. bronchiseptica* (n=83), *C. perfringens* (n=222), *E. rhusiopathiae* (n=2), haemolytic *E. coli* (n=303), non-haemolytic *E. coli* (n=214), *G. parasuis* (n=122), *K. pneumoniae* (n=52), *S. enterica* (n=89), *S. hyicus* (n=17) and *S. suis* (n=355) isolates from 2021-2023 was subjected to WGS. Table 9.3 shows all significant changes in resistance mechanisms over a 1-year period (2023 vs. 2022) and a 2-year period (2023 vs. 2021), while a full list of resistance mechanisms detected in isolates from 2021-2023 can be found on DK-VET's homepage (https://www.vetssi.dk/).

Most pathogenic bacteria isolated from pigs in 2023 displayed similar frequencies of resistance mechanisms as in 2022 and 2021. However, 22 pathogen-resistance mechanism combinations were associated with significantly increased frequencies, whereas one was associated with a significantly decreased frequency.

In 2023, *aph(3')-Ia* encoding resistance to neomycin was present in 62.9% of the haemolytic *E. coli* isolates and in 14.3% of the non-haemolytic *E. coli* isolates. No other resistance genes known to confer resistance to neomycin were detected in *E. coli*. Interestingly, *aph(3')-Ia* was also present in 8.3% of the *K. pneumoniae* isolates from 2023, and in 38.7% of the *S. enterica* isolates from 2023, respectively. In addition, *aph(3')-Ia* was present in 2.0% of the *G. parasuis* isolates from 2021 but absent in *G. parasuis* isolates from 2022 and 2023.

Table 9.3 Statistically significant temporal changes in resistance mechanisms identified through whole genome sequencing of pathogenic bacteria from pigs, Denmark, 2023 vs. 2022 and 2023 vs. 2021 DANMAP 2023

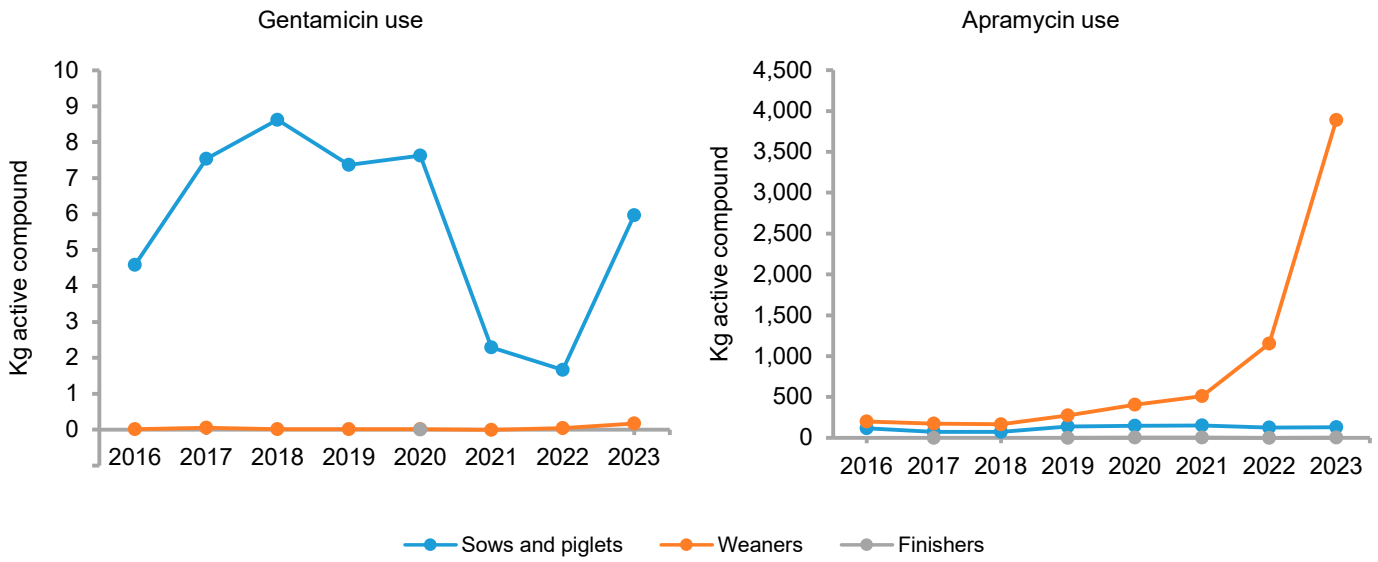
Pathogen	Resistance gene/mutation	Class	Phenotype	2021	2022	2023	2023 vs. 2022	2023 vs. 2021
				Presence (%)	Presence (%)	Presence (%)	P value	P value
Cp	*ant(6)-Ib*	Aminoglycoside	Streptomycin	73.8%	85.7%	87.8%	0.80	0.03
	tet(44)	Tetracycline	Doxycycline, Tetracycline, Minocycline	73.8%	85.7%	87.8%	0.7989	0.0298
H-Ec	*aac(3)-IV*	Aminoglycoside	Apramycin, Gentamicin, Tobramycin	9.6%	21.8%	25.8%	0.5081	0.0037
	aph(3')-Ia	Aminoglycoside	Neomycin, Kanamycin, Lividomycin, Paromomycin, Ribostamycin	30.8%	34.5%	62.9%	0.0001	0.0000
	aph(4)-Ia	Aminoglycoside	Hygromycin	9.6%	20.0%	21.3%	0.8611	0.0269
	$bla_{TEM-127}$	Beta-lactam	Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin	0.0%	0.9%	4.5%	0.1749	0.0436
	cmlA1	Amphenicol	Chloramphenicol	14.4%	7.3%	18.0%	0.0279	0.5580
	mef(C)	Macrolide	Erythromycin	0.0%	0.0%	4.5%	0.0385	0.0436
	mph(G)	Macrolide	Erythromycin	0.0%	0.0%	4.5%	0.0385	0.0436
	tet(B)	Tetracycline	Doxycycline, Tetracycline, Minocycline	21.2%	35.5%	38.2%	0.7677	0.0110
NH-Ec	*floR*	Amphenicol	Chloramphenicol, Florfenicol	22.8%	11.5%	28.6%	0.0264	0.4669
	lnu(F)	Lincosamide	Lincomycin	5.4%	0.0%	8.6%	0.0375	0.5334
	mef(C)	Macrolide	Erythromycin	3.3%	1.9%	12.9%	0.0426	0.0315
	mph(B)	Macrolide	Erythromycin, Spiramycin, Telithromycin	8.7%	7.7%	0.0%	0.0308	0.0104
	mph(G)	Macrolide	Erythromycin	3.3%	1.9%	12.9%	0.0426	0.0315
Kp	*aph(3'')-Ib*	Aminoglycoside	Streptomycin	8.3%	37.5%	54.2%	0.3487	0.0111
	aph(6)-Id	Aminoglycoside	Streptomycin	8.3%	37.5%	54.2%	0.3487	0.0111
Se	*aac(3)-IV*	Aminoglycoside	Apramycin, Gentamicin, Tobramycin	0.0%	7.7%	25.8%	0.0509	0.0177
	aph(4)-Ia	Aminoglycoside	Hygromycin	0.0%	7.7%	25.8%	0.0509	0.0177
	bla_{TEM-1B}	Beta-lactam	Amoxicillin, Ampicillin, Cephalothin, Piperacillin, Ticarcillin	63.2%	59.0%	87.1%	0.0155	0.0776
	sul1	Folate pathway antagonist	Sulfamethoxazole	31.6%	20.5%	51.6%	0.0107	0.2420
	tet(B)	Tetracycline	Doxycycline, Tetracycline, Minocycline	47.4%	59.0%	77.4%	0.1285	0.0371
Ss	*erm*(B)	Macrolide, Lincosamide, Streptogramin B	Erythromycin, Lincomycin, Clindamycin, Quinupristin, Pristinamycin IA, Virginiamycin S	58.3%	64.8%	74.3%	0.1272	0.0201

