Prevalence of Antibiotic Resistance Marker Genes (ARMG) in Selected Environments in Norway - Reindeer

M-909|2017

December 2017

genøk

GenØk-Centre for Biosafety, Tromsø, Norway
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Suggested citation: Nordgård, L. et al (2017) Prevalence of Antibiotic Resistance Marker Genes (ARMG) in Selected Environments in Norway – Reindeer. Project report, M-909|2017, GenØk, Tromsø, Norway.

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This project was commissioned by the Norwegian Environment Agency

Development of bacterial resistance to antibiotics is a growing problem in the world. The national strategy of the Norwegian government against antibiotic resistance for 2015-2020 highlights that this issue must be considered in a holistic perspective, where human and animal health and the environment interact and must be seen in context to each other. The presence of resistant bacteria in different natural environments, such as soil, fresh water, sea sediments and wild animals, has only been sporadically studied, although they may contribute to the development of resistance of clinical importance. There is therefore a need for more knowledge about antibiotic-resistant bacteria in different natural environments in general and in Norway in particular. The national strategy is based on the report "*Antibiotikaresistens-kunnskapshull og aktuelle tiltak (2014*)" prepared by an expert group. In this report, the need to assess the risk of a possible deliberate release of genetically modified organisms (GMOs) with antibiotic resistance marker genes (ARMG) is identified as one of the areas where more information is necessary.

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Acknowledgement
We thank Berit Tømmerås for assistance in the lab and Katrine Jaklin for editorial work.

Content

Summary

This project was commissioned by the Norwegian Environment Agency, and granted with 0,175 mill.NOK,, in order to map the naturally occurring prevalence of two antimicrobial resistance marker genes (ARMG), coding for kanamycin resistance, used in plant gene technology in Norwegian wild animals. The two genes are; neomycin phosphotransferase II (*NptII/aph(3`)-IIa*) and neomycin phosphotransferase III (*NptIII/aph(3`) IIIa (1, 2).*

Faecal samples from reindeers from respectively three different grazing districts have been analysed. The samples were collected over a period of three years (2013-2015). The reason for the choice of the reindeer-grazing district is based on assumed differences in exposure of drivers for antimicrobial resistance. The total number of samples from each district per year, ranged from 7-17 samples. In total 76 samples were analysed for the presence of ARMG.From the 76 samples analyzed, none of the samples tested positive for *nptII* and three samples tested positive for *nptIII*. The results indicate that the natural occurrence/background of *nptII* and *nptIII* genes in the selected samples are not prevalent during the testing period.

Norsk sammendrag

Dette prosjektet ble initiert på oppdrag av Miljødirektoratet, og bevilget 0,175 mill NOK, for å kartlegge den naturlige forekomsten av to antibiotikaresistensmarkørgener (ARMG), neomycin phosphortansferase II (*nptII/aph(3`)-IIa)* og neomycin phosphotransferase III (*nptIII/aph(3`) IIIa),*, hos viltlevende arter i norsk natur.

I dette prosjektet har vi studert feces-prøver fra reinsdyr i tre ulike reinbeitedistrikter. Prøvene ble hentet inn i en periode over tre år (2013-2015). Bakgrunnen for valg av reinbeitedistrikt er basert på antatt forskjeller i eksponering av human aktivitet og drivere for resistensutvikling. Det totale antall prøver fra hvert distrikt per år varierte fra 7-17 prøver. Totalt ble det analysert 76 prøver for forekomst av ARMG. Av de totalt 76 prøvene som ble analyser var ingen positive for *nptII* og kun tre prøver testet positiv for *nptIII*. Resultatene viser at den naturlige forekomsten/bakgrunnen av *nptII* og *nptIII* gener i de utvalgte miljøene er lav eller under deteksjonsgrensen i perioden vi har hentet prøver.

Abbreviations/descriptions

AMR	Antimicrobial resistance
ARB	Antimicrobial resistant bacteria
ARG	Antimicrobial resistance gene
ARMG	Antimicrobial resistance marker gene
bp	Base pair
DNA	Deoxyribonucleic acid
g	Gram
GM	Genetically modified
GMO	Genetically modified organism
HGT	Horizontal gene transfer
KmR	Kanamycin resistance
ml	Millilitre
ng	Nano gram
NORM/VET	Nasjonalt overvåkningssystem for antibiotikaresistens hos mikrober fra fôr, dyr og næringsmidler
NptII (Aph(3')-IIa	Neomycin phosphotransferase II gene
NptIII (Aph(3')-IIIa	Neomycin phosphotransferase III gene
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
WWTP	Waste water treatment plant
µl	Micro litre

Background

Over the last decade, increased resistance against antibiotics (AB) among bacteria in many different ecological niches like soil, wastewater treatment plants (WWTPs), river water, drinking water, seawater, sediments etc. has emerged (3-11). In these different environments, antibiotics, antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARGs) have routinely been observed.

The environment can be seen as a melting point for antimicrobial resistance, and the reasons for this are complex. First of all, it is important to remember that the occurrence of antimicrobial resistance in the environment may also result from the production of antimicrobials by certain groups of microorganisms in different ecological niches (12-14). In addition, the increased use of antibiotics in general combined with exposure to additional resistance promoting substances, such as disinfectants, biocides and some heavy metals is significant (6, 15, 16).

Antimicrobial resistance (AMR) is usually studied in bacteria from humans and domestic animals. However, for further insight into the dissemination and occurrence of AMR wild life has been suggested as a good source of information. It is well known that antimicrobial resistant bacteria are able to spread from contaminated sources like WWTPs, farm handling and other human sources to other ecological niches and probably also wild animals (17, 18). Wild animals are not naturally exposed to antimicrobials, but can interact with human waste and infrastructure in urban areas and function as a reservoir of ARB and ARG as well as provide dissemination over long distances (19).

The presence of ARMGs in genetically modified (GM) plants and possible large-scale release in the environment, or the use as GM plants in food or feed, has raised concerns over the past years regarding possible risks for human health and the environment (20-22). *NptII*, one of the most abundantly used ARMG in plant gene technology, encodes the neomycin phosphotransferase enzyme, that confers resistance to kanamycin, neomycin and geneticin. Kanamycin is presently not licensed for the treatment of infectious diseases in humans or animals in Norway, mainly because of adverse side effects. The development of resistance to other antibiotics may however make it more important in the future (23). *NptIII* is among the most prevalent aminoglycoside phosphotransferases in Gram-positive bacteria and the gene also gives resistance to amikacin, licidomycin and isepamicin which are more clinically relevant than kanamycin (24).

Both *nptII* and *nptIII* are present in a range of synthetic vectors and in naturally occurring plasmids and transposons, and may be horizontally transferred between different bacterial cells and strains (1, 25-29). One of the main concerns regarding the use of ARMGs in GM plants, is the risk of horizontal gene transfer (HGT) of plant-derived ARM genes to soil or gut bacteria

resulting in a reduced antimicrobial treatment of animal and human infectious diseases. This risk has been claimed to be very low, but cannot be excluded (30, 31). Although an event may be rare, it may have an ecological impact if the transferred gene alters the fitness of the recipient bacteria or cell (32). In this context, the need to monitor the presence and level of antibiotics and ARMGs in different ecological niches has been identified as one of the areas where more information is necessary. Norway is one of the most restrictive countries with regard to import and use of GMOs, and none of these plants are grown in Norway today. This might be changed in the future, and to be able to evaluate the significance of any release of GMOs containing ARMGs it is important to map and monitor the already existing background level of the specific ARMGs in microorganisms in different Norwegian environments.

The aim of this study was to describe the natural background load of antibiotic resistance marker genes (ARMGs) in fecal samples from reindeers grazing in three different Norwegian areas with different exposure of human activities. Reindeers live in the northern parts of the world, are highly migratory and travel in large herds. They are eating mostly grass-like plants and shrubs in summer, and lichen, which carpets the snow-covered forests, in the winter. Depending on their grazing area, some reindeers may interact with human waste and infrastructure while others are not exposed to human activity at all. In this study samples from three different reindeer-grazing districts, with assumed differences in exposure of human activity and drivers for antimicrobial resistance, have been analysed. The methodological approach for detection of ARMGs in this study is based on previous projects published by Nordgård et al. (22, 33).

Photo: adobestock.com/ belov3097. Reindeer used in the tourism industry in Tromsø.

Methods

Three different grazing areas in Norway were chosen with the intention to establish a baseline frequency of *nptII* and *nptIII* in feces samples from reindeers. The different areas, indicated in figure 1, were selected based on estimated different degrees of exposure from human activity and resistant driver substances.

Area 1: Tønsvik (Tromsdalen/Mauken, Tromsø) is considered as highly exposed to human activity. Parts of the herd that graze here are involved in a tourist enterprise with frequent interactions with people from all over the world in the winter season. Moreover, there is an industry and offshore harbor located in old Grøtsund fort in Tønsvik. We therefore also assume that resistance drivers may be present in the environment.

Area 2: Røros (Riast-Hylling) is considered as an area subject to some human contact, but less than in area 1.

Area 3: Varangerhalvøya (Rakkonjarga) is a large sub-arctic to low-arctic mountain territory with very little or no human activity.

All three areas selected in this study are already established sampling areas for various studies of reindeers within the project “"Infeksjonssjukdommer hos rein i kystnære beiteområder" (UiT/Reindriftens utviklingsfond; 2013-2016) led by Professor Morten Tryland (UiT). In this project, there was a joint sampling and data call for the various subprojects at already established monitoring stations for climate research. Agreements for collection of the sampling material were made with reindeer herders in the various districts.

The number of samples analyzed are listed in table 1. Polymerase chain reaction (PCR) was used for detection of the specific resistance genes. The methods are described in the appendix.

Table 1: Number of samples included in this study

	2015	2014	2013
Tønsvika	17	7	-
Røros	17	-	-
Tana	9	12	14

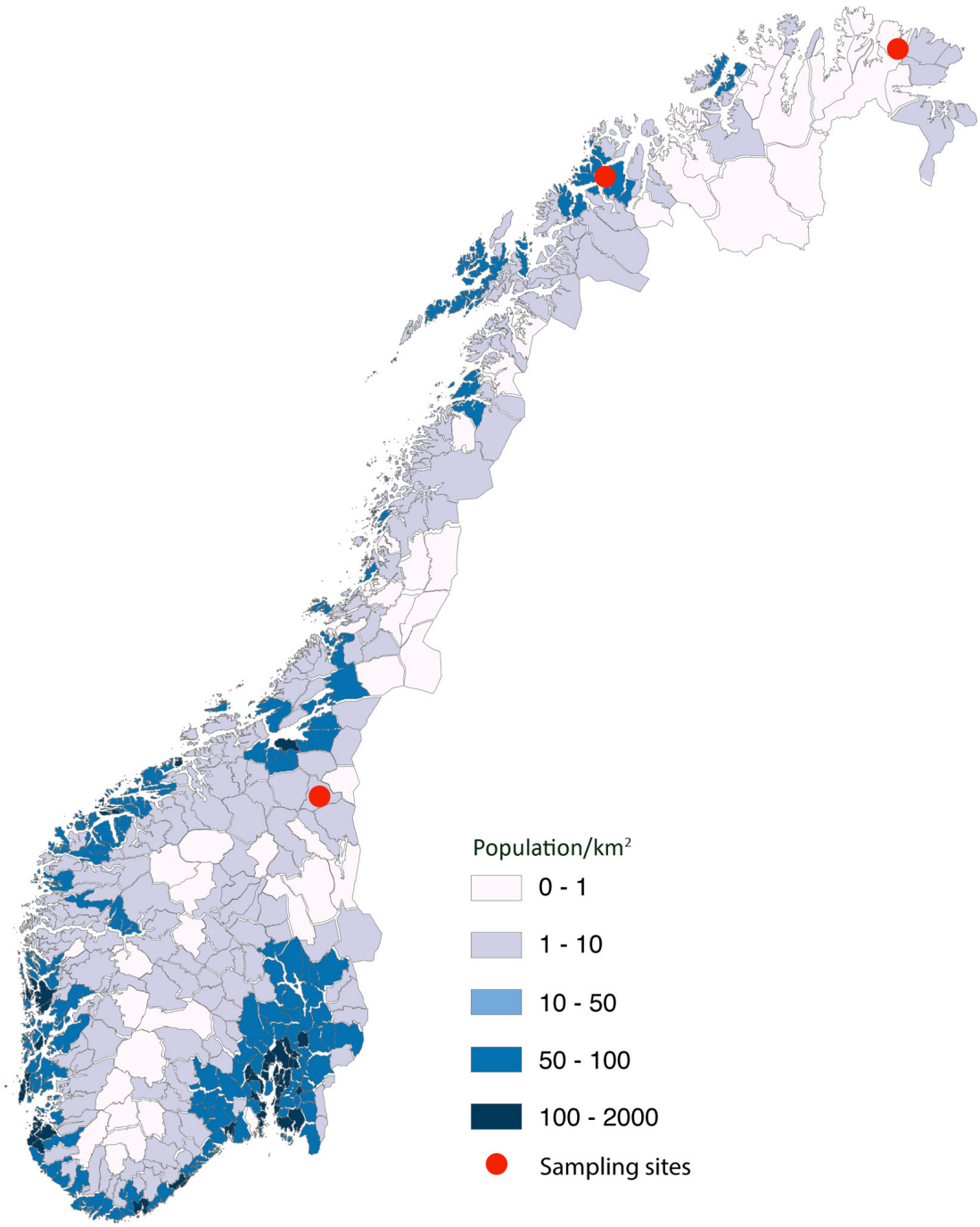

Figure 1: Illustrates the samples sites in red. The different colours of the map indicates population density. Tana, the northern most sampling site is located in an area with low population density, and Røros and Tønsvika have intermediate and high population densities, respectively.
Source: Statistics Norway. "Folkemengde og befolkningsendringar", Creative Commons Attribution-Share Alike 4.0 International license

Results

Each of the individual samples were analysed in duplicates by PCR, for the presence of *nptII* and *nptIII*. Two different primer sets were used for both *nptII* and *nptIII* (Table 2).

Table 2: PCR based detection of *nptII* and *nptIII* genes in different environmental samples

District	Number of samples tested (n=76)	16S positive (571bp) (n=75)	NptII short positive (129bp) (n=0)	NptII full positive (795bp) (n=0)	NptIII short positive (82bp) (n=1)	NptIII full positive (523bp) (n=2)
Tana 2015	9	8/9	0/9	0/9	0/9	1/9
Tana 2014	12	12/12	0/12	0/12	0/12	0/12
Tana 2013	15	14/14	0/14	0/14	0/14	0/14
Røros 2015	17	17/17	0/17	0/17	0/17	0/17
Tønsvik 2015	17	17/17	0/17	0/17	1/17	1/17
Tønsvik 2014	7	7/7	0/7	0/7	0/7	0/7

Photo: adobestock.com/Juhku

Discussion

To provide information on kanamycin resistance in the total bacterial assemblage in fecal samples from reindeers grazing in three different parts of Norway, total DNA was extracted and examined by PCR for the prevalence of *nptII* and *nptIII*. The results indicates that these specific antibiotic resistance marker genes (ARMG) are not prevalent in the samples tested in this study.

The 16sRNA gene was used as an amplification inhibition control in this screening study. All samples, except one, tested positive for 16sRNA by PCR. The *NptII* gene was not detected in any of the samples tested. The detection limit of a given molecular method is rarely zero, which means that the gene was not detected or that it was under detection limit of the specific method. From the 76 samples analyzed, three samples (3,9%) tested positive for the presence of *nptIII* gene, two from Tønsvika and one from Tana. The samples that are *nptIII* positive will be submitted for full-length sequencing to gather information of resistance gene sequence variation, in a follow up study.

A review of the scientific literature retrieves only few studies that have investigated the prevalence of *nptII* and *nptIII* genes in total DNA isolated from different environmental samples (21, 22, 24, 34-36). These studies show only a very low prevalence of these genes from total DNA isolated from non-clinical environments like soil, river water, sewage and manure, which supports our findings. In addition, the results are supported by the fact that none of our sample areas are affected by GM plant cultivation or release. The prevalence of these specific marker genes in total DNA isolated from fecal samples from wild animals has not been described in the literature, which makes the comparison of our results with other similar studies difficult.

Phenotypic antimicrobial resistance to different classes of antibiotics in faecal bacterial communities of different wild animals (birds, mammals and others) and in different countries is described in the literature (17, 19, 37-41). Among the different types of antibiotics tested for, kanamycin resistance has also been reported. In a study from 2005 (41), a low percentage of antimicrobial resistance in faecal *enterococci* from 77 wild animals in Portugal was demonstrated. In another study, faecal bacterial isolates from wild western lowland gorillas in Gabon, antimicrobial resistance against 12 different antibiotics was observed, and among them also kanamycin resistance (38). High level of kanamycin resistance were also observed in approximately 14% of *E.coli* isolates tested from wild birds species in Poland (37). These kanamycin resistant isolates harboured the *nptIII gene*. A study from Spain by Guerrero-Ramos, a low percentage of high level resistance (HLR) to kanamycin was observed in enterococci species tested from wild game meet in Spain. All *enterococcal* isolates showing high level kanamycin resistance contained the *nptIII* gene (19). High level resistance (HLR) to kanamycin with the presence of the *nptIII* gene, was also observed in *enterococcal* species

from wild animals in Spain (39). Some recent publication have also reported multidrug-resistant bacteria in wild birds and mammals from remote areas with no exposure of antibiotics (42, 43), which may indicate that AMR is a wider environmental issue of public health concern. In many of the studies of wild animals, the sample sizes are small. However, the prevalence of AMR in environmental samples indicates the need to monitor the antimicrobial resistance in wildlife and environments that are not directly exposed to antibiotic or antimicrobial resistance genes.

Only a few studies have been conducted related to antimicrobial resistance in fecal samples from reindeers. In Norway a joint NORM/NORM-VET report is issued annually with the focus to present data on the occurrence of antimicrobial resistance and the usage of antimicrobial agents in humans and animals for every year. Since the prevalence of antimicrobial resistance among certain bacteria of the normal enteric microflora can serve as an indicator for the selective antimicrobial pressure in various populations, studies on *E.coli* strains from fecal samples also from reindeers has been conducted. The NORM/NORM-VET report from 2003 (44), moderate occurrence of antimicrobial resistance among *E. coli* from fecal samples from wild reindeers was reported. In another study conducted in 2012, 107 *E. coli* isolates was isolated from faecal samples from reindeer. The results indicated that the prevalence of resistant *E. coli* in reindeers is low (45).
E. coli has also been isolated from 42 wild reindeer by Lillehaug et al in 2005 (46). The results in this study, demonstrated that ten (24%) of the reindeer isolates were resistant to one or more of the antibiotics tested for. In general, the antibiotic resistance pattern found in this study indicated low levels of resistant strains among the *E. coli* isolates tested.
In a study by Ramstad et al (47), *E. coli* strains from reindeers, living in remote environments in Norway and Svalbard was characterized for antimicrobial resistance. The main conclusion from this study was that known resistance genes were detected. Some strains also had several resistance genes closely arranged in their genome and some had resistance genes arranged in conjunction with replicons that may indicate plasmid location. In addition, resistance regions with high homology to plasmid regions previously described in bacteria from humans and/or animals were identified, in several strains. The prevalence of kanamycin resistance and *nptII* genes in the *E.*coli isolates has, however, not been investigated in these studies.

In general, comparison to previous results has been difficult in this report, since we could not find any other studies in the literature that has investigated the prevalence of ARMG in wild animals. This study was designed to meet the requirements from the assignment text to describe the prevalence ARMGs in reindeers. Cultivable bacteria were not included in this study due the natural movement of the reindeer in the winter season and the limited project period. By only using one methodological approach, there are certainly limitations. Cultivation of bacteria from reindeer faeces on selective media and further screening of kanamycin resistant colonies for the specific ARMGs, will be included in follow up studies. However, the PCR results of total DNA extracted from fecal samples also includes the bacteria that we are

not able to cultivate. This illustrates that each method and approach has its strengths and weaknesses, and the importance of combining different methods.

Limitations

There are many limitations/uncertainties related to how to assess ARMGs in natural environments. Many of these uncertainties are due to technical limitations, data interpretation and the fact that this study only represent a "snapshot" of the environment tested. In this study, some of the limitations are also due to the limited project period and can easily be followed up.

Sampling and sample size: samples from more than three reindeer grazing areas could be included to increase the number of samples.
Isolation of total DNA for PCR: may need optimization. Low yields and issues with amplification suggest the presence of inhibitors which may have reduced the probability of detecting the specific genes in our samples.
Positive resistance-gene-specific amplicons has not yet been confirmed by further sequencing due to limited project period, but will be included in follow up studies. The sequencing analysis will give information of resistance gene sequence variability in these wild life samples.
Quantitative PCR: could be included to determine the abundances of the specific ARMGs
Cultivation based methods: Cultivable bacteria was not included in this study due the natural movement of the reindeer in the winter season and the limited project period. This will be included in follow up studies.

Conclusions

This study suggests that the naturally occurring background level of *nptII* and *nptIII* genes in reindeers from three different areas in Norway is low or below the detection limit. From the 76 samples analysed, none of the samples tested positive for the *nptII* gene, and three of the samples, two from Tønsvika and one from Tana, tested positive for the presence of the *nptIII* gene. Given the low levels of these specific genes it cannot be excluded that the introduction of new/external sources of such genes will not increase the prevalence level over time. With the limited number of experimental studies available to resolve the uncertainties arising from introduction of ARMGs, there are still significant knowledge gaps, as well as uncertainties regarding ARMGs in natural environments.

Further information on the natural occurrence of *nptII/nptIII* in different relevant Norwegian environments is necessary to be able to provide quantitative assessment of the possible risk of introducing ARMGs into Norwegian environments. In addition, more experimental and epidemiological data are needed on the distribution of ARMGs and ARGs in general, in wildlife.

Photo: adobestock.com/Sam

Appendix

Total DNA isolation from reindeer stool samples

Duplicates of 0.2 g frozen samples was used to isolate the total DNA from reindeer faeces. DNA from the aliquots was extracted using the QIAmp DNA stool kit for pathogen detection (Qiagen), according to the manufacturer's instructions. In addition, DNeasy Power Clean Clenup kit from Qiagen, was used according to the manufacturers' protocol.

Quantity and quality of the purified DNA were determined using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The eluted DNA extracts were stored at -20°C until further analysis. 50/100 ng of the resulting DNA solution served as template for the *nptII/nptIII* screening as well as for the 16S rRNA PCR assay.

DNA isolation from bacterial control strains

Different *Acinetobacter baylyi* (*A. baylyi*) isolates served as controls in this study (see table 4). The bacterial isolates were extracted using Quickextract^{tm} DNA Extraction Solution 1.0 (Epicentre Biotechnologies) according to the manufacturer`s instructions, and the concentration and quality were assessed using Nano Drop. The DNA extracts were stored at -20°C until further analysis. One µl of a 50ng/µl dilution of the resulting DNA solution served as template for the *nptII/nptIII* screening as well as for the 16S rRNA PCR assay.

PCR amplification of the DNA extraction control 16S rRNA

The eluted DNA from environmental samples and bacterial colonies served as template for the bacterial 16S rRNA gene to confirm the general absence of PCR inhibitors and the successful isolation of bacterial DNA. The reactions were performed in a total volume of 20 µl containing the following: 1 µl of each primer (Sigma Aldrich) at 10 µM concentration, 10 µl mastermix (DreamTaq PCR Mastermix, Thermo Fisher), 5 µl water and 50/100ng template DNA. Maximum 3 µl template DNA was added to each reaction and the volume in each tube was adjusted with ddH_2O up to a final volume of 20 µl. Positive and negative controls (listed in table 4) were included in each PCR set-up.

The PCR conditions were as follows: 1 cycle of initial denaturation at 95˚C for 2 min, 30 cycles of denaturation at 95 ˚C for 30 s and annealing at 60 ˚C for 30 s and elongation at 72 ˚C for 40 s, one cycle of final elongation at 72˚C for 5 min. Primers and controls are listed in Table 3 and 4. The PCR products were run on 1 % SeaKem® LE Agarose

SeaKem agarose-gels (Lonza) stained with Gelred (Invirtogen). Quantitative DNA Marker ϕx174 BsuRI (HaeIII) Marker 9 (Thermo Sscientific) was used to show the proteins sizes. Visualization was performed using Gel Doc^{TM}XR 170-8170 (BioRad) with Quantity One 1-D analysis software version 4.5.2.

PCR amplification of *nptII* and *nptIII* genes in the *KmR* isolates and environmental samples

To determine if *nptII* and/or *nptIII* genes were present in the total DNA from the environmental samples and the kanamycin resistant bacterial colonies, two primer pairs, amplifying one short and one long fragment of DNA, were used for both *nptII* and *nptIII* (Table 3). The reactions were performed in a total volume of 20 µl containing the following: 1 µl of each primer at 10 µM concentration, 10 µl mastermix (DreamTaq PCR Mastermix, Thermo Fisher), 5 µl water and 50/100ng template DNA. Maximum 3 µl template DNA was added to each reaction, and the final volume was adjusted up to 20 µl with ddH_2O. Primers and controls are listed in Table 3 and 4, respectively.

For the amplification of *nptII* (long and short) and *nptIII* short, the PCR conditions were as follows: 1 cycle of initial denaturation at 95˚C for 2 min; 30 cycles of denaturation at 95 ˚C for 30 s, annealing at 60 ˚C for 30s and elongation at 72 ˚C for 40 s; and one cycle of final elongation at 72˚C for 5 min. For *nptIII* long the following pcr programme was used: 1 cycle of initial denaturation for 3 min at 94 ˚C; 35 cycles of denaturing at 94 ˚C for 40s, annealing at 55 ˚C for 40s and elongation at 72 ˚C for 40 s and a final extension step at 72 ˚C for 2 min. The long PCR products were run on 1 % SeaKem® LE Agarose-gels (Lonza) stained with Gelred, while the shorter fragments were run on 2 % agarose gels. The ϕx174 bsuri ladder from Thermo scientific was used. Visualization was performed using Gel Doc with Quantity One software.

Table 3. PCR primers (Eurogentech)

Target	Name	Size	Primer sequence **(5'-3' direction)**	Reference
16S rRNA	16S_F 16S_R	571 bp	TGGAGAGTTTGATCMTGGCTCAG CTTTACGCCCARTRAWTCC	(Woegerbauer et al. 2014)
nptII short	NptII_F NptII_R	129 BP	GATCTCCTGTCATCTCACCTTGCT TCGCTCGATGCGATGTTTC	(Woegerbauer et al. 2014)
nptII long	NptII:Full_F NptII:Full_R	795 bp	ATGATTGAACAAGATGGATTGC TCAGAAGAACTCGTCAAGAAGG	(Woegerbauer et al 2014)
nptIII short	NptIII_F NptIII_R	82 bp	ACATATCGGATTGTCCCTATACGAA TCGGCCAGATCGTTATTCAGTA	(Woegerbauer et al. 2014)
nptIII long	NptIII_Full_F NptIII_Full_R	523 bp	GGCTAAAATGAGAATATCACCGG CTTTAAAAAATCATACAGCTCGCG	(Vakulenko et al. 2003)

Controls used in this study

Table 4. Bacterial strains used as controls in this study

Strain	Relevance	Control	
***A. baylyi* ADP1200 Com+ (1200)**	Km (S)	Negative control	**(48)**
***A. baylyi* ADP1200 Com + Km^R**	Km (R), *nptII*	Positive control	**(48)**
***A. baylyi* BD413 JV28-Km^R**	Km (R), *nptII*	Positive control	**(49)**
***A. baylyi* ADP1200Com+Km_+**	Km (R), *nptIII*	Positive control	**(48)**
Water	DNAse/RNAse free water (Sigma)	Negative control	**-**

*all our bacterial strains controls were kindly provided from the Department of Pharmacy, UiT The Arctic University of Norway

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