

Title: NEO-TRACK: NEOMycin-resistant *Escherichia coli* TRACKing in European livestock

Partners names and institutes:

UCPH: Mattia Pirolo (PI, mapi@sund.ku.dk), Peter Damborg (co-PI, pedam@sund.ku.dk) and Luca Guardabassi (lg@sund.ku.dk)

ANSES: Jean Yves Madec (jean-yves.madec@anses.fr) and Marisa Haenni (marisa.haenni@anses.fr)

APHA: Manal AbuOun (manal.abuoun@apha.gov.uk), Nicholas Duggett (nick.duggett@apha.gov.uk), Muna Anjum (muna.anjum@apha.gov.uk) and Francesca Martelli (francesca.martelli@apha.gov.uk)

SVA: Mikaela Klahr Fritz (mikaela.klahr.fritz@sva.se) and Oskar Nilsson (oskar.nilsson@sva.se)

WBVR: Kees Veldman (kees.veldman@wur.nl) and Michael Brouwer (mike.brouwer@wur.nl)

Project summary

In Denmark, a recent increase in neomycin use was followed by a dramatic raise in neomycin resistance to nearly 50% among porcine clinical *Escherichia coli*. Similarly, in France around 50% of *E. coli* from cattle are neomycin-resistant. Such figures have raised concerns about the effectiveness of this first-choice antibiotic for the treatment of enteric diseases in livestock. Recently, UCPH partners identified two neomycin resistance determinants located on distinct plasmid scaffolds circulating in two predominant *E. coli* lineages (ST100 and CC10). We hypothesize that the spread of these lineages and mobile elements extends beyond hosts and geographical borders. Accordingly, NEO-TRACK aims at investigating the distribution of neomycin-resistant *E. coli* lineages and their plasmids among livestock in the five European countries representing CoVetLab (Denmark, France, Netherlands, Sweden and United Kingdom).

We will employ short- and long-read sequencing to perform a comparative characterisation of neomycin-resistant strains collected either prospectively and/or retrospectively from livestock animals in each CoVetLab partner country. We will also develop screening tools to rapidly identify the most prevalent neomycin resistance plasmids in *E. coli* isolated from different animal species. These tools will facilitate screening in larger strain collections, enabling effective tracking of the spread of epidemiologically successful clones and plasmids. We will also arrange short-term missions among early career researchers to foster knowledge exchange and collaboration between institutions.

NEO-TRACK will provide insights into the dynamics of the spread of neomycin-resistant *E. coli* in European livestock. The bioinformatic pipeline and screening tools developed by this project will support large-scale investigations and aid in monitoring and management of neomycin resistance in livestock animals.

Full proposal

Theoretical foundation and scientific challenge

Escherichia coli is one of the most common Gram-negative opportunistic bacteria in animals and humans, causing a large variety of gastro-intestinal and extra-intestinal diseases, such as enteritis, bacteraemia, and urinary tract infection. Within livestock animals, enterotoxigenic *E. coli* (ETEC) is the most common cause of colibacillosis, including neonatal and post-weaning diarrhoea (PWD) in pigs, diarrhoea in newborn calves and various systemic infections in poultry. Moreover, *E. coli* is a reservoir for exchange of antimicrobial resistance (AMR) genes and mobile genetic elements (MGE) across different hosts.

Neomycin (NEO), an aminoglycoside antibiotic, is approved in Europe for treatment and control of colibacillosis in cattle, pigs, sheep, goats and poultry by the oral route and to treat bovine mastitis by intramammary administration [1]. NEO resistance is usually mediated by aminoglycoside-modifying enzymes such as aminoglycoside 3' O-phosphotransferases [*aph(3')*] [2,3]. The *aph(3')-Ia* gene (previously *aphA1*) is the most commonly reported NEO resistance gene in commensal and pathogenic *E. coli* isolated from pigs [4-6]. NEO resistance genes are usually located on MGEs such as transposons, facilitating their horizontal movement between different clonal lineages and bacterial species [4,7].

The proportion of phenotypically NEO-resistant *E. coli* in pig and cattle, irrespective of the age, within each CoVetLab partner country is provided in Figure 1.

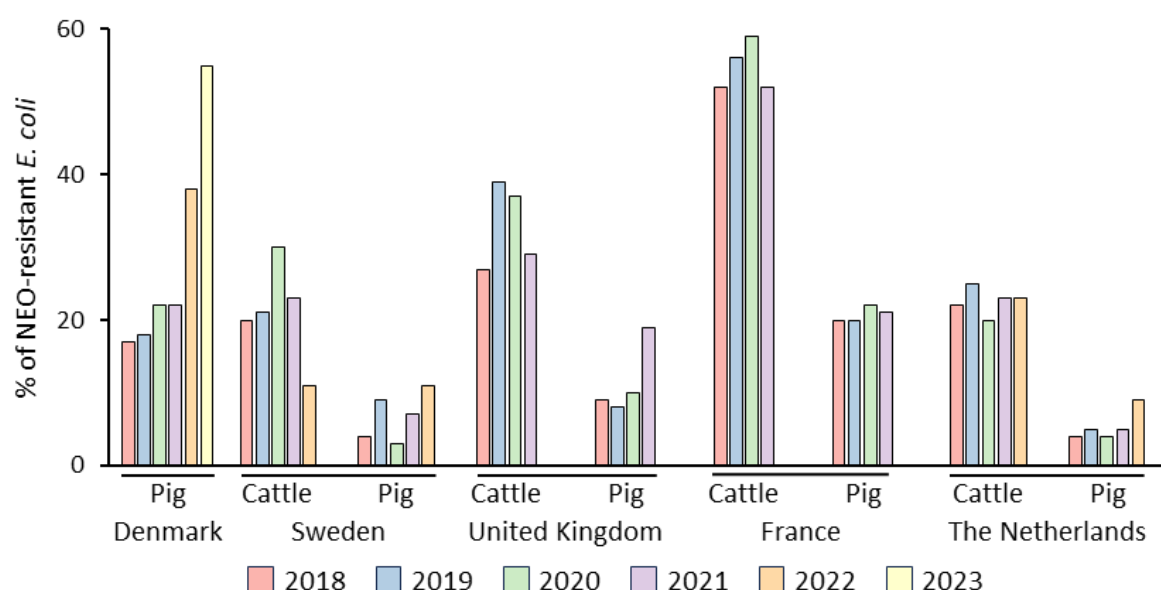


Figure 1. Proportion of NEO-resistant *E. coli* from cattle and pig (all age) in CoVetLab partner countries in 2018-2023.

In Denmark, NEO was used for the treatment of PWD in pigs until its withdrawal in 2008 and was subsequently reintroduced in 2017 [8]. This led to a spike in NEO use in pig farming, especially after the EU restrictions on colistin and zinc oxide. As a result, the prevalence of NEO resistance among clinical *E. coli* isolates surged to 20% in 2020 [9,10], and reached 55% in the first half of 2023 [11]. In France, the proportion of NEO-resistant *E. coli* shows a stable trend over the last 10 years. Specifically, more than 50% of *E. coli* from diarrheic calves and approximately 20% from diseased pigs are NEO-resistant [12]. In the United Kingdom, a composite proportion of 19% NEO-resistant *E. coli* across all livestock animal species was observed in 2021 [13]. This figure is most pronounced among pre-weaned and neonatal calves, where the prevalence of NEO-resistant *E. coli* showed a stable trend of approximately 30-35%. In Sweden, NEO resistance among clinical *E. coli* isolates reached 11% in both pigs and calves in 2022 [14]. While this percentage is comparatively lower than in other CoVetLab partner countries, an increasing prevalence of NEO-resistant *E. coli* has been observed within the Swedish pig population (from 4% in 2018 to 11% in 2022). Over the last five years, the proportion of NEO-resistant *E. coli* isolates from cattle in the Netherlands has been 20-25% [15]. Concomitantly, the proportion of NEO-resistant *E. coli* within the Dutch pig population increased from 4-5% over the period 2018-2021 to 9% in 2022.

With the aim of addressing the current spread of NEO-resistant *E. coli* in Denmark, UCPH partners have recently characterised by whole-genome sequencing (WGS) a historical collection of 128 NEO-resistant clinical *E. coli* isolates from Danish pig farms [16]. Results from the study evidenced that the rise in NEO resistance is attributable to the spread of *aph(3')-Ia* and, to a lesser extent *aph(3')-Ib*, located on distinct plasmid scaffolds circulating within two major clonal lineages, namely sequence type (ST) 100 and clonal complex (CC) 10. More specifically, the spread of *aph(3')-Ia* was associated to multiple acquisitions of at least five distinct conjugative plasmids by phylogenetically distinct *E. coli* lineages, whereas the dissemination of *aph(3')-Ib* was mediated by the acquisition of a small non-conjugative plasmid.

Building upon the recent results from Denmark and considering the overall high level of NEO resistance in livestock farming within the CoVetLab partner countries, we hypothesize that the spread of NEO-resistant *E. coli* lineages and mobile elements extends beyond hosts and borders. Consequently, the primary objective of NEO-TRACK is to investigate the dynamics of NEO resistance and the associated genetic elements in *E. coli* from different livestock sources and geographical locations. By employing a comprehensive genomic analysis, we aim at elucidating the mechanisms driving the successful spread of NEO-resistant *E. coli* by mapping the distribution of NEO-resistant *E. coli* lineages and plasmids in CoVetLab partner countries. As a secondary objective, NEO-TRACK aims at developing screening tools to rapidly identify the most prevalent plasmids conferring NEO-resistance in *E. coli*. These innovative tools will facilitate screening in larger strain collections, enabling rapid and cost-effective tracking of the spread of NEO-resistant plasmids.

Relevance, implementation and dissemination

NEO-TRACK will contribute significantly to understanding the inter-host dissemination of NEO resistance in European livestock. This will have several implications and will provide important data for stakeholders in public health, veterinary medicine, and agriculture:

- 1) Our international collaborative approach will identify patterns of transmission of NEO-resistant lineages and plasmids within and between countries.
- 2) By analysing strains collected over several years, we will address the evolution of NEO-resistant clones within each livestock sector.
- 3) The inclusion of strains from different hosts will allow to investigate if host-specific adaptation events occurred over time.
- 4) By investigating the co-occurrence of antimicrobial resistance genes (ARGs) within NEO resistance plasmids, especially of ARGs conferring resistance to antimicrobials of critical human importance, we will identify plasmids of particular importance for future surveillance.
- 5) We will develop and test different screening tools for effective tracking of important NEO-resistant plasmids, including those carrying multiple ARGs of veterinary and human clinical relevance. These tools will support large-scale investigations and aid in monitoring and management of NEO resistance in livestock.

NEO-TRACK will involve partners from all CoVetLab countries. This international collaboration is needed to enhance the robustness, generalisability, and validity of findings. The collaborative nature of this project will promote the exchange of bioinformatic tools (*i.e.*, those required for *in silico* analysis of clones and plasmids) protocols (*i.e.*, for NEO resistance screening and primer design), and resources (the genome collection of NEO-resistant strains will be shared among CoVetLab partners). In addition, by fostering a solid network of collaboration, also through short-term missions among early career researchers, NEO-TRACK will facilitate access to larger grants opportunities in the field of veterinary microbiology and AMR surveillance.

The project will be promoted widely using X (formerly Twitter) and LinkedIn accounts from CoVetLab partners. At the end of the project, genomic data (raw reads, genome assemblies and plasmid sequences) will be uploaded in public databases such as the European Nucleotide Archive. Metadata, MIC profiles, protocols and primers from the screening tools developed in the project will be also publicly available in Zenodo (<https://zenodo.org/>). Results from the project will be published in well-established peer-reviewed journals and disseminated at renowned international conferences, such as the European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) and Antimicrobial Resistance in Animals and the Environment (ARAE).

Work plan

NEO-TRACK is structured into three work packages (WPs) as outlined below. A schematic overview of the main activities proposed in NEO-TRACK is provided in Figure 2.

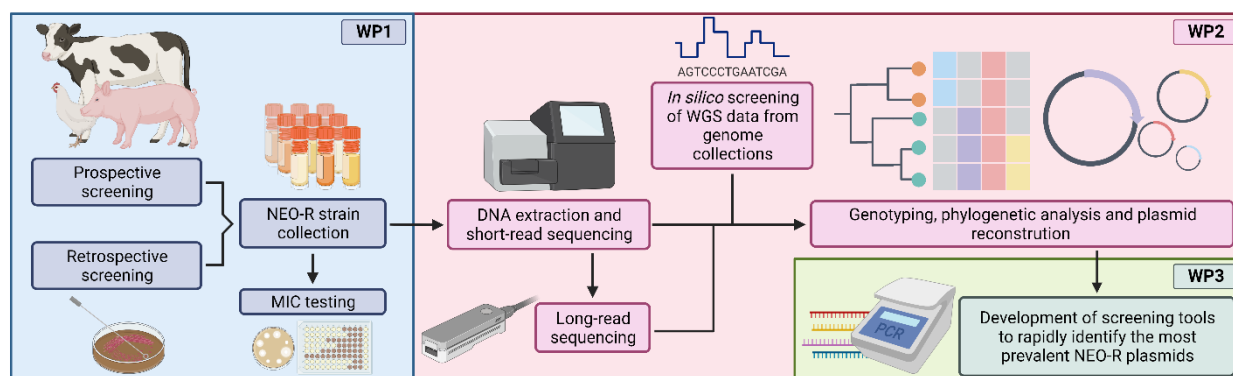


Figure 2. Schematic overview of the main activities in NEO-TRACK.

WP1. Identifying and sequencing NEO-resistant strains for the project
T1.1. Retrospective collection of NEO-resistant strains
CoVetLab partners will screen existing collections of clinical and non-clinical strains collected from livestock animals for NEO resistance. The screening procedure will be standardized and comprise a culture step on a selective medium (e.g. MacConkey agar supplemented with NEO) and a genotypic confirmation step by PCR targeting the predominant NEO resistance genes (<i>i.e.</i> , <i>aph(3')-Ia</i> , <i>aph(3')-Ib</i> , <i>aph(3')-IIa</i> , <i>aph(3')-IIIa</i> , etc.). For each strain identified from the retrospective screening, the NEO minimal inhibitory concentration (MIC) will be evaluated by standard methods. The same applies to strains identified by the <i>in silico</i> screening described below in T2.2 of WP2. Details on existing strain collections that will be screened by CoVetLab partners is provided in Table 1.
T1.2. Prospective screening for NEO-resistant strains
Two approaches will be followed to prospectively screen NEO-resistant strains from different livestock sources. First, within the EU AMR surveillance framework, incoming livestock samples will be screened for the presence of NEO-resistant strains. Second, all clinical NEO-resistant isolates from veterinary diagnostic laboratories included in the national surveillance programmes will be prospectively collected and stored within CoVetLab institutions. PCR-screening for NEO resistance genes and MIC testing of strains will be conducted as described in T1.1. Details on the prospective screening planned by CoVetLab partners is provided in Table 1.
WP2. WGS analysis
T2.1. DNA extraction and sequencing
The DNA from NEO-resistant strains identified in WP1 will be extracted using protocols available in each CoVetLab partner institute. All strains will be subjected to short-read sequencing on Illumina platforms. In addition, a subset of NEO-resistant strains from all CoVetLab partners will be selected for long-read sequencing using Oxford Nanopore Technologies (ONT). Isolates will be selected based on 1) genotyping results (<i>i.e.</i> , belonging to the most common lineages), 2) carriage of ARGs (<i>i.e.</i> , isolates with multiple ARGs will be prioritized) and 3) high coverage of previously identified plasmids carrying NEO resistance (see T2.3 for details).
T2.2. In silico screening, genotyping and NEO-R determinants prediction
<i>In silico</i> screening of WGS data (short- and long-reads genomes) from previously sequenced livestock and non-livestock strains for the presence of NEO resistance determinants will enrich the pool of data of WGS analyses. Details from existing genome collections that will be screened by CoVetLab partners are provided in Table 1. For each genome, the multi-locus sequence type, phylogroup and serogroup will be assigned using <i>mlst</i> (https://github.com/tseemann/mlst), <i>ClermonTyping</i> (http://clermontyping.iame-research.center/) and <i>SerotypeFinder</i> (https://cge.food.dtu.dk/services/SerotypeFinder/), respectively. Clonal complex clustering will be performed using <i>PHYLOViZ</i> (https://www.phyloviz.net/) or <i>sourmash</i> (https://sourmash.readthedocs.io). The presence of NEO and other resistance determinants will be predicted <i>in silico</i> by aligning genomes against reference AMR genes databases (e.g., <i>ResFinder</i> , <i>CARD</i>).
T2.3. NEO-R plasmid reconstruction and phylogenetic analysis
Long-read sequencing data from NEO-resistant strains will be used to reconstruct complete genomes and plasmids harbouring NEO resistance determinants. To predict the carriage of NEO plasmids within our genome collection, we will map short-read data against NEO plasmid sequences. Through this systematic analysis, we will address the association between the most prevalent lineages and predominant plasmids. Phylogenetic distances between NEO-resistant strains will be inferred by core-genome and single-nucleotide polymorphism analyses. These insights will be used to assess transmission of NEO-resistant strains across diverse bacterial species, hosts, and countries. A genome-wide association analysis will be carried out to address the genetic evolution of NEO-resistant lineages within the same hosts. In addition, we will search different public repositories (NCBI, Enterobase, etc.) for genomic data that will match the lineages identified within our collection of NEO-resistant strains. These data will expand the host range included in our analysis and will be used to investigate host adaptation events.
WP3. Screening tools for rapid identification of NEO resistance plasmids
T3.1. Targets and tools selection

Upon exploring plasmid replicon-specific conserved sequences from long-read sequence data, screening tools for the most predominant plasmids will be developed. Plasmids carrying NEO resistance genes in addition to multiple other ARGs will be prioritized in target selection, making our tools useful for monitoring the dissemination of new multi-drug resistant vectors associated with animal farming. Both single and multiplex PCR will be evaluated as screening tools. Primers will be designed and checked *in silico* for cross-reactivity based on the available sequences from the strain and plasmid collection.

T3.2. Validation of screening tools

The screening tools designed in T3.1 will be validated under laboratory conditions. The analytical specificity of primers and reaction conditions will be tested on a representative collection of strains selected for their presence/absence of targets. To address the reproducibility of the test, each CoVetLab partner will participate in the validation step.

Gantt chart

Work Packages (WP) and Tasks (T)	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11	M12
WP1. Identifying and sequencing NEO-resistant strains for the project												
T1.1. Retrospective collection of NEO-resistant strains												
T1.2. Prospective screening for NEO-resistant strains												
WP2. WGS analysis												
T2.1. DNA extraction and sequencing												
T2.2. In silico screening, genotyping and NEO-R determinants prediction												
T2.3. NEO-R plasmid reconstruction and phylogenetic analysis												
WP3. Screening tools for rapid identification of NEO resistance plasmids												
T3.1. Targets and tools selection												
T3.2. Validation of screening tools												
Preparation of the final report												

Budget

Expenses	UCPH	ANSES	APHA ^a	SVA	WBVR	Total
Technical assistance	12,000	3,600	14,900	12,000	6,000	48,500
Laboratory expenses ^b	2,000	6,000	1,600	1,000	5,000	15,600
Short- and long-reads sequencing	5,000	10,400	3,500	6,000	8,000	32,900
Short-term mission for early career researcher	1,000	-	-	1,000	1,000	3,000
Total	20,000	20,000	20,000	20,000	20,000	100,000

^aExpenses for APHA were converted from GBP to EUR on the 30/08/2023.

^bConsumables for the retrospective and prospective screening of NEO-resistant strains and screening tools validation.

All expenses are expressed in EUR.

Budget justification

The budget has been carefully designed to align with the objectives of NEO-TRACK. There are no plans to transfer funds from one institute to another.

Following the recommendation from the CoVetLab consortium, the budget allocated to WGS has been reduced from 55% of the total budget of the Stage 1 submission to 33% of Stage 2. This substantial proportion of the budget is justified, since a comprehensive genomic analysis is necessary to map and understand the diversity and evolution of NEO-resistant lineages and plasmids. In other words, without a largely sequence-based approach it would not be possible to track properly the dissemination of neomycin resistance across hosts, countries, and bacteria. In order to expand the evidence base of the project, partners will include previously obtained sequence data in the analyses.

We will also allocate budget to support short-term missions for early career researchers who will learn methods such as bioinformatic analyses from other partner institutions and thereby contribute to build capacity in their home research environment.

Table 1. Details on retrospective and prospective screening, and existing WGS data from each CoVetLab partner

Task	UCPH	ANSES	APHA	SVA	WBVR
T1.1. Retrospective screening for NEO-resistant strains	~400 <i>E. coli</i> and <i>Enterobacteriaceae</i> from companion animals. All recent (from 2017) clinical <i>E. coli</i> and <i>Enterobacteriaceae</i> from livestock animals stored at SEGES (https://www.seges.dk/).	- 100 mcr-1-positive <i>E. coli</i> from bovine - 80 AmpC-producing <i>E. coli</i> from bovine - 250 ESBL-producing <i>E. coli</i> from bovine - 50 ESBL-producing <i>E. coli</i> from cats/dogs - 100 <i>Klebsiella pneumoniae</i> from cats/dogs - 120 <i>Enterobacter hormaechei</i> from cats/dogs and horses.	MIC screen a selection of <i>E. coli</i> for NEO resistance (isolates from WGS collections)	~200 non-ETEC strains from pigs collected during National surveillance.	>300 <i>E. coli</i> non-clinical NEO-resistant isolates from livestock collected between 2001 and 2007.
T1.2. Prospective screening for NEO-resistant strains	-	We will conduct a prospective study specifically on NEO-R <i>E. coli</i> from veterinary labs involved in the national surveillance network (Resapath).	-	We will save all NEO-R isolates from clinical samples coming to SVA. We will investigate the possibility to include NEO within the panel of antibiotics tested during the future sampling campaigns of not only diseased but also healthy pigs.	We will prospectively screen caecal samples collected at slaughter for national AMR monitoring purposes. Samples from broilers and veal calves will also be included in this screening.
T2.1. <i>In silico</i> screening of existing WGS data	- >500 short-reads genomes of porcine clinical <i>E. coli</i> collected in 2023 sequenced at SSI. - >500 short-reads genomes of clinical <i>E. coli</i> from human infection from 2019 to 2023 sequenced at Rigshospitalet, Copenhagen - ~150 short-reads genomes of <i>E. coli</i> from companion animals.	>1,000 short-reads and ~100 long-reads genomes of <i>E. coli</i> from livestock animals	>1,000 short-reads and 49 long-reads genomes of <i>E. coli</i> from livestock animals (of which 105 positive for <i>aph(3)-la</i> and 77 <i>aph(3)-la</i> -positive ESBL <i>E. coli</i> genomes from different livestock animals from the National surveillance program or livestock research projects.	- ETEC (~100) and non-ETEC (~25) from pigs - ~200 <i>aph(3)</i> -positive genomes from clinical ESBL-producing <i>E. coli</i> from different animal species (predominantly dogs and horses) - ~25 <i>E. coli</i> genomes from the EU-harmonised monitoring of healthy animals and meat	>1,700 <i>E. coli</i> genomes including: - ESBL-producing <i>E. coli</i> from livestock animals (~500 short-reads and ~150 long-reads genomes) from 2018-2021 - ESBL-producing <i>E. coli</i> from the national AMR monitoring of 2021-22 (~450 short-reads and ~200 long-reads genomes) - <i>E. coli</i> from the national monitoring of 2006-18 (~600 short-reads genomes)

References

- [1] European Medicine Agency (EMA), 2022. Committee for veterinary medicinal products: neomycin. Available at https://www.ema.europa.eu/en/documents/mrl-report/neomycin-summary-report-3-committee-veterinary-medicinal-products_en.pdf (accessed 30/08/2023).
- [2] Sun H, Li S, Xie Z, Yang F, Sun Y, Zhu Y, Zhao X, Jiang S, 2012. A novel multidrug resistance plasmid isolated from an *Escherichia coli* strain resistant to aminoglycosides. *J Antimicrob Chemother* 67:1635–1638.
- [3] Garneau-Tsodikova S, Labby KJ, 2016. Mechanisms of resistance to aminoglycoside antibiotics: Overview and perspectives. *Medchemcomm* 7:11–27.
- [4] Reid CJ, Wyrsh ER, Roy Chowdhury P, Zingali T, Liu M, Darling AE, Chapman TA, Djordjevic SP, 2017. Porcine commensal *Escherichia coli*: a reservoir for class 1 integrons associated with IS26. *Microb Genom* 3:e000143.
- [5] Brilhante M, Perreten V, Donà V, 2019. Multidrug resistance and multivirulence plasmids in enterotoxigenic and hybrid Shiga toxin-producing/enterotoxigenic *Escherichia coli* isolated from diarrheic pigs in Switzerland. *Vet J* 244:60–68.
- [6] García V, Gambino M, Pedersen K, Haugegaard S, Olsen JE, Herrero-Fresno A, 2020. F4- and F18-positive enterotoxigenic *Escherichia coli* isolates from diarrhea of postweaning pigs: Genomic characterization. *Appl Environ Microbiol* 86:e01913-20.
- [7] Wyrsh ER, Reid CJ, DeMaere MZ, Liu MY, Chapman TA, Roy Chowdhury P, Djordjevic SP, 2019. Complete sequences of multiple-drug resistant IncHI2 ST3 plasmids in *Escherichia coli* of porcine origin in Australia. *Front Sustain Food Syst* 3:18.
- [8] Jensen J, Kyvsgaard NC, Battisti A, Baptiste KE, 2018. Environmental and public health related risk of veterinary zinc in pig production – Using Denmark as an example. *Environ Int* 114:181–190.
- [9] Subramani P, Pirolo M, Haugegaard S, Skarbye AP, Conrady B, Pedersen KS, Guardabassi L, Damborg P, 2023a. Neomycin resistance in clinical *Escherichia coli* from Danish weaner pigs is associated with recent neomycin use and presence of F4 or F18 fimbriae. *Prev Vet Med* 212:105852.
- [10] DANMAP, 2022. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. ISSN: 1600–2032.
- [11] SEGES, 2023. Current resistance assessment. Available at: https://svineproduktion.dk/services/~link.aspx?_id=382DEB84D0544179B39D38911AA80F61&_z=z (accessed 30/08/2023).
- [12] ANSES, 2022. Resapath - French surveillance network for antimicrobial resistance in bacteria from diseased animals, 2021 Annual report, Lyon et Ploufragan-Plouzané-Niort, France, November 2022, report, 46 pp.
- [13] UK-VARSS, 2022. Veterinary antibiotic resistance and sales surveillance report. New Haw, Addlestone: Veterinary Medicines Directorate.
- [14] SWEDRES-SVARM, 2022. Sales of antibiotics and occurrence of resistance in Sweden. Solna/Uppsala ISSN2001-7901.
- [15] Royal GD. Overzicht antibioticumresistentie van ziekteverwekkers. Available at <https://www.gddiergezondheid.nl/nl/Diergezondheid/Antibioticumgevoeligheid> (accessed 30/08/2023).
- [16] Subramani P, Menichincheri G, Pirolo M, Arcari G, Kudirkiene E, Polani R, Carattoli A, Damborg P, Guardabassi L, 2023b. Genetic background of neomycin resistance in clinical *Escherichia coli* isolated from Danish pig farms. Accepted in *Appl Environ Microbiol*.