Mapping of antimicrobial resistance in marine mammals: Targeted PCR and metagenomic analysis

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Background

Antibiotic resistance, particularly antibiotic resistance among bacteria, is globally recognised as a threat to modern medicine, and thus a threat to human and animal health (World Health Organization, 2015). There is increasing appreciation for the fact that the environment plays a dynamic role in the issue of antibiotic resistance. It has become clear that the environment is both a source and a recipient of antibiotic resistance genes (ARGs) and resistant bacteria (ARB).

The national strategy of the Norwegian government against antibiotic resistance for 2015-2020 highlights that this issue must be considered in a holistic perspective. It is aligned with the One Health principle, and acknowledges that human and animal health and the environment interact and must be seen in context to each other. The identification and monitoring of the presence of ARB and ARRs in different environments is highlighted as one of the areas where more information is needed. The presence of resistant bacteria in different natural environments, such as soil, fresh water, sea sediments and wild animals, has however only been sporadically studied, although they may contribute to the development of resistance of clinical importance. This implies that there is therefore a need for more knowledge about antimicrobial resistant bacteria (ARB) and antimicrobial resistant genes (ARGs) in different natural environments in Norway.

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Summary

In this study faecal samples were collected from ten selected whales for analysis of antimicrobial resistance. The study is a part of a larger project and therefore builds on previous experiments where a culture-based approach was used to investigate the MIC values of bacteria isolates from 55 whale samples and 5 seals (Venter, 2019). Here we have extended the culture-based approach by a non-culture approach. Hence, this study combines the strengths of both a culture-based approach where resistance phenotypes can be investigated, and non-culture-based approach, which is not limited to microorganisms that can only grow under laboratory conditions.

Findings from previous experiments were published by Venter in 2019. Faecal samples from 60 marine mammals (55 minke whales and 5 seals) were tested for phenotypic resistance to a selection of antibiotics (ampicillin, cefotaxime, vancomycin, kanamycin, tetracycline, ciprofloxacin, erythromycin and trimethoprim). MICs were determined for 239 isolates from whale samples and 46 isolates from seal samples. For each antibiotic, isolates which were capable of growing at the maximum concentration were tested (32 mg/L). The MIC values for ampicillin and tetracycline were low compared to studies of other environmental samples. The highest number of isolates with an MIC of ≥32 mg/L was seen for trimethoprim, which also had an overall MIC range which was high compared to similar studies. This was followed by molecular testing for a selection of antibiotic resistance genes (ARGs) using standard PCR methods. The selected ARGs were *mecA, tetA, ermB, nptII, qnrs, dfrA1, vanA*, as well as two genes which code for multidrug resistance (MDR) efflux pumps, *acrB* and *mexD*. Only *nptII* failed to produce any amplicons. There was not a strong association between high MIC values and the detection of resistance genes (except for vancomycin and *VanA*), though this is likely due to the small selection of genes screened for.

Based on the findings from the first part of the project (Venter, 2019), we decided to continue the research with the most interesting samples based on bacteria isolates with the highest or most interesting MIC values. These findings were correlated back to the original whale samples, and it was decided to select samples from ten whales. From these selected whales total microbial DNA was extracted for targeted PCR for selected ARGs; *mecA, tetA, tetB, tetM, qnrS, erm(B), dfrA1, nptII,* and *nptIII. MDR efflux pumps; mexB*, *mexD, acrB* and *acrD*, were also included in this essay, and several of the analyzed samples were positive for one or more genes coding for antibiotic MDR efflux.

In addition to targeted PCR, the extracted microbial DNA from the whale faecal samples was subjected to shotgun metagenomics analysis, using the HiSeq platform of Illumina. The findings from the

metagenomic analysis shows that variants of *van* and *tet* genes, coffering resistance to vancomycin and tetracycline, respectively, were among the most prevalent. In addition, it was found one or more genes coding for antibiotic MDR efflux in the samples. The detected *tet* genes are different variants to those screened for in the targeted PCR analysis, and show the importance of combining different methodology in the case of environmental microbiology.

The results of this study reveal a snapshot of the antimicrobial susceptibility and resistance genes present in samples collected from marine mammals, and contribute to a growing body of evidence connecting the environment to dissemination of antibiotic resistance. The present study is limited in terms of scale, and continued research is required to fully understand the role of marine mammals in the context of maintenance and spread of antimicrobial resistance.

Sammendrag på norsk

I dette studiet har vi analysert antimikrobiell resistens i avføringsprøver fra 10 utvalgte vågehval. Denne studien bygger på tidligere eksperimenter der en kulturbasert tilnærming ble brukt for å undersøke MIC-verdiene til bakterieisolater fra 55 hval- og 5 selprøver, se rapport publisert av Venter (2019). Her har vi utvidet den kulturbaserte tilnærmingen med en ikke-kulturell tilnærming. Denne studien kombinerer dermed styrkene til både en kulturbasert tilnærming, som gjør det mulig å undersøke resistensfenotyper og ikke-kulturbasert tilnærming, som ikke er begrenset til mikroorganismer som kun vokser under laboratorieforhold.

I studie publisert av Venter i 2019, ble fekalprøver fra 60 marine pattedyr (55 vågehval og 5 sel) testet for fenotypisk resistens mot et utvalg av antibiotika (ampicillin, cefotaxime, vancomycin, kanamycin, tetracyklin, ciprofloxacin, erythromycin og trimetoprim). MIC ble fastslått for 285 av isolatene, 239 fra hvalprøver og 46 isolater fra selprøver. For hvert antibiotikum fantes det isolater som var i stand til å vokse i den maksimale konsentrasjonen som ble testet (32 mg/l). MIC-verdiene for ampicillin og tetracyklin var lave sammenlignet med studier av andre miljøprøver. Det høyeste antallet isolater med en MIC på ≥32 mg / L ble sett for trimethoprim, som også hadde et samlet MIC-område som var høyt sammenlignet med lignende studier. Dette ble fulgt av molekylær testing for et utvalg av antibiotikaresistensgener (ARGs) ved bruk av standard PCR-metoder. De utvalgte ARG var *mecA, tetA, ermB, nptII, qnrs, dfrA1, vanA*, samt to gener som koder for multidrug resistens (MDR) effluks pumper, *acrB* og *mexD*. Kun i testene for *nptII* ble det ikke produsert noen amplikoner (PCR-produkt). Det var ikke en sterk sammenheng mellom høye MIC-verdier og deteksjon av resistensgener (unntatt for vancomycin og *VanA*), men dette skyldes sannsynligvis det begrensede utvalget av gener som ble analysert.

Basert på funnene til Venter (2019), bestemte vi oss her for å fortsette analysene med de mest interessante prøvene basert på bakterieisolater med de høyeste eller mest interessante MIC-verdiene. Disse funnene ble korrelert med de opprinnelige hvalprøvene, og det ble besluttet å velge prøver fra ti hvaler. Fra disse utvalgte hvalene ble totalt mikrobielt DNA ekstrahert for målrettet PCR for følgende utvalgte antimikrobielle resistensgener (ARGs); *mecA, tetA, tetB, tetM, qnrS, erm (B), dfrA1*, *nptII*, og *nptIII*. De fire genene som koder for efflukspumper, *mexB, mexD, acrB* og *acrD*, ble også inkludert, og flere av de analyserte prøvene var positive for en eller flere av genene som koder for MDR efflux.

I tillegg til målrettet PCR ble det ekstraherte DNAet fra hval prøvene brukt for metagenomisk analyse ved HiSeq-plattformen til Illumina. Disse resultatene viser at varianter av *van*- og *tet*-gener, som gir resistens mot henholdsvis vancomycin og tetracykliner, var blant de mest utbredte. I metagenom-

undersøkelsen ble det også funnet varianter av MDR effluks pumper. De påviste *tet*-genene er av forskjellige gen-varianter enn de som ble screenet for i den målrettet PCR-analyse, og viser viktigheten av å kombinere flere metoder innen studier av ARG i miljøet. I metagenom-undersøkelsen ble det også funnet varianter av MDR effluks pumper.

Resultatene i denne studien viser et øyeblikksbilde av antibiotikaresistensen og av de ARG som er tilstede i prøver fra marine pattedyr. Studien er begrenset i omfang, men bidrar til økt kunnskap om forekomst og spredning av antibiotikaresistens i naturmiljøet. Det er nødvendig med videre forskning for å fullt ut forstå rollen marine pattedyr har i sammenheng med opprettholdelse og spredning av antimikrobiell resistens.

Introduction

In the decades since humans began the mass production of antimicrobial products (especially antibiotics (AB)), there has been an increase in cases of antimicrobial resistance the clinical level, which interferes with our ability to treat disease. A large and diverse range of antibiotic resistance genes (ARG) and antibiotic resistant bacteria (ARB) is found in the environment, which also could be found there before humans started using antimicrobial products, and it is likely that clinical antimicrobial resistance has its origins in environmental reservoirs (D'Costa *et al.*, 2011; Larsson *et al.*, 2018; Perry *et al.*, 2016). The capacity of microorganisms to share genetic material via horizontal gene transfer (HGT) means that genes present in the environmental resistome have the potential to move between bacterial communities, potentially transferring antimicrobial resistance genes to human and animal pathogens (Finley et al., 2013, Larsson, 2014). Drivers of antimicrobial resistance, such as antibiotics, biocides, and heavy metals, likely exert selective pressure in the environment which drives an increase in the prevalence and spread of ARGs and ARBs. Anthropogenic activities and the environment intersect in several ways when considering the issue of AB, ARBs and ARGs, as illustrated in figure 1.

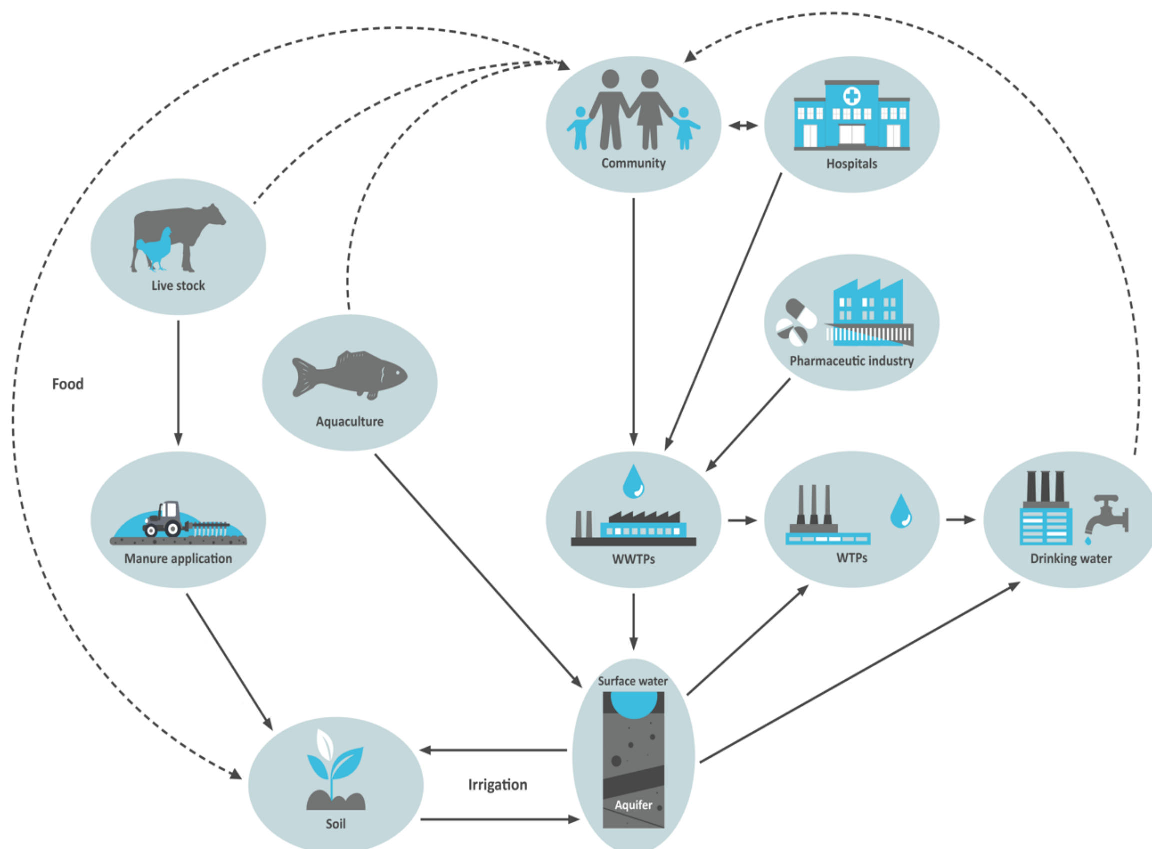

Figure 1: A representation of the intersections between human activity and environmental compartments in terms of the spread of antibiotics and antimicrobial resistance determinants (Illustration: Cathrine Brynjulfsen. All icons are designed by following contributors at Freepik.com: Macrovector, Ibrandify, Zirconicusso). First published in Nordgård et al., 2017a).

The presence of ARB 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 antibiotic resistance of clinical importance. There is therefore a crucial need for more knowledge about ARB and ARGs in different natural environments in Norway. There is also a need for better understanding of how antimicrobial resistance drivers present in the environment impact the prevalence and spread of resistance, how this intersects with spread facilitated by wildlife and water, and connects with human activities (VKM, 2018). Establishing a causal link between exposure to anthropogenic activities and resistance in animals has proven difficult (VKM, 2018). A recent project screened for resistance in marine mussels, and while this was detected, it did not correlate with areas of increased human activity (Svanevik et al., 2018).

Minke whales are highly mobile animals, migrating enormous distances from areas in the North Atlantic (around the coast of Norway and Greenland), to as far south as the waters around the South-Eastern United States and the Caribbean (Risch et al., 2014). Migratory animals are of particular interest when considering environmental reservoirs of ARB and ARGs, because in addition to being exposed to multiple sources of antimicrobials and drivers of resistance, they may also play a role in the spread of resistant bacteria and genes between different environments (Viana et al., 2016, VKM, 2018). Minke whales, with their extensive migration patterns, thus make good candidates for investigation of this concept.

Materials and Methods

Sampling

Minke whale samples were collected by Lotta Lindblom from the Institute of Marine Research in 2017 and 2018. The samples were collected in the Barents Sea (outside the Bear Island) and transferred to GenØk, where they were kept frozen until use. More information regarding the whales from which the samples were taken can be found in appendix 1. Other samples from the whales will be investigated by UiT- The Arctic University of Norway with regard to demographics, reproduction and diet, while the University of Oslo will perform the ecotoxicology studies (personal communication with Tore Haug (UiT)).

Methods

A brief summary of the methods is presented in the figure below. In the first part of this study three main phases of work were carried out (Venter, 2019): resuscitation of bacteria from samples, determination of minimum inhibitory concentration of various antibiotics, and screening for selected ARGs. In this study two phases of work were carried out: total DNA extraction from whale samples, and the isolated DNA was used either for PCR based detection of ARGs in total DNA or metagenomics analyses.

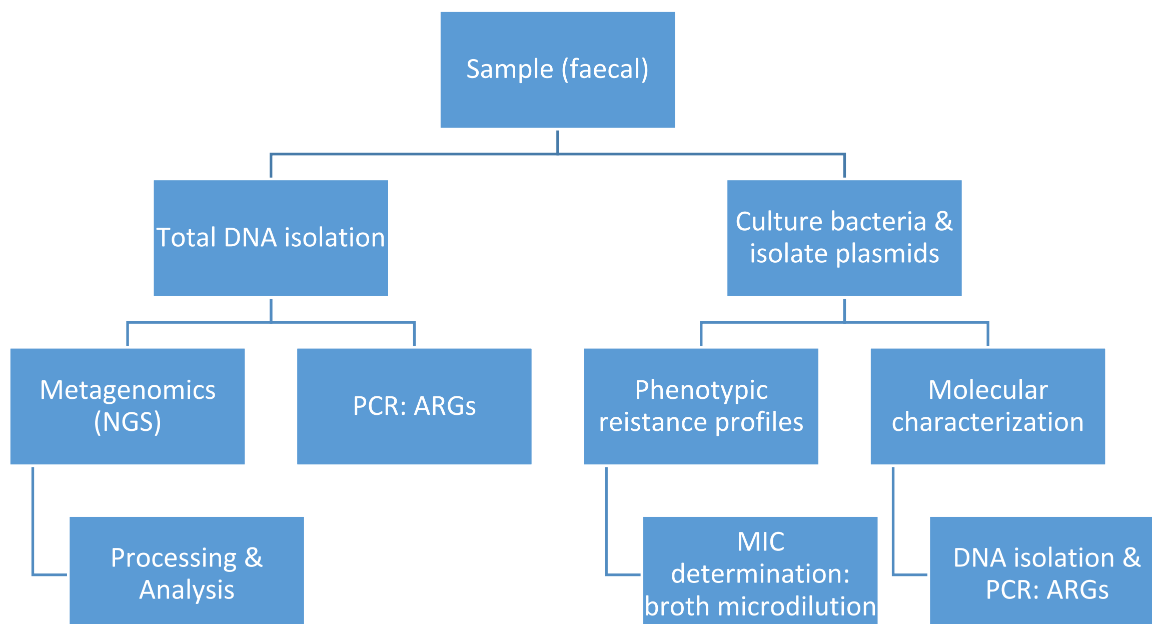

Figure 2. Methods used for molecular and phenotypic characterization of antimicrobial resistance.

For methods used in the culture-based approach, as resuscitation of bacteria from frozen samples, phenotypic characterization (Broth microdilution), and molecular characterization: DNA Isolation and PCR of ARGs, see Venter 2019.

Total DNA extraction from whale faeces

Based on interesting findings from the culture-approach (Venter, 2019), we decided to focus on the bacteria isolates with the highest or most interesting MIC values, and correlate these samples back to the original whale samples. These faecal samples were selected for more thorough analysis. The first step of the analysis was total DNA isolation from the samples. Total DNA was extracted from selected whale faeces samples with number 26, 30, 36, 37, 49, 50, 51, 86, 96 and 105 (see appendix 1) using the QIAamp DNA stool kit (Qiagen) according to the manufacturer's instructions. DNA was extracted in parallels of two from each faeces sample. Quantity and quality of the purified DNA were determined using a NanoDrop™ 2000 spectrophotometer (ThermoFisher Scientific, Wilmington, DE, USA). Additionally, DNA concentrations were measured using Qubit Fluorometric quantification (ThermoFisher Scientific, Waltham, MA, USA) employing the dsDNA High Sensitivity Assay as described by the Manufacturer. The eluted DNA extracts were stored at -20°C until further analysis.

PCR based detection of ARGs in total DNA

PCR experiments for the amplification of specific resistance genes were performed with DNA isolated directly from whale feces as described above. The selected resistance genes that were tested for were: *mecA, tetA, tetB, tetM qnrS, Erm(B), dfrA1, mexB, mexD, acrB, acrD, nptII,* and *nptIII.* Primers and additional information on the amplification of the selected genes are described in table 1, same as used in Venter (2019), and additional primers used is presented in table 2*.* The 16S rRNA gene was amplified as a control of the extracted DNA to confirm the general absence of PCR inhibitors and the presence of bacterial DNA. In general, all reactions were performed in a total volume of 20 µl containing the following: 1 µl of each specific primer (Sigma Aldrich) at 10 µM concentration, 10 µl mastermix (DreamTaq PCR Mastermix, Thermo Fisher), 6 µl water and 2 µl template DNA.

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 sec, annealing at 54-62 ˚C for 30 sec (see table 1 and 2 for specific annealing temperature) and elongation at 72 ˚C for 40 sec. Finally, one cycle of elongation at 72˚C for

5 min was added after the described 30 cycles. All PCRs were carried out on a BioRad ThermoCycloS1000. The PCR products were run on 2% agarose gels, using E-Gel® 1 Kb plus DNA ladder (all supplied by Invitrogen, Norway), for visualization. All PCR analyses included no-template controls and positive controls and were run in parallels of three.

Table 1. Selected ARGs and the primers used for their detection (Venter, 2019).

Antibiotic	Gene	Primer Sequences (5' - 3')	Annealing temp (°C)	Amplicon size (bp)	Reference
Tetracycline	*TetA*	CCTGATTATGCCGGTGCT TGGCGTAGTCGACAGCAG	61	200	(Szczepanowski et al., 2009)
Ampicillin	*mecA*	AAAAAGATGGCAAAGATATTCAA TTCTTCGTTACTCATGCCATACA	56	185	(Szczepanowski et al., 2009)
Kanamycin	*nptII*	ATGATTGAACAAGATGGATTGC TCAGAAGAACTCGTCAAGAAGG	55	364	(Börjesson et al., 2009)
Erythromycin	*Erm(B)*	GATACCGTTTACGAAATGG GAATCGAGACTTGAGTGTGC	58	364	(Chen et al., 2007)
Ciprofloxacin	*qnrS*	ATCAAGTGAGTAATCGTATGTACT CACCTCGACTTAAGTCTGAC	61	171	(Berglund et al., 2014)
Trimetoprim	*dfrA1*	ATGGAGTGCCAAAGGTGAAC TATCTCCCCACCACCTGAAA	62	241	(Grape et al., 2007)
Vancomycin	*VanA*	GGGAAAACGACAATTGC GTACAATGCGGCCGTTA	54	732	(Biavasco et al., 2007)
MDR efflux pump, RND family	*acrB*	ATATCCTACGATTGCACCGC GGTACCCGTGGAGTCACTGT	62	160	(Szczepanowski et al., 2009)
MDR efflux pump, RND family	*mexD*	TCAACGGTCTGGGTAACTCC GTCGATCAACAGGCGCAGT	62	182	(Szczepanowski et al., 2009)

Table 2. Additional selected ARGs and the primers used for their detection in this study of total DNA.

Antibiotic	Gene	Primer Sequences (5' - 3')	Annealing temp (°C)	Amplicon size (bp)	Reference
Tetracycline	*TetB*	TACGTGAATTTATTGCTTCGG ATACAGCATCCAAAGCGCAC	61	206	(Aminov et al, 2002)
	TetM	GCTTATTCCGGGGAAATTGT CGGGTCACTGTCGGