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Antimicrobial resistance in the Norwegian environment - red fox as an indicator

Veterinærinstituttet
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Content

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Sammendrag

Denne rapporten er laget på oppdrag av Miljødirektoratet for å kartlegge forekomst av antibiotikaresistens i miljø ved prøvetaking av tarminnhold fra rever felt under jakt i Norge i 2016. Reven er et rovdyr og øverst i næringskjeden. Den spiser blant annet fugler og smågnagere, men kan også spise matavfall og være i kontakt med kloakk eller gjødsel, og få i seg antibiotikaresistente bakterier fra alle disse. Reven anses derfor å være en god indikator for forekomst av antibiotikaresistens i miljøet.

De mottatte prøvene ble delt i tre ulike grupper basert på human populasjonstetthet for å reflektere mulig forskjeller i eksponering for drivere for resistensutvikling. Bakteriearten *Escherichia coli* (*E. coli*), som er en del av revens naturlige tarmflora, ble brukt som indikatorbakterie for å estimere forekomst av antibiotikaresistens. I tillegg ble viktige resistensformer undersøkt med selektiv metodikk.

Forekomsten av resistente *E. coli* var lav, 92,3 % av isolatene var følsomme for alle antibiotika som inngikk i testpanelet. Imidlertid varierte forekomsten mellom de tre gruppene. I områdene med medium og høy populasjonstetthet var det henholdsvis 4,7 % og 15,2 % av isolatene som var resistent mot minst ett av de antibiotika som inngikk i testpanelet. Det var statistisk signifikant forskjell mellom områdene med medium og høy populasjonstetthet. Multiresistens, definert som isolater resistente mot tre eller flere substanser i testpanelet, ble kun funnet i 2,4 % av isolatene.

Både kinolonresistente *E. coli* og *E. coli* resistent mot tredje generasjons cefalosporiner var uvanlig i områder med lav populasjonstetthet, mens begge disse resistensformene ble hyppigere funnet i områder med medium og høy populasjonstetthet.

Den selektive screeningen for kinolonresistente *E. coli* viste en relativ hyppig forekomst i de sydøstlige delene av Norge. Resultatene av den selektive screeningen for resistens mot tredje generasjons cefalosporiner viste at de resistente isolatene fra områder med medium og høy populasjonstetthet i hovedsak var assosiert med $bla_{CTX\text{-}M}$ gener, mens de resistente isolatene fra lavt eksponerte områder kun var isolater som hadde oppregulert AmpC produksjon som følge av mutasjoner i kromosomale gener.

Resultatene fra denne undersøkelsen indikerer at human populasjonstetthet har betydning for forekomst av antibiotika resistens (AMR) i norsk villfauna.

Summary

This report is commissioned by the Ministry of Climate and Environment to map the occurrence of antimicrobial resistance in the environment by sampling intestinal content of foxes hunted in Norway in 2016. The red fox is a top predator species, and can acquire AMR bacteria from consumption of prey, as well as it may interact with human waste and infrastructure (e.g. garbage and sewage). Further on, the red fox is distributed throughout Norway, hence a good indicator species for monitoring AMR in its environmental habitat.

The received samples were divided into three different groups based on human population density to reflect possible exposure to drivers for antimicrobial resistance related to human activity. The bacterial species *Escherichia coli* (*E. coli*), which is part of the normal intestinal microbiota of the fox, was used as indicator bacteria to assess the occurrence of antimicrobial resistance. Additionally, resistance to some selected critically important antimicrobial agents was investigated by the use of selective screening.

The occurrence of resistance in *E. coli* indicator bacteria was low as 92.3% were susceptible to all antimicrobials included in the test panel. However, the occurrence of AMR differed significantly between the medium and high population density areas with 4.7% and 15.2% resistant to at least one antimicrobial substance, respectively. Multidrug resistance (i.e. isolates resistant to ≥3 antimicrobial substances) was only detected in (2.4%) of the isolates.

Occurrence of both quinolone resistant *E. coli* and *E. coli* resistant to 3. generation cephalosporins detected by selective screening were uncommon in the low population density areas, whereas these resistance forms were more frequently detected in foxes in areas with medium and high population density.

The selective screening for quinolone resistant *E. coli* showed a relatively frequent occurrence in the southeastern parts of Norway. Furthermore, the selective screening of resistance to 3. generation cephalosporins showed that the resistance in isolates from low exposure areas was only due to chromosomal mutations resulting in an up-regulation of the chromosomal *ampC* gene. In medium and high population density areas, $bla_{CTX\text{-}M}$ genes were detected in four out of ten of the isolates resistant to 3. generation cephalosporins.

The results from the present study indicate that human population density, which reflects human activities, is a driver for the occurrence of antimicrobial resistance (AMR) in Norwegian wildlife.

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 this 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 was commissioned to investigate the occurrence of antimicrobial resistance in the environment, using bacteria isolated from red foxes (*Vulpes vulpes*) 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.

Wild animals are potential reservoirs of AMR bacteria. The red fox is prevalent and widely distributed throughout Norway, hence a good indicator species for monitoring AMR in the environment. In urban areas, red foxes may interact with human waste and infrastructure (e.g. garbage and sewage). Furthermore, the red fox is a top predator species, and can possibly acquire AMR bacteria from consumption of prey. The red foxes is highly adaptive concerning preferred habitat, particularly with regards to human population density, ranging from non-inhabited, remote areas to large cities. This broad geographical distribution enabled us to study the occurrence of AMR bacteria in foxes living in areas with different human population densities, which probably reflect possible differences in occurrence of, and exposure to resistance drivers.

The aim of this study was to estimate the occurrence of AMR bacteria in wild red foxes in three areas with an assumed different exposure to AMR drivers in Norway. The resistant bacteria were to be further investigated and the relevant resistance genes characterized.

Methods

Faecal samples from 387 foxes selected from the Norwegian monitoring programme for *Echinococcus multilocularis* were examined. One sample per fox was analysed.

The samples were divided in three different groups based on the population density in the municipality where the fox was sampled, with an assumed difference in exposure AMR drivers as follows;

- low exposure = <five inhabitants per km^2, (N=98)
- medium exposure = between five and 200 inhabitants per km^2, (N=200)
- high exposure = >200 inhabitants per km^2, (N=89)

The grouping of municipalities is visualized in Figure 1.

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 13 different antimicrobials,
2. Selective screening for *E. coli* resistant to 3. generation cephalosporins, carbapenems, quinolones or colistin, and for enterococci resistant to vancomycin.

All isolates were susceptibility tested by using a broth microdilution method. PCR and sequencing were used for detection of relevant resistance genes. Further details concerning the methodologies applied in this report are described in the appendix section.

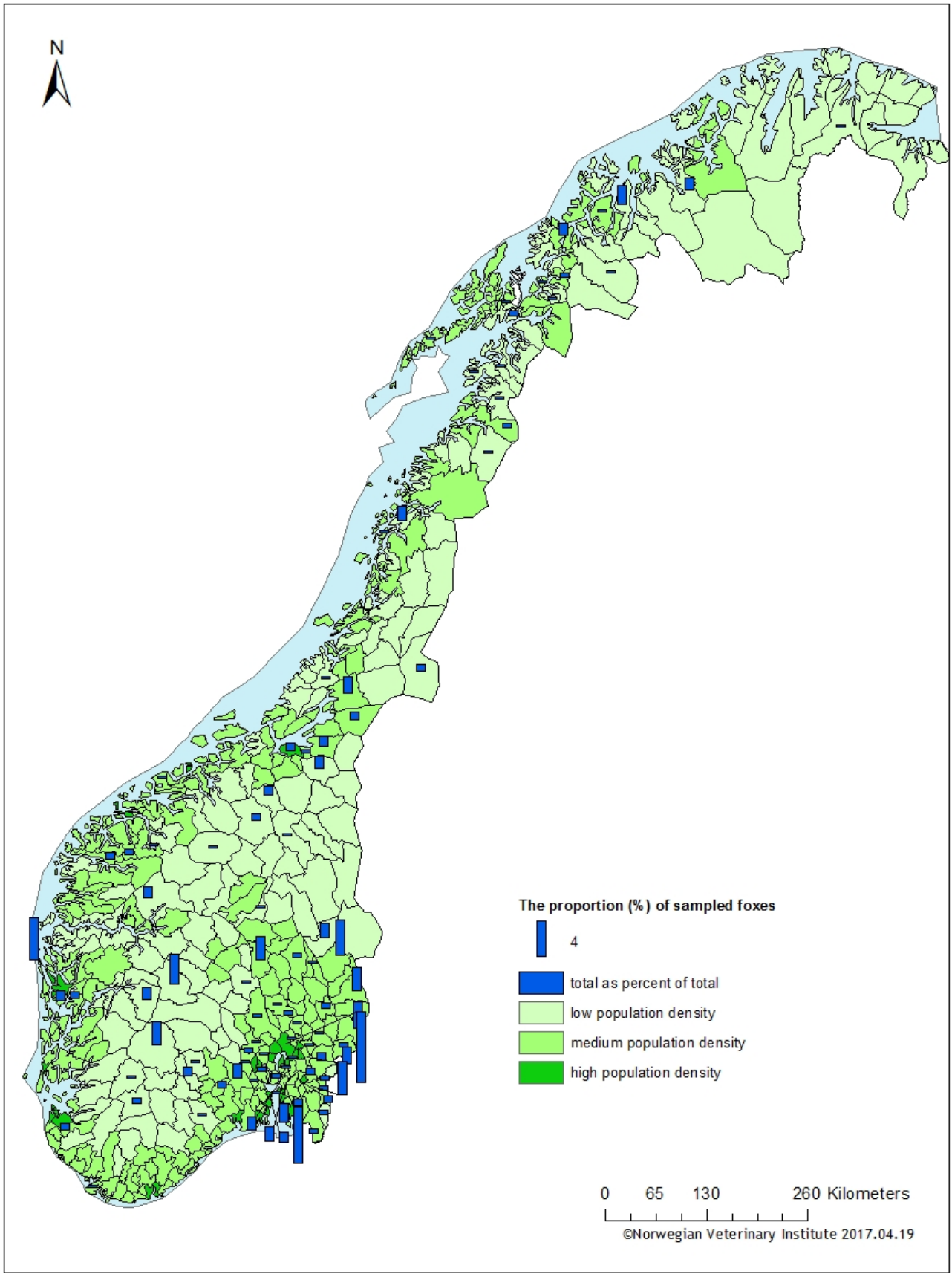

Figure 1. The proportion of foxes hunted and sampled (N=387) in the municipalities. Each municipality was categorized according to the human population density and an assumed equivalent exposure level of AMR drivers in; low exposure (n=89), medium exposure (n=171), and high exposure (n=66), as described in the material and methods section.

Results

Indicator *E. coli*

In total, *E. coli* was isolated from 326 out of 387 faecal samples from wild red foxes. The total number of isolates included for further susceptibility testing in the three exposure areas low, medium and high, were 89, 171 and 66, respectively. In total, 92.3% of the isolates were susceptible to all antimicrobial agents included in the panel. The occurrence of isolates resistant to at least one antimicrobial was 15.2% in the high exposure areas, whereas it was 7.9% and 4.7% in the low and medium exposed areas, respectively (Figure 2). Only the difference between the high and medium exposed areas was significant while differences between the other areas were non-significant.

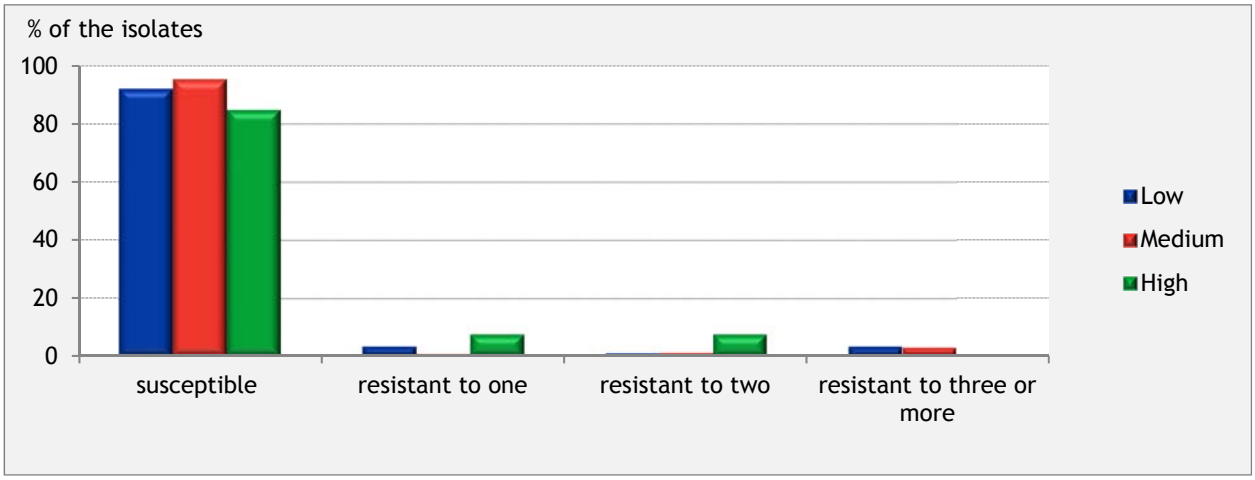

Figure 2. The antimicrobial resistance profiles among E. coli isolates (N=326) from wild red foxes in Norway. The samples were categorized according to the human population density to an assumed equivalent exposure level of AMR drivers in; low exposure (n=89), medium exposure (n=171), and high exposure (n=66) areas, as described in the material and methods section.

The occurrence of resistance to the different substances by exposure area is shown in Figure 3. Detailed results from the susceptibility testing are presented in the appendix section, Tables A2a and A2b.

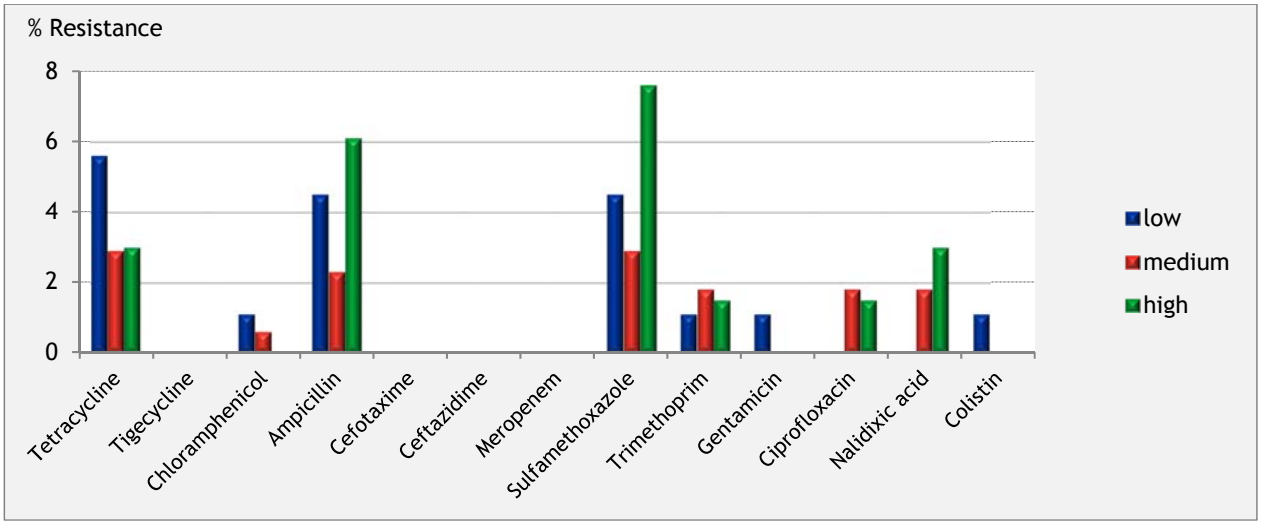

Figure 3. The prevalence of resistant *E. coli* isolates (N=326) originating from wild red foxes in Norway. The samples were categorized according to the human population density to an assumed equivalent exposure level of AMR drivers in; low exposure (n=89), medium exposure (n=171), and high exposure (n=66), as described in the material and methods section.