Abbreviations: Cp, *Clostridium perfringens*; H-Ec, haemolytic *Escherichia coli*; NH-Ec, non-haemolytic *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Se, *Salmonella enterica*; Ss, *Streptococcus suis*

aac(3)-IV encoding resistance to gentamicin was present in 25.8% of the haemolytic *E. coli* isolates, in 7.1% of the non-haemolytic *E. coli* isolates, in 12.5% of the *K. pneumoniae* isolates and in 25.8% of the *S. enterica* isolates. We also identified three less frequent gentamicin resistance genes in haemolytic *E. coli*, including *aac(3)-IId* (6.7%), *aac(3)-IVa* (1.1%) *and ant(2")-Ia* (2.2%). The use of gentamicin in pigs is negligible and cannot explain the rapidly increasing resistance to this drug (Figure 9.3). Interestingly, tthree of the four gentamicin resistance genes found in haemolytic *E. coli, aac(3)-IId, aac(3)-IV and aac(3)-IVa,* also confer resistance to apramycin, which is increasingly used in weaners (Figure 9.3). These observations suggest a causal relationship between increased use of apramycin and increased resistance to gentamicin in haemolytic *E. coli.*

As in previous years, WGS demonstrated a low level of resistance mechanisms towards carbapenems, 3rd, 4th and 5th generation cephalosporins, oxazolidinones and polymyxins.

Figure 9.3 Gentamicin and apramycin use in pigs by age group, Denmark, 2016-2023 DANMAP 2023



9.4 WGS-based prediction of AMR

WGS-based prediction of AMR was assessed by determining the concordance, sensitivity, specificity, positive predictive value, negative predictive value, major error rate and very major error rate between the results obtained through AST and WGS using the genotype-to-phenotype translations in the ResFinder 4.1 database. Table 9.4 shows aggregated results, while results for all pathogen-drug combinations can be found on DK-VET's homepage (https://www.vetssi.dk/). The observed concordance was 99.6% for *A. pleuropneumoniae,* 79.6% for *B. bronchiseptica,* 94.2% for haemolytic *E. coli,* 94.3% for non-haemolytic *E. coli,* 76.0% for *K. pneumoniae,* 97.3% for *S. enterica,* 91.5% for *S. hyicus* and 94.5% for *S. suis.*

Table 9.4 Diagnostic performance of ResFinder 4.1 as an antimicrobial resistance prediction tool for pathogenic bacteria from pigs, Denmark, 2021-2023 DANMAP 2023

Pathogen	Antimicrobial agent	P+/G+	P-/G-	G+/P-	G-/P+	Concordance	Sensitivity	Specificity	PPV	NPV	ME rate	VME rate
Ap	All	8	2,810	2	8	99.6	50.0	99.9	80.0	99.7	0.1	50.0
Bb	All	3	176	1	45	79.6	6.3	99.4	75.0	79.6	0.6	93.8
H-Ec	All	1,403	2,492	162	80	94.2	94.6	93.9	89.6	96.9	6.1	5.4
NH-Ec	All	917	1,836	108	57	94.3	94.1	94.4	89.5	97.0	5.6	5.9
Kp	All	65	311	101	18	76.0	78.3	75.5	39.2	94.5	24.5	21.7
Se	All	374	450	13	10	97.3	97.4	97.2	96.6	97.8	2.8	2.6
Sh	All	44	63	1	9	91.5	83.0	98.4	97.8	87.5	1.6	17.0
Ss	All	229	1,581	4	102	94.5	69.2	99.7	98.3	93.9	0.3	30.8

Data are based on epidemiological cut-offs (ECOFFs) and clinical breakpoints when ECOFFs are unavailable (https://www.vetssi.dk/).
Abbreviations: Ap, *Actinobacillus pleuropneumoniae*; Bb, *Bordetella bronchiseptica*; H-Ec, haemolytic *Escherichia coli*; NH-Ec, non-haemolytic *Escherichia coli*; Kp, *Klebsiella pneumoniae*; Se, *Salmonella enterica*; Sh, *Staphylococcus hyicus*; Ss, *Streptococcus suis*; P+, resistant phenotype; P-, susceptible phenotype; G+, resistant gentotype; G-, susceptible gentotype; PPV, positive predictive value; NPV, negative predictive value; ME, major error; VME, very major error

9.5 Conclusions and perspectives

Haemolytic *E. coli* displayed significantly increased resistance to florfenicol, gentamicin, neomycin, spectinomycin, streptomycin and tetracycline, while non-haemolytic *E. coli* displayed significantly increased resistance to florfenicol and significantly decreased resistance to amoxicillin-clavulanic acid. In addition, *S. enterica* and *S. hyicus* displayed significantly increased resistance to gentamicin and tiamulin, respectively.

The increasing frequency of neomycin and gentamicin resistance in haemolytic *E. coli* is worrisome and should be monitored closely in the coming years. Of note, our interpretation of neomycin resistance was based on ECOFFs, which do not necessarily indicate whether a drug is clinically active. Future studies should therefore seek to establish animal-specific clinical breakpoints to antimicrobial agents of veterinary importance by considering what happens to the drug within a specific animal and body site (pharmacokinetics).

WGS demonstrated that resistance towards carbapenems, 3rd, 4th and 5th generation cephalosporins, oxazolidinones and polymyxins remained at a low level.

WGS-based prediction of AMR using the genotype-to-phenotype translations in the ResFinder 4.1 database seems to be a promising tool for pathogenic bacteria from pigs. However, it is not always possible to compare AST and WGS results due to the lack of ECOFFs and clinical breakpoints for many antimicrobial agents of veterinary importance, and due to limited knowledge on mechanisms conferring resistance to these drugs. In addition, ResFinder 4.1 does not always list all the phenotypes for a given resistance mechanism. For example, it predicts that *aac(3)-IV* confers resistance to gentamicin and tobramycin but not to apramycin, even though this gene is known to encode resistance to apramycin. Finally, the ResFinder 4.1 genotype-to-phenotype translation scheme for point mutations in *K. pneumoniae* is under development and the phenotypes are currently based on antimicrobial classes rather than agents, which might explain some of its poor performance in this species. Closing these gaps could substantially improve the usefulness of WGS for AMR prediction and surveillance in pathogenic bacteria from animals. WGS is also a useful tool for monitoring resistance mechanisms in pathogenic bacteria, for which AST is unavailable, and for tracing the spread of specific resistance genes and pathogenic bacteria within and between animal and human populations.

Lina M. Cavaco, Mikkel Lindegaard, Ute W. Sönksen, Pia T. Hansen, Svend Haugegaard, Charlotte M. Salomonsen, Peter Damborg and Jesper Larsen
For further information: Jesper Larsen, jrl@ssi.dk