

Antibiotic resistance in terrestrial wild mammal species in Norway - roe deer and wild reindeer as indicators species



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ISSN 1890-3290

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Commissioned by
The Norwegian Environment Agency



Reference
M-969|2018

Design Cover: Reine Linjer
Photo front page: Knut Madslie

Sammendrag

Antimikrobiell resistens (AMR) er et voksende problem over hele verden, og er også utbredt i miljøet. Bruk av antibiotika til mennesker og husdyr kan ha direkte innvirkning på miljøet, og ville dyr kan bidra til spredning av antibiotikaresistente bakterier og gener. Det er uklart hvilken rolle antibiotikaresistens i ville dyr har, til tross for at utbredelse og spredning av antibiotikaresistens er av stor betydning for menneskers helse. Nylige studier i Norge av antibiotikaresistens i bakterier fra villfugl og rødrev viser at slike studier er viktig for å oppnå en større forståelse av problemstillingen, og at ville dyr kan brukes som indikatorarter for overvåking av antibiotikaresistens i miljøet.

Målet med denne studien var å estimere forekomsten av AMR-bakterier hos to arter av hjortevilt (villrein og rådyr) som har en antatt forskjellig eksponering for menneskelige aktiviteter (og AMR-drivere) i Norge. Undersøkelsen viser at det generelt er lav forekomst av antimikrobiell resistens hos *E. coli* i tarmfloraen hos villrein og rådyr i Norge. *E. coli* ble isolert og sensitivitetstestet fra 230 av 265 (86.8%) villrein og fra 274 av 301 (91%) rådyr. Blant disse var hhv. 96.5% fra villrein og 93.8% fra rådyr fullt følsomme mot alle antibakterielle substanser det ble testet mot. Forekomst av resistens mot mer enn én antibakteriell substans var sjelden. Resistens mot streptomycin var det vanligste funnet og ble påvist i henholdsvis 1,7 % av *E. coli* isolatene fra villrein og i 5.1% av *E. coli* isolatene fra rådyr. Det er uklart hvordan denne resistensen mot streptomycin har oppstått, det kan være spredning fra husdyr eller menneskelig aktivitet, eller også på grunn av en eller flere naturlig forekommende AMR-drivere i miljøet.

Fra kun ett rådyr ble det påvist *E. coli* resistent mot 3. generasjons cefalosporiner (0.3%). Resistensen var forårsaket av det ESBL (ekstendert spektrum beta-laktamaser)-kodende genet *bla*_{CTX-M-1}. Dette er det første funnet av ESBL-produserende *E. coli* hos hjortevilt i Norge. Funnet var fra et rådyr jaktet i et område nær en av de største byene i Norge. Selv om ESBL-produserende *E. coli* nylig er rapportert funnet hos rødrev i Norge, er funnet noe overraskende. Det viser at bakterier resistente mot kritisk viktige antimikrobielle midler kan være tilstede til tross for et lite eller manglende seleksjonspress.

Flere og mer omfattende studier er nødvendig for å skaffe mer kunnskap om forekomsten og betydningen av antibiotikaresistens i ville dyr/miljøet, og i ett «En helse» perspektiv.

Summary

Antimicrobial resistance (AMR) is an emerging problem worldwide, and is widely spread in the environment. The use of antibiotics in humans, livestock or agriculture may have a direct impact on wildlife, and wild animals may provide a biological mechanism for the spread of antibiotic resistant genes. The wild reservoirs of AMR remain poorly understood, even if the occurrence and transmission are of paramount relevance to human health. Recent studies in Norway in wild birds and red foxes highlighted the importance of wild animals for the study of AMR, and its possible use as sentinel species for surveillance.

The aim of this study was to estimate the occurrence of AMR bacteria in two deer species (wild reindeer and roe deer), which have assumed different exposure to human activities (and AMR drivers) in Norway. This survey indicates that there in general is a low occurrence of AMR among *E. coli* of the intestinal microbiota of wild reindeer and roe deer in Norway. *E. coli* was isolated and susceptibility tested from 230 out of 265 (86.8%) wild reindeer, and from 274 out of 301 (91%) roe deer. Among the isolates obtained from wild reindeer and from roe deer, 96.5% and 93.8%, respectively, were fully susceptible to all the tested substances. Resistance to more than one substance tested for was rare. Resistance to streptomycin was the most commonly occurring resistance form and was detected in 1.7% of the *E. coli* isolates from wild reindeer and in 5.1% of the *E. coli* isolates from roe deer. Resistance to streptomycin cannot be easily explained, except for contamination from livestock animals or humans, or it being a natural form of resistance. The overall occurrence of *E. coli* resistant to 3rd generation cephalosporins was 0.3% in roe deer, and was mediated by *bla*_{CTX-M-1}. This is the first finding of an ESBL from a wild cervid in Norway. This isolate originated from a roe deer hunted in an area near one of the largest cities in Norway. While ESBL

has been recently reported in red foxes in Norway, its occurrence is surprising given the low selection pressure in Norway and highlights the existence of bacteria resistant to critically important antimicrobials in spite of absence of selection pressure.

Longitudinal and spatial broad studies should be prioritized in order to better understand this problem and elucidate the role of wildlife species in the spread of AMR in a One Health perspective, especially in ecosystems with relatively simple and well-characterized potential inputs of AMR, such as Norway.

Background

The Norwegian government has issued a national strategy against antimicrobial resistance for 2015-2020 [1], where it is emphasized that this problem needs a holistic approach, where human and animal health and the environment needs to be assessed in relation to each other. The respective Ministries are to follow up the strategy. Based on this, the Norwegian Environment Agency received an assignment from the Ministry of Climate and Environment to map reservoirs of antimicrobial resistance (AMR) in the Norwegian environment. The Norwegian Veterinary Institute (NVI) was commissioned to investigate the occurrence of antimicrobial resistance in terrestrial wild mammals, using bacteria isolated from wild, free-ranging roe deer (*Capreolus capreolus*) and reindeer (*Rangifer tarandus tarandus*) as an indicator.

Antimicrobial resistance (AMR) is an emerging problem all over the world, and the overall consumption of antimicrobials is considered as the major cause of this situation. Resistant bacteria and transferable resistance genes within bacterial populations may spread to other hosts. However, other factors may also be of importance for AMR dissemination. Chemical substances such as disinfectants, pesticides and other biocides, and heavy metals can for instance contribute to AMR development. All these factors are hereafter termed resistance drivers.

Moreover, there is a continuous exchange of bacteria between different niches in the ecosystem and resistant bacteria in different environments like soil, freshwater, the sea, sediments and wildlife can thus contribute to AMR dissemination. It is therefore a need to gain more knowledge of the occurrence of AMR in different environmental niches. Additionally, such new knowledge needs to be assessed for possible relationships with exposure to antimicrobials and other potential resistance drivers.

In Norway people account for 89% of antibiotic consumption [2]. In population dense areas there may be a higher probability of excretion of antibiotics and antibiotic resistant bacteria into the environment e.g. through garbage / waste and sewage, as well as from hospitals and other health institutions. The impact of the use of pesticides in agriculture settings remains poorly understood, and can greatly affect the accumulation / spread of resistance in the natural environment.

It is nonetheless recognised today that AMR is widespread in the environment and the result of the use of antibiotics in humans, livestock or agriculture may have a direct impact on wildlife. Such animals may provide a biological mechanism for the spread of antibiotic resistance genes.

The wild reservoirs of resistance remain, however, poorly understood, even if its origins and mechanisms are of paramount relevance to human health because of the increasing importance of zoonotic diseases as well as the need for predicting emerging resistant pathogens.

In wild animals, one of the first reports of AMR dates back to 1978, in Japanese wild birds [3], but since then and especially in the last two decades, several studies have identified AMR in different wildlife species (for a review on AMR in wildlife please refer to [4-7]). Even in remote ecosystems untouched by anthropogenic pressures such as the Arctic, AMR has been described in wildlife species [8, 9].

Despite these studies, the lack of continuous time and spatial surveillance of AMR in wildlife species has made it difficult to understand the mechanisms and variations in distribution and sources of AMR, and how these affects the global situation on resistance in humans, animals and the environment.

To better understand the dynamics of AMR in the environment, several studies have begun to consider the role of wildlife as bioindicators or sentinels for AMR [4, 10-13]. While birds often have been chosen as a study species, several studies have focused in terrestrial mammals such mice (*A. sylvaticus*), voles (*M. agrestis*, *M. glareolus*), red deer (*Cervus elaphus*), roe deer, red foxes (*V. vulpes*), Iberian wolves (*C. lupus signatus*), Iberian lynx (*Lynx pardinus*).

In Norway, a recent study on AMR in red foxes correlated the occurrence of resistance in the foxes with a gradient of human +population density (*Mo et al., submitted 2017; [14]*), showing the potential of using this species as a sentinel for AMR spread in the environment.

Both reindeer and roe deer are relatively stationary, though some have seasonal migration. While wild reindeer, mainly located in high mountain areas, likely have little contact with human activity, roe deer on the other hand inhabit areas of both low and high human population density (e.g. Oslo). Roe deer might hence be more prone to have contact with not only humans but also garbage and sewages. By monitoring AMR in wild reindeer and roe deer, we may be able to uncover differences between these two deer species, and relate them to human contact and its impact for the spread of AMR in the environment.

The aim of this study was to estimate the occurrence of AMR bacteria in two deer species (wild reindeer and roe deer), which have assumed different exposure to human activities (and AMR drivers) in Norway.

Material & Methods

Sampling

To obtain knowledge of the health status of Norwegian populations of wild ungulates, NVI carries since 1998 a yearly health surveillance program for cervids and musk ox (HOP) [15], on assignment from the Norwegian Environment Agency.

Regarding sampling, this study took advantage of the ongoing CWD eradication/surveillance program. The CWD program is based on the analysis of brain samples, but in 2017 an additional faecal sample was collected during the wild reindeer and roe deer hunt. Additionally, faecal samples from roe deer were collected from road fatalities in Oslo metropolitan area during necropsy by the Norwegian Veterinary Institute. Faecal samples from 301 roe deer and 265 wild reindeer were examined. One sample per animal was analysed.

The location of collected samples is presented in Figure 1.

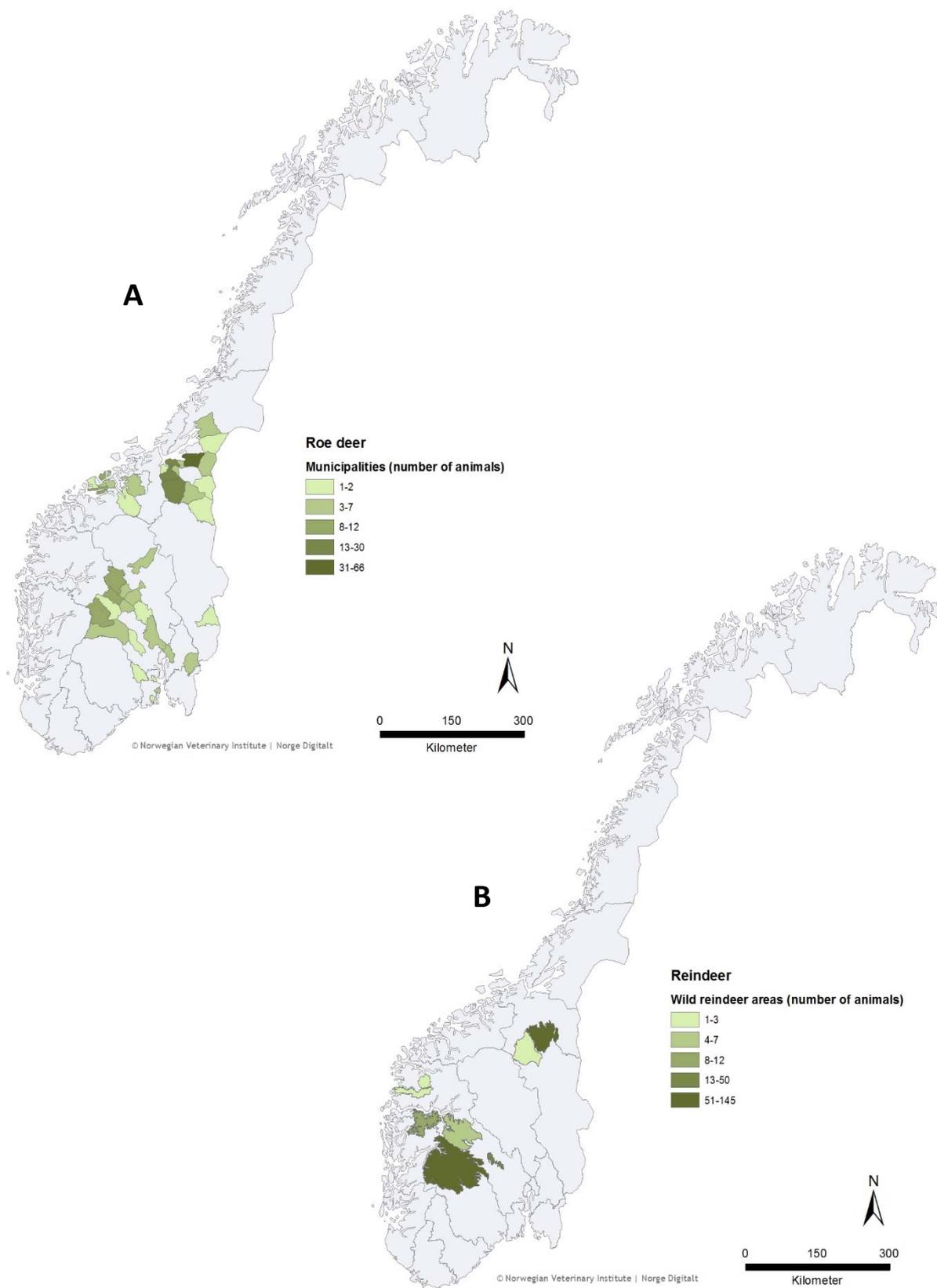


Figure 1. The locations of sampled roe deer (n=301), (A), and wild reindeer (n=265), (B). Number of samples collected per municipalities and wild reindeer area is coloured from light green to dark green, darker nuances show increased number of animals investigated

Laboratory methods

Two strategies for detection of resistant bacteria were used:

1. Non-selective culturing and inclusion of one randomly chosen *E. coli* (indicator *E. coli*) from each sample for testing of resistance against 15 different antimicrobials,
2. Selective screening for *E. coli* resistant to 3rd generation cephalosporins, carbapenems and quinolones.

Indicator E. coli

Faecal swabs were plated on MacConkey agar. The agar plates were incubated at 41.5±0.5°C for 24-48 hours. A single colony displaying typical *E. coli* morphology was randomly selected and sub-cultured on blood-agar. The isolates were confirmed as *E. coli* by a positive indole test.

Pre-enrichment of sample material

The swabs used for plating on MacConkey agar was then inoculated in 5 mL buffered peptone water (BPW-ISO) and incubated at 37±1°C for 20±2 hours.

3. generation cephalosporinase-producing E. coli

A total of 10 µL of the overnight enrichment was plated on MacConkey agar supplemented with 1 mg/L cefotaxime and MacConkey agar supplemented with 2 mg/L ceftazidime [16]. The agar plates were incubated at 41.5±0.5°C for 24-48 hours. Presumptive cephalosporin-resistant *E. coli* were sub-cultured on blood agar, and confirmed as *E. coli* using MALDI-TOF.

Quinolone-resistant E. coli

A total of 10 µL of the overnight enrichment was plated on MacConkey agar supplemented with 0.06 mg/L ciprofloxacin. The agar plates were incubated at 41.5±0.5°C for 24-48 hours. Presumptive QREC were sub-cultured on blood agar, and confirmed as *E. coli* using MALDI-TOF MS.

Carbapenemase-producing E. coli (CPE)

A total of 10 µL of the overnight pre-enrichment were plated onto chromID™ CARBA and chromID™ OXA-48 agar (bioMérieux, Marcy l'Etoile, France)[16]. The agar plates were incubated at 37±1°C for 24-48h. Presumptive CPE were sub-cultured on blood agar, and confirmed as *E. coli* using MALDI-TOF MS.

Susceptibility testing

Antimicrobial susceptibility testing was performed on all isolates. Minimum inhibitory concentration (MIC) values were determined using broth microdilution (Sensititre, TREK diagnostics LTD, Thermo Scientific), except for determination of streptomycin MICs that was performed with gradient strips (bioMérieux). *E. coli* were tested on the EUVSEC panel. *E. coli* displaying resistance to 3rd generation cephalosporins were additionally subjected to testing with the EUVSEC2 panel to determine the beta-lactam resistance profile. Susceptible *E. coli* ATCC 25922 was included as quality control in the susceptibility testing. In addition, *E. coli* K5-20 (AmpC, *bla*_{CMY-2}) and *E. coli* K8-1 (ESBL, *bla*_{CTX-M-15}) were included as quality controls for the EUVSEC2 panel and *E. coli* 2003-10-681 and *E. coli* 2002-10-702 were included as controls for the streptomycin MIC determination tests.

Detection of resistance genes

Isolates displaying resistance to critically important antimicrobials were investigated further and their resistance mechanisms confirmed. These included *E. coli* resistant to 3rd generation cephalosporins or to colistin. *E. coli* isolates displaying cephalosporin resistance with an AmpC phenotype were subjected to real-time PCR for detection of *bla*_{CMY-2} using previously published primers and probe [17]. If the real-time PCR result was negative, the isolates were subjected to PCR for detection of mutations in the promoter/attenuator region of the chromosomal *ampC* gene [18] and a multiplex PCR for detection of plasmid-mediated AmpC genes [19]. *E. coli* isolates displaying cephalosporin resistance with an ESBL phenotype were subjected to PCR for detection of *bla*_{TEM}, *bla*_{SHV} and *bla*_{CTX-M} genes [20, 21]. All PCR amplicons were sequenced to determine the gene variant responsible for the resistance phenotypes. A multiplex PCR for detection of *mcr-1* and *mcr-2*, encoding plasmid-mediated colistin resistance, was performed on two colistin resistant isolates [22].

Data processing

Susceptibility data were recorded and stored in the sample registration system at NVI as discrete MIC values. MIC-values for streptomycin obtained with gradient strips were transformed to the next MIC-value above the recorded value for intermediate values not included in Table 1. Data management and analysis was performed in SAS-PC System® v 9.4 for Windows (SAS Institute Inc., Cary, NC, USA). The 95% confidence intervals were calculated by the exact binomial test using R version 3.3.1 for Windows (R Development Core Team, 2016).

Results

Indicator *E. coli*

In total, *E. coli* was isolated and susceptibility tested from 274 out of 301 (91%) faecal samples from roe deer, and from 230 out of 265 (86.8%) faecal samples from wild reindeer. One isolate per positive sample was susceptibility tested. The occurrence of resistance to the different antimicrobial substances by species is shown in Figure 3. Detailed results from the susceptibility testing are presented in Table 1.

The occurrence of resistance was low among the isolates as shown in Table 1 and Figure 2. Among the isolates obtained from wild reindeer and from roe deer, 96.5% and 93.8%, respectively, were fully susceptible to all the tested substances. Resistance to more than one substance was rare with 0.4% of the isolates from roe deer resistant to two substances and 1.1% of the isolates from roe deer resistant to three substances (streptomycin and sulfamethoxazole and tetracycline or ampicillin).

Resistance to streptomycin was the most commonly occurring resistance form and was detected in 1.7% of the *E. coli* isolates from wild reindeer and in 5.1% of the of the *E. coli* isolates from roe deer.

A few isolates had MIC values to colistin above the cut-off value. These isolates were investigated by PCR for the colistin resistance encoding genes *mcr-1* and *mcr-2*, but came out negative for both genes.

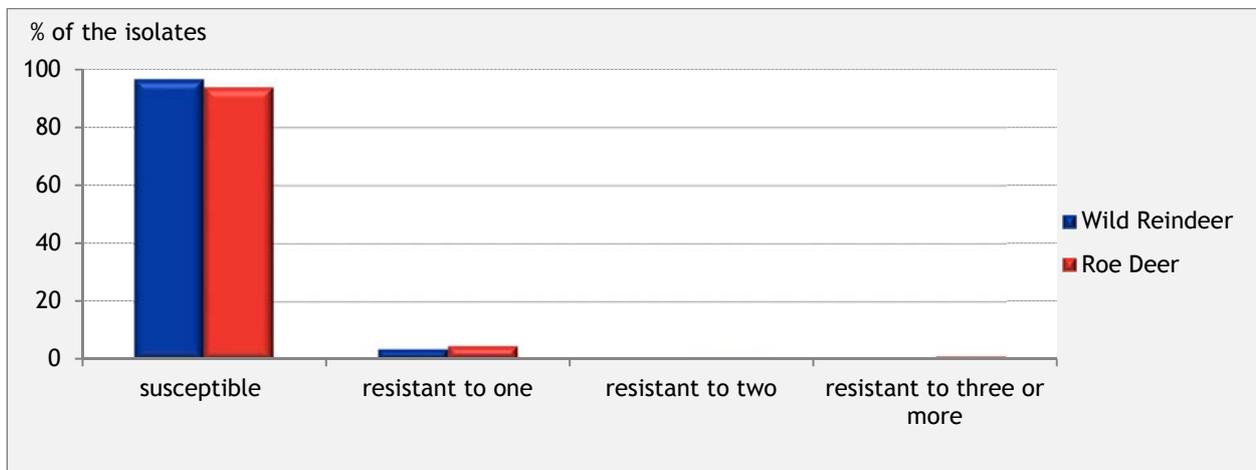


Figure 2. The antimicrobial resistance profiles among *E. coli* isolates (N=504) from wild reindeer and roe deer in Norway.

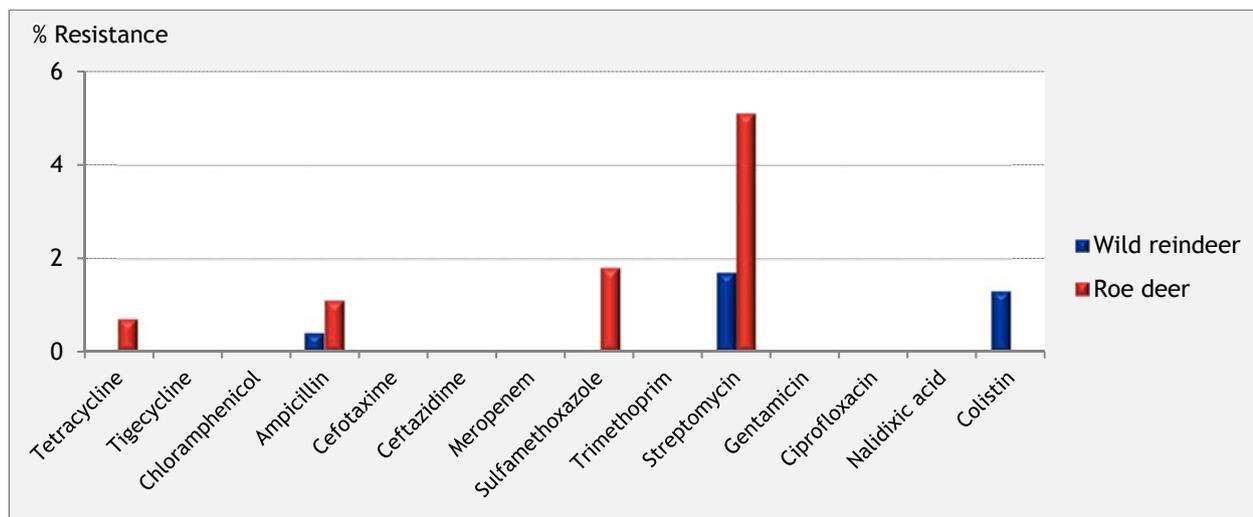


Figure 3. The prevalence of resistant *E. coli* isolates (N=504) originating from wild reindeer and roe deer in Norway.

Selective screening for resistance to important antimicrobials

Selective screening for *E. coli* resistant to 3rd generation cephalosporins and quinolones was performed on a total of 262 and 291 faecal samples from reindeer and roe deer, respectively. The selective screening for *E. coli* resistant to carbapenems was performed for 262 and 240 faecal samples from reindeer and roe deer, respectively.

The overall occurrence of *E. coli* resistant to 3rd generation cephalosporins was 0.0% (95% CI: 0.0-1.4) in wild reindeer and 0.3% (95% CI: 0.0-1.3) in roe deer. Only one isolate was from roe deer was resistant to 3rd generation cephalosporins. The genetic mechanism behind resistance to cephalosporins was investigated by PCR and sequencing and the *bla*_{CTX-M-1} gene was identified. Susceptibility testing showed that the isolate was resistant to beta-lactams and cefalosporins only, and not to any other classes of antimicrobials.

The overall occurrence of quinolone resistant *E. coli* was 0.8% (95% CI: 0.0-2.7) in wild reindeer and 0.3% (95% CI: 0.0-1.9) in roe deer. Two isolates from reindeer samples had increased tolerance to quinolones and the two isolates exhibited MICs above the cut-off values for both nalidixic acid and ciprofloxacin. The mechanism behind quinolone resistant in these isolates is probably due to mutations in chromosomally located genes. No co-resistance to other antimicrobials were found for the two isolates. From roe deer, one sample was positive for quinolone resistant *E. coli*. The MIC value for nalidixic acid was above cut-off, but for ciprofloxacin below cut-off. Mutation(s) in chromosomally located genes is probably the reason for increased tolerance to quinolones. Susceptibility data also showed that the isolate was resistant to streptomycin.

The overall occurrence of carbapenem resistant *Enterobacteriaceae* was 0% in both wild reindeer and roe deer samples indicating prevalences below 1.5% and 1.3%, in wild reindeer and roe deer, respectively.

Table 1. Antimicrobial resistance in indicator *E. coli* isolated from faecal samples of roe deer (n=274) and wild reindeer (n=230) in 2017.

Substance	Sample	Resistance (%) [95% CI]	Distribution (%) of MIC values (mg/L)*														
			0.015	0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256
Tetracycline	Roe deer	0.7 [0.1-2.6]									93.1	5.8	0.4				0.7
	Wild reindeer	0.0 [0.0-1.6]									96.5	3.5					
Tigecycline	Roe deer	0.0 [0.0-1.3]					97.1	2.9									
	Wild reindeer	0.0 [0.0-1.6]					98.7	1.3									
Chloramphenicol	Roe deer	0.0 [0.0-1.3]										97.4	2.6				
	Wild reindeer	0.0 [0.0-1.6]										97.0	3.0				
Ampicillin	Roe deer	1.1 [0.2-3.2]							0.4	28.1	63.9	6.6			0.4	0.7	
	Wild reindeer	0.4 [0.0-2.4]							0.9	29.1	57.4	12.2	0.4				
Cefotaxime	Roe deer	0.0 [0.0-1.3]					100										
	Wild reindeer	0.0 [0.0-1.6]					100										
Ceftazidime	Roe deer	0.0 [0.0-1.3]						100									
	Wild reindeer	0.0 [0.0-1.6]						100									
Meropenem	Roe deer	0.0 [0.0-1.3]		100													
	Wild reindeer	0.0 [0.0-2.4]		99.6	0.4												
Sulfamethoxazole	Roe deer	1.8 [0.6-4.2]										96.4	1.8				1.8
	Wild reindeer	0.0 [0.0-1.6]										98.3	1.3	0.4			
Trimethoprim	Roe deer	0.0 [0.0-1.3]					90.9	8.4	0.7								
	Wild reindeer	0.0 [0.0-1.6]					91.7	7.0	1.3								
Azithromycin	Roe deer	ND									19.3	46.4	32.8	1.5			
	Wild reindeer	ND									35.3	37.0	26.1	1.7			
Gentamicin	Roe deer	0.0 [0.0-1.3]						61.7	35.0	3.3							
	Wild reindeer	0.0 [0.0-1.6]					0.4	76.1	18.7	4.8							
Ciprofloxacin	Roe deer	0.0 [0.0-1.3]	78.5	20.8	0.7												
	Wild reindeer	0.0 [0.0-1.6]	82.2	17.4	0.4												
Nalidixic acid	Roe deer	0.0 [0.0-1.3]									98.9	1.1					
	Wild reindeer	0.0 [0.0-1.6]									100						
Colistin	Roe deer	0.0 [0.0-1.3]							98.5	1.5							
	Wild reindeer	1.6 [0.0-4.3]							97.8	0.9	0.9	0.4					
Streptomycin	Roe deer	5.1 [2.8-8.4]					0.7	35.4	55.5	2.9	0.4	0.7	2.9	1.1	0.4		
	Wild reindeer	1.7 [2.8-4.41]					2.6	11.7	4.8	34.4	41.7	2.6	0.4	0.4	1.3		

*Bold vertical lines denote epidemiological cut-off values for resistance. ND=cut-off not defined by EUCAST. CI=confidence interval. White fields denote range of dilutions tested for each antimicrobial substance. MIC values higher than the highest concentration tested are given as the lowest MIC value above the range. MIC values equal to or lower than the lowest concentration tested are given as the lowest concentration tested.

Discussion

This survey indicates that there in general is a low occurrence of antimicrobial resistance among *E. coli* of the intestinal microbiota of wild reindeer and roe deer in Norway.

Comparison to previous Norwegian results is possible for resistance data from wild reindeer, as two studies have been conducted earlier [23, 24]. Comparison to these studies is possible due to equal sampling procedure and inclusion criteria for isolates to be tested (indicator bacteria/faecal *E. coli* from healthy animals). In both the previous surveys the overall resistance rates were relatively low with 76.2% and 89.7% of the isolates being susceptible to tested substances [23, 24]. Similar results were also found in this study, as 96.5% of the tested isolates were fully susceptible to all substances included in the test panel. Comparison of results from these three studies has to take into consideration some small changes made in the panel of antimicrobial substances tested. However, these small changes will not have any major influence on the comparability.

The first survey on antimicrobial resistance from wild reindeer was performed in 2003 in Forollhogna wild reindeer area. A total of 42 *E. coli* isolates, all from different animals, were susceptibility tested using a broth microdilution method [23]. The resistance rates were in general low, however, a remarkably high occurrence of resistance to streptomycin was reported as 24% of the isolates were classified as resistant. The genetic mechanism for streptomycin resistance has been investigated and the genes responsible for resistance were the same as those commonly occurring in bacterial isolates from livestock and humans (data not published/*M Sunde personal communication*). One of the isolates was in-depth characterized and the *aadA1* gene, located within a class 1 integron, was responsible for streptomycin resistance. This is a common genetic element in resistant *Enterobacteriaceae* from humans and animals. However, the general structure of the integron was somewhat different in the reindeer isolate [25]. In addition to streptomycin resistance, a few isolates resistant to sulfamethoxazole and oxytetracycline were found.

The second surveys was conducted in 2012, and animals from the following three wild reindeer areas were sampled; Forollhogna, Rondane and Nordfjella. A total of 107 *E. coli* isolates, all from different animals, were susceptibility tested with a broth microdilution method. The overall resistance rates were low, but also among these isolates resistance to streptomycin occurred and was the most frequently found resistance form, with 6.5% of the isolates being resistant. Resistance to tetracycline, sulfamethoxazole and ampicillin was also found in a few isolates. In addition, one *E. coli* was classified as resistant to cephalosporins. Chromosomal mutations leading to upregulated AmpC production was found to be the mechanism behind this cephalosporin resistance [24].

Results from the present and previous studies carried out in Norway show that resistance to streptomycin occur to some extent among *E. coli* from wild reindeer. There is no clear explanation for these findings. One possible explanation is contamination from livestock animals (eg. domestic sheep grazing in reindeer habitats during summer) or humans, or it can be a natural form of resistance appearing as a response to a kind of selection pressure from the natural environment of the animals, like plants, fungi or moss. The usage of streptomycin in humans in Norway is minimal and the findings of streptomycin resistant *E. coli* from wild reindeers will not represent an important potential reservoir for resistant bacteria to humans and/or animals.

Comparison to previous Norwegian results is also possible for resistance data from roe deer, as one study have been conducted earlier [26]. Comparison to this study is possible due to equal sampling procedure and inclusion criteria for isolates to be tested (indicator bacteria/faecal *E. coli* from healthy animals). However, the study from 2005 only tested 44 *E. coli* isolates (from 45 different animals). In general, the results obtained in the two studies regarding resistance frequencies and resistance forms are similar.

Among the *E. coli* isolates from roe deer, one was identified as resistant to extended spectrum cephalosporins. Resistance to cephalosporines was mediated by the ESBL (extended spectrum beta-lactamase) encoding gene *bla*_{CTX-M-1}. This is the first finding of an ESBL producing *E. coli* from a wild living

cervid in Norway. A few studies from other countries have detected *E. coli* resistant to extended spectrum cephalosporins from deer; one finding from a red deer in Spain [12] and one finding from a red deer Poland [27]. The isolate from the Spanish study also carried *bla*_{CTX-M-1} [12]. In Norway, ESBL producing *E. coli* have earlier been detected from the intestinal flora of red foxes (*Mo et al., submitted 2017, [14]*) and from marine bivalve molluscs [28]. The finding of ESBL producing *E. coli* from various environmental niches in Norway indicates a possible “endemic” occurrence, albeit at very low frequency, in the environment. Such occurrence is surprising as the selection pressure from cephalosporin usage to humans and animals is low in Norway. The findings demonstrate that bacteria resistant to critically important antimicrobials can be present in environment samples, in spite of absence of selection pressure.

Internationally, it has to our knowledge not been performed such comprehensive and representative studies of AMR in wild reindeer as in the present study. A few studies have investigated *E. coli* isolates from roe deer, though the number of isolates included/animals sampled have been lower [27]. However, other studies have also shown that wildlife has the potential to serve as an environmental reservoir for AMR as previously described in the background of this study. In addition, an association between human population densities and occurrence of AMR in wildlife has been described, showing that wild animals living in highly populated areas are more likely to carry AMR bacteria compared to animals living in remote areas [29]. The only ESBL producing isolate detected in this study originated from a roe deer hunted in an area near one of the largest cities in Norway. In addition, wild animals living in areas with high livestock density have been shown to be more likely to be colonized with AMR *E. coli* compared to wild animals living in remote areas [30, 31].

The present study takes advantage of two different approaches for detecting and describing antimicrobial resistance in wild reindeer and roe deer. Both methods give important and complementary information. AMR in indicator *E. coli* is an international standardized method for investigating occurrence and follow trends in bacteria from feed, food and animals. For a low prevalent country as Norway, selective methods are necessary to follow the situation considering resistance to important antimicrobials, such as 3rd generation cephalosporins.

In Norway, a comprehensive study on antimicrobial resistance in wild red fox was recently conducted using the same sampling procedure and inclusion criteria for bacterial isolates (*Mo et al., submitted 2017, [14]*). Comparison of results from the two surveys showed that resistance frequencies for indicator *E. coli* were similar in red fox and cervids as 92.3% versus 94.8% of the isolates, respectively, were susceptible to all antimicrobial substances tested. However, more samples with isolates exhibiting resistance to critically important antimicrobials, such as 3rd generation cephalosporins and fluoroquinolones, were found from wild red foxes when selective screening was performed.

A recent report from the Scientific Opinion of the Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety [32] have concluded that it is most likely that some heavy metals such as copper, zinc and cadmium as well as some disinfectants such as phenols and quaternary ammonium compounds have a potential as resistance drivers. Information of possible contamination of such substances in different areas of Norway is limited. However, the use of and contamination with many such metals and compounds may also be correlated to population density.

To conclude; the results of the present study indicate that there is a low occurrence of antimicrobial resistance among *E. coli* of the intestinal microbiota of wild reindeer and roe deer in Norway. A low, but continuous occurrence of resistance to streptomycin in samples from rein deer is demonstrated. Furthermore, the first finding of an ESBL producing *E. coli* from a wild, free-ranging cervid was documented.

Future perspectives

Most AMR studies in wildlife are survey based and/or small scale, so one can only speculate on possible sources of AMR or the impact of wildlife AMR on clinical resistance. The long-term monitoring of wildlife species for AMR may therefore be an important tool for national and international AMR surveillance strategies. Wildlife can function as sentinel/early-warning systems for AMR spread and help characterize/understand the dynamic and mechanisms for resistance in the environment.

Ecosystems with relatively simple and well-characterized potential inputs of AMR, such as the case of Norway, can provide tractable and realistic systems for studying AMR in the natural environment. Especially in pristine environments such as the Arctic, it is of paramount importance to understand the spread of AMR and the risk/impact it may pose for the health and conservation of these wild species.

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Appendix

Definitions and classification of resistances used in this report

Epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, accessed February 2018) were used to categorize the isolates as susceptible or resistant, except for azithromycin for *E. coli* where only MIC-values are presented as no cut-off values have been defined (Table A1.). EUCAST definitions of clinical breakpoints and epidemiological cut-off values are presented at the web page: <http://www.srga.org/Eucastwt/eucastdefinitions.htm>. The terms and usage of this way of classification of resistance are further explained below.

Epidemiological cut-off values

ECOFFs are mainly used by epidemiologists and could indicate emerging resistance in the bacterial populations. Based on the distribution of the MIC or the inhibition zone diameter distribution, each bacterial population could, in an ideal case, be divided into two populations by a biphasic curve as shown in the example below (Figure A1). The curve to the left (blue) shows the susceptible or wild type distribution whereas the curve to the right (red) shows the resistant or non-wild type distribution. The green line indicates a possible ECOFF value applicable to the distributions in the example.

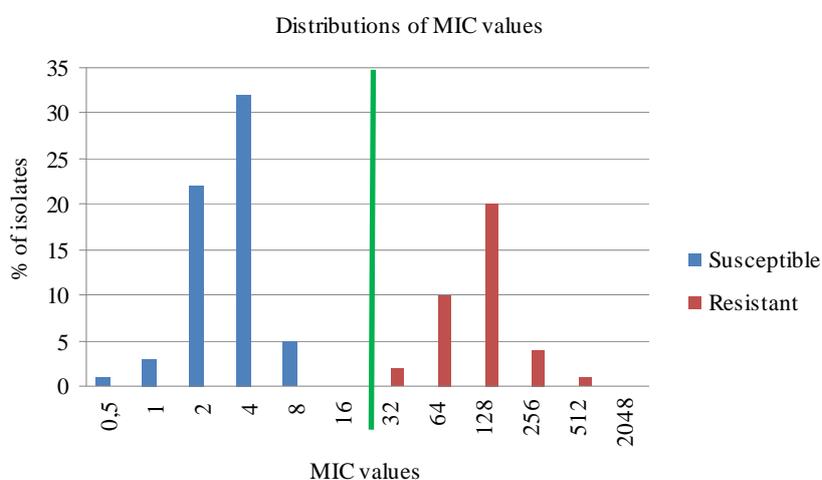


Figure A1. Example of a MIC-distribution of a bacterial population to an antimicrobial substance, blue staples = wild type or susceptible population, red staples = non-wild type or resistant population) and a possible epidemiological cut-off value, line in green.

However, for several bacterial populations and corresponding tested antimicrobial substances these distributions may be overlapping. A part of the population within the overlapping area may carry resistance mechanisms and others not. In the area with the non-wild type distribution, new resistance mechanisms are responsible for the resistance either alone or in addition to the resistance mechanisms present at lower MIC values. In order to establish MIC values for each specific bacterial population and antimicrobial substance, large amounts of data are needed.

Table A1. The epidemiological cut-off values (ECOFFs) used to define the isolates as susceptible or resistant for each substance included in the *E. coli* test panel.

Antimicrobial	ECOFF (mg/L)	Antimicrobial	ECOFF (mg/L)
Ampicillin	> 8	Meropenem	> 0.125
Azithromycin*	ND	Nalidixic acid	> 16
Cefotaxime	> 0.25	Streptomycin	> 16
Ceftazidime	> 0.5	Sulfamethoxazole	> 64
Chloramphenicol	> 16	Tetracycline	> 8
Ciprofloxacin	> 0.064	Tigecycline	> 0.5
Colistin	> 2	Trimethoprim	> 2
Gentamicin	> 2		

* ND = Not defined by EUCAST

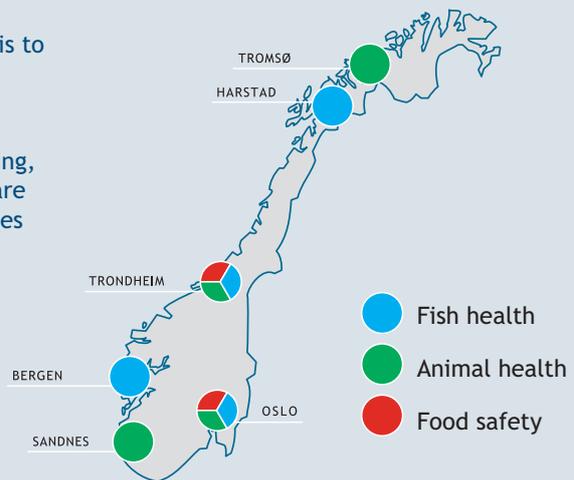
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