Resistance to sulfamethoxazole, ampicillin and tetracycline occurred most frequently. None of the indicator *E. coli* displayed resistance to the 3. generation cephalosporins cefotaxime and ceftazidime. Resistance to the quinolones ciprofloxacin and nalidixic acid occurred only in isolates from foxes hunted in areas with medium or high population density. Ciprofloxacin resistance was detected in 1.8% and 1.5% of the isolates, respectively, while resistance to nalidixic acid was detected in 1.8% and 3% of the isolates, respectively. One isolate resistant to colistin was detected among the samples from the low exposure area. None of the plasmid-mediated colistin resistance genes *mcr-1* or *mcr-2* were identified, indicating that the resistance phenotype probably is due to chromosomal mutations.

Multidrug resistance (i.e. resistance to ≥3 antimicrobial substances) was detected in eight (2.4%) of the isolates, and were from the low and medium exposure area (Figure 2). Resistance towards ampicillin, sulfamethoxazole and tetracycline was most frequent among these.

Bacteria resistant to important antimicrobials

Selective screening for *E. coli* resistant to 3. generation cephalosporins, carbapenems, quinolones or colistin, and for enterococci resistant to vancomycin was performed on a total of 387 faecal samples. *E. coli* displaying resistance towards carbapenems or colistin or enterococci displaying resistance towards vancomycin were not detected.

The overall occurrence of *E. coli* resistant to 3. generation cephalosporins was 3.4%, differing between the exposure areas as followed; 3.4%, 3.5% and 6.0% in the low, medium and high exposure areas, respectively. In the low exposure areas, resistance to 3. generation cephalosporins was found to be due to chromosomal mutations resulting in an up-regulation of the chromosomal *ampC* gene (Table 1). In the medium and high exposure areas, however, the $bla_{CTX\text{-}M}$ genes were detected in four out of ten isolates (Table 1).

The overall occurrence of quinolone resistant *E. coli* was 12.9%, with the lowest occurrence in the low exposure areas with 9.2% compared to medium and high exposed areas with 14.5% and 16.9%, respectively (Figure 5). In total, 32 out of the 50 detected quinolone resistant isolates (64%) originated from foxes hunted in the south eastern part of Norway. However, there was a higher number of samples (51.6%) obtained from this part of Norway.

Table 1. Overview of genes encoding 3. generation cephalosporin resistance in *E. coli* obtained by selective screening of faecal samples from red foxes in the three defined exposure areas low, medium and high.

Resistance genotype	Exposure area		
	Low	Medium	High
$bla_{CMY\text{-}2}$			1
$bla_{CTX\text{-}M\text{-}14}$			1
$bla_{CTX\text{-}M\text{-}1}$		1	
$bla_{CTX\text{-}M\text{-}15}$		1	1
Up-regulated chromosomal *ampC*	3	4	1

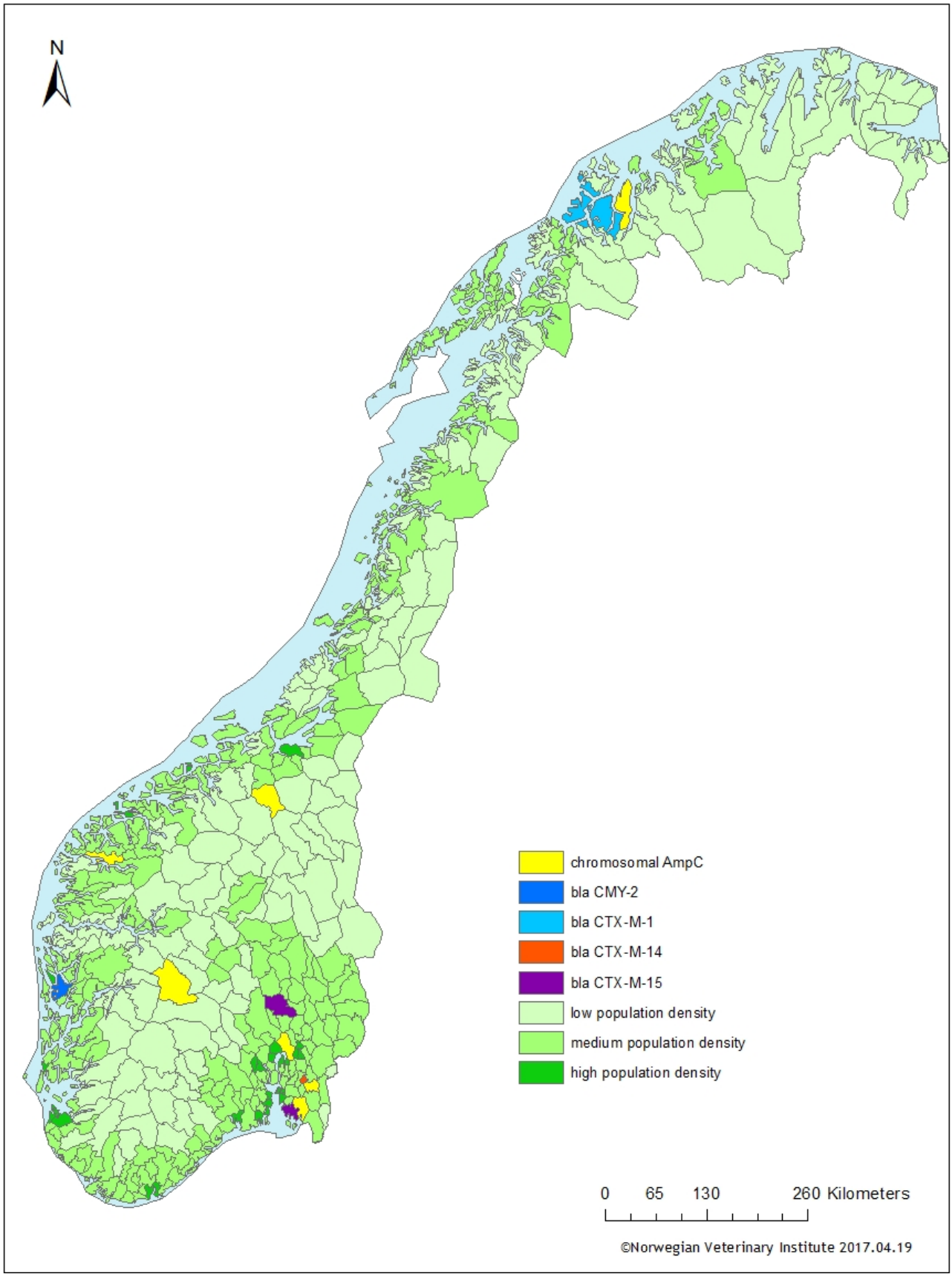

Figure 4. Distribution of genes encoding resistance to 3. generation cephalosporins in *E. coli* isolated from red foxes. Each municipality was categorized according to the human population density and an assumed equivalent exposure level of AMR drivers in; low exposure (n=89), medium exposure (n=171), and high exposure (n=66), as described in the material and methods section.

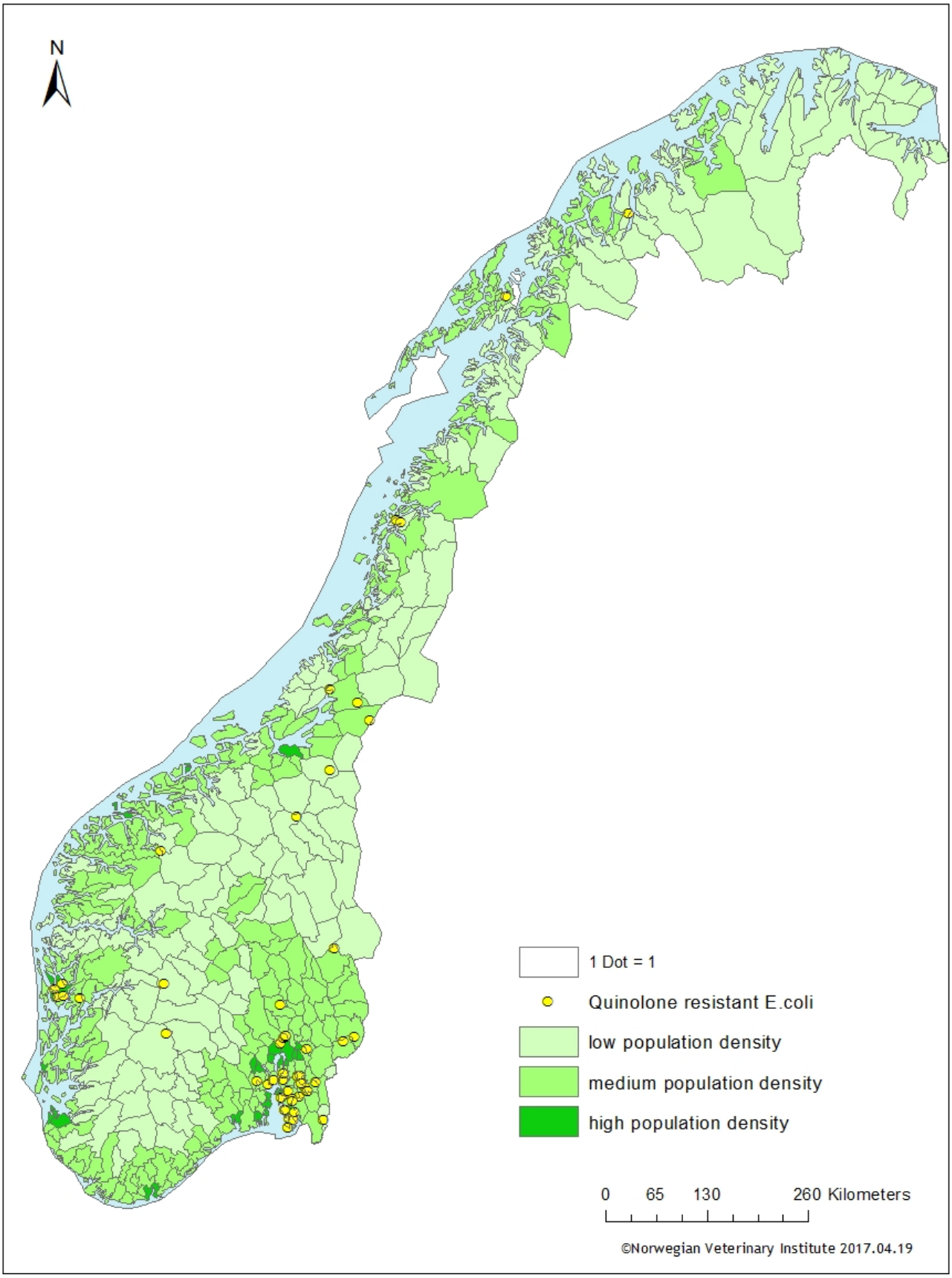

Figure 5. Occurrence of quinolone resistant *E. coli* (resistant to nalidixic acid or nalidixic acid and ciprofloxacin) in faecal red fox samples. Each municipality was categorized according to the human population density and an assumed equivalent exposure level of AMR drivers in; low exposure (n=89), medium exposure (n=171), and high exposure (n=66), as described in the material and methods section.

Discussion

This survey indicates that there in general is a low occurrence of antimicrobial resistance among *E. coli* of the intestinal microbiota of wild red foxes in Norway. Differences were observed between the investigated areas, indicating that the occurrence of AMR in *E. coli* from red foxes is higher in areas with a higher human population density and in areas close to the larger cities than in less populated areas. A similar difference between the studied areas was observed with regard to the occurrence of *E. coli* resistant to both 3. generation cephalosporins and quinolones. Both were uncommon in the low exposure areas, whereas more frequently detected in foxes from medium and high exposure areas, thus supporting that these areas are associated with a higher exposure to resistance drivers. Moreover, the results showed that the resistance to 3. generation cephalosporins in isolates from low exposure areas was only due to chromosomal mutations resulting in an up-regulation of the chromosomal *ampC* gene. In medium and high population density areas, $bla_{CTX\text{-}M}$ genes were detected in four out of the ten isolates resistant to 3. generation cephalosporins. These findings are further supported by the human AMR situation as the occurrence of *E. coli* resistant to 3. generation cephalosporins in humans are commonly associated with $bla_{CTX\text{-}M}$-genes [2].

Interestingly, the multidrug resistant isolates found among the indicator *E. coli* was isolated in the low and medium exposure areas, and the most frequent detected resistance was towards substances commonly used in agriculture. However, only a few isolates were detected and further studies are needed to investigate whether there is a true association.

The occurrence of quinolone resistant *E. coli* among the samples were more common than the occurrence of *E. coli* resistant to 3. generation cephalosporins. There was a clustering of the quinolone resistant isolates in the south eastern parts of Norway. However, this is an area of high population density, and the findings are likely to be associated with this matter. Finding of quinolone resistant *E. coli* in animal samples using selective screening has been shown to be quite common, though with variations associated with animal species [2-4].

Comparison to previous Norwegian results is difficult, as antimicrobial resistance in red fox has, to our knowledge, only been studied once, in 2010 [5]. The results then were not particularly different from the present study; 90.9% of the isolates were susceptible to all antimicrobial agents tested for compared to 92.3% in the present study. However, comparison has to take into consideration changes made in the panel of antimicrobial agents tested. Moreover, the previous study included only a limited number of samples from only one county in Norway, and the results are therefore not directly comparable to the present study. Internationally, it has to our knowledge not been performed such comprehensive and representative studies of AMR in red foxes as in the present study. However, other studies have also shown that wildlife has the potential to serve as an environmental reservoir for AMR [6-10]. 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 [11]. Also, 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 [12, 13].

The study was designed according to the three human population density categories as described in the appendix section, and meeting the requirements from the assignment text. A large proportion of the Norwegian population is living in the south eastern part of the country, especially in Oslo and the surrounding areas. The categorization between the medium and high exposure areas was set taking size of cities into consideration, so that very small cities were not categorized together with the largest Norwegian cities, and to reflect the high exposure areas with high human activity and low agricultural activity. Using the red fox samples from the *Echinococcus multilocularis* surveillance programme enabled a nationwide study. The surveillance programme is depending on voluntary participation of hunters, and neither hunters nor hunting results can be expected to be randomly distributed. As a result, the number of samples in each density category is not equal, with most samples from the medium population dense

areas. Moreover, some parts of Norway were not well represented, such as the south coastal area from which no samples were included. Still, the number of samples was sufficient to study the possible influence of human activity on AMR distribution to the environment.

The density of agriculture and different production animals is to some extent correlated to population density. However, the animal population density is more diverse and complex including a number of species. Therefore, the results are not analysed to assess if AMR might be associated to these factors alone.

The present study takes advantage of two different approaches for detecting and describing antimicrobial resistance in red fox. 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 3. generation cephalosporins.

A recent report [14] from the Scientific Opinion of the Panel on Microbial Ecology of the Norwegian Scientific Committee for Food Safety 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 human population density is associated to the prevalence of AMR in Norwegian wildlife, and consequently that human activity includes drivers for AMR occurrence accessible to the wild fauna. Foxes living in urban areas are more likely to be exposed to AMR bacteria (and possibly also antimicrobial agents as waste through garbage and sewage) from predating rodents, birds etc. contaminated with such AMR bacteria, and to waste water from hospitals and other facilities.

Future perspectives

This study emphasizes the possible association between population density and the occurrence of AMR in wildlife. The surveillance programme for *Echinococcus multilocularis* will continue in the following years and represents a unique possibility to use the collected material to monitor the occurrence of AMR in the Norwegian wild fauna represented by red foxes. The environmental burden of resistance in Norway could be continuously estimated and monitored in order to detect changes and future trends in the AMR pattern. Given the several active and passive surveillance and research activities conducted in different wildlife species in Norway, we believe that continuous monitoring of selected samples from different species, ecological niches and geographical areas could help to better understand the environmental burden of resistance in Norway, hence contributing to a better control of this problem in both humans and animals according to a true one health approach.

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Appendix

Classification of exposure areas

Three areas assumed to have different levels of exposure to antimicrobial usage or other resistance drivers were defined as followed:

- Low exposure: Areas with a low population density (below 5 inhabitants per square kilometre).
- Medium exposure: Areas with more than 5 inhabitants per square kilometre but less than 200 inhabitants per square kilometre.
- High exposure: Areas with more than 200 inhabitants per square kilometre.

Data regarding the estimated population density in 2015 (number of inhabitants per square kilometre and municipality) were derived from the Statistics Norway (accessed 19.09.2016).

The rationale for using the human population density instead of trying to combine the human population density and animal population density was based on the fact that the animal population density is more diverse including a number of species and by including the density of human population density alone the animal population is to a large extent covered. The breakpoints for assessing the areas as low, medium and high was assessed according to the knowledge that some areas like mountainous areas are very low populated and other municipalities should include the larger cities where agricultural areas are less likely to be present.

In total, 387 swabs of faecal samples from red foxes collected within the Norwegian surveillance programme of *Echinococcus multilocularis* in 2016 were included. This surveillance programme is upon request from the Norwegian Food Safety Authority (NFSA) and based on analyses of faecal samples from red foxes during the ordinary hunting season. Hunters from the entire country submit samples, and about 600 samples are examined each year. The faecal samples were in 2016 also analysed within the Norwegian Surveillance programme of antimicrobial resistance in feed food and animals (NORM-VET), which is also commissioned by the NFSA. The NFSA authorized use of the results from NORM-VET and to perform extended analyses of the faecal samples for the purpose of this study.

The samples received from the hunters are registered in the sample registration system (PJS) at the Norwegian Veterinary Institute with information regarding the municipality in which the fox was shot. Thereby, the fox could be allocated to one of the defined areas of exposure as described above. Most of the samples originated from the medium exposed area. Therefore, a representative random sample from this category was selected, in which at least one sample from each municipality was included. Thereafter, the number of samples included was proportional to the number of samples available for each municipality.

Laboratory methods

Indicator E. coli

Faecal swabs were plated on MacConkey agar. The agar plates were incubated at 41.5±0.5ºC 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.

Vancomycin-resistant enterococci (VRE)

Faecal swabs were plated on Slanetz and Bartley agar supplemented with 4 mg/L vancomycin. Agar plates were incubated at 41.5±0.5ºC for 48 hours. Typical colonies were sub-cultured on blood agar and confirmed as *Enterococcus faecium* or *Enterococcus faecalis* using MALDI-TOF MS.

Pre-enrichment of sample material

The swabs used for plating on MacConkey and Slanetz and Bartley was then inoculated in 5 mL buffered peptone water (BPW-ISO) and incubated at 37±1ºC 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 [15]. The agar plates were incubated at 41.5±0.5ºC 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±0.5ºC 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) [15] The agar plates were incubated at 37±1ºC for 24-48h. Presumptive CPE were sub-cultured on blood agar, and confirmed as *E. coli* using MALDI-TOF MS.

Colistin-resistant E. coli
A total of 10 µL of the overnight enrichment was plated on Superpolymyxin agar [16]. The agar plates were incubated at 41.5±0.5ºC for 24-48 hours. Presumptive colistin-resistant isolates 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). *E. coli* were tested on the EUVSEC panel. *E. coli* displaying resistance to 3. 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.

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 3. 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 one colistin-resistant isolate [22].

Data processing
Susceptibility data were recorded and stored in the sample registration system at NVI as discrete MIC values. 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).

Definitions and classification of resistances used in this report
Epidemiological cut-off values recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, accessed 19.05.2016) 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.

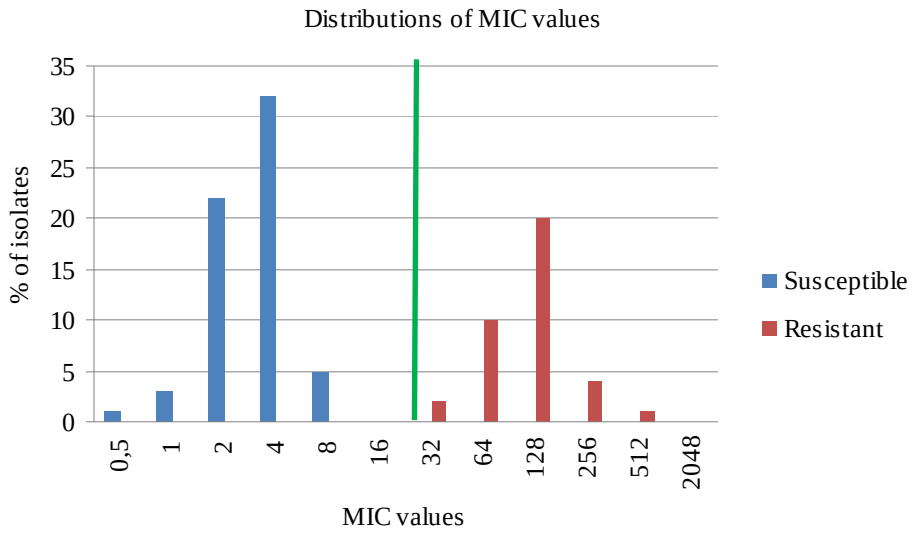

Figure A1. Example of a MIC-distribution of a bacterial population to an antimicrobial substance, blue staples = wild type or susceptible population, red staples = non-wild type or resistant population) and a possible epidemiological cut-off value, line in green.

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 agent, 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	Gentamicin	> 2
Azithromycin*	ND	Meropenem	> 0.125
Cefotaxime	> 0.25	Nalidixic acid	> 16
Ceftazidime	> 0.5	Sulfamethoxazole	> 64
Chloramphenicol	> 16	Tetracycline	> 8
Ciprofloxacin	> 0.06	Tigecycline	> 0.5
Colistin	> 2	Trimethoprim	> 2

* ND = Not defined by EUCAST.

TableA2a. Antimicrobial resistance in indicator *Escherichia coli* (n=326) isolated from faecal samples of red foxes (n=387) in 2016. The samples were categorized according to the human population density to an assumed equivalent exposure level of AMR drivers in low exposure (L), (n=89), medium exposure (M), (n=171), and high exposure (H) (n=66) as described in the material and methods section.

Substance	Category	Resistance (%)[95% CI]	Distribution (%) of MIC values (mg/L)*															
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	≥ 512
Tetracycline	L	5.6 [1.8-12.6]								94.4					3.4	2.2		
	M	2.9 [1.0-6.7]								97.1					1.8	1.2		
	H	3.0 [0.4-10.5]								93.9	3.0				1.5	1.5		
Tigecycline	L	0.0 [0.0-4.1]					96.6	3.4										
	M	0.0 [0.0-2.1]					97.1	2.9										
	H	0.0 [0.0-5.4]					98.5	1.5										
Chloramphenicol	L	1.1 [0.0-6.1]										98.9				1.1		
	M	0.6 [0.0-3.2]										98.2	1.2			0.6		
	H	0.0 [0.0-5.4]										98.5	1.5					
Ampicillin	L	4.5 [1.2-11.1]							1.1	29.2	53.9	11.2			4.5			
	M	2.3 [0.6-5.9]								33.9	59.6	4.1			2.3			
	H	6.1 [1.7-14.8]								34.8	51.5	7.6	1.5		4.5			
Cefotaxime	L	0.0 [0.0-4.1]					100											
	M	0.0 [0.0-2.1]					100											
	H	0.0 [0.0-5.4]					100											
Ceftazidime	L	0.0 [0.0-4.1]						100										
	M	0.0 [0.0-2.1]						100										
	H	0.0 [0.0-5.4]						100										
Meropenem	L	0.0 [0.0-4.1]		100														
	M	0.0 [0.0-2.1]		100														
	H	0.0 [0.0-5.4]		100														
Sulfamethoxazole	L	4.5 [1.2-11.1]										99.4	1.1			1.1		3.4
	M	2.9 [1.0-6.7]										96.5	0.6					2.9
	H	7.6 [2.5-16.8]										90.9	1.5					7.6

Table A2b. Antimicrobial resistance in indicator *Escherichia coli* (n=326) isolated from faecal samples of red foxes (n=387) in 2016. The samples were categorized according to the human population density to an assumed equivalent exposure level of AMR drivers in low exposure (L), (n=89), medium exposure (M), (n=171), and high exposure (H) (n=66) as described in the material and methods section.

Substance	Category.	Resistance (%) [95% CI]	Distribution (%) of MIC values (mg/L)*															
			0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	≥ 512
Trimethoprim	L	1.1 [0.0-6.1]					89.9	9.0							1.1			
	M	1.8 [0.4-5.9]					95.9	2.3							1.8			
	H	1.5 [0.0-8.2]					87.9	9.1	1.5						1.5			
Azithromycin	L	ND								56.2	34.8	7.9	1.1					
	M	ND								52.6	39.8	7.6						
	H	ND								50.0	34.8	15.2						
Gentamicin	L	1.1 [0.0-6.1]						65.2	29.2	4.5	1.1							
	M	0.0 [0.0-2.1]						73.1	24.6	2.3								
	H	0.0 [0.0-5.4]						60.6	30.3	9.1								
Ciprofloxacin	L	0.0 [0.0-4.1]	92.1	6.7	1.1													
	M	1.8 [0.4-5.9]	89.5	8.8			1.2					0.6						
	H	1.5 [0.0-8.2]	81.8	15.2	1.5		1.5											
Nalidixic acid	L	0.0 [0.0-4.1]									98.9	1.1						
	M	1.8 [0.4-5.9]									97.1	1.2				1.8		
	H	3.0 [0.4-10.5]									97.0			1.5		1.5		
Colistin	L	1.1 [0.0-6.1]						1.1	97.8		1.1							
	M	0.0 [0.0-2.1]						0.6	98.8	0.6								
	H	0.0 [0.0-5.4]							98.5	1.5								

*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 agent. 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.

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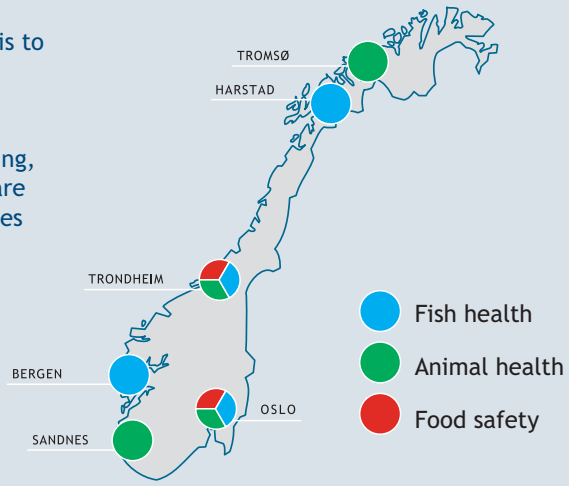

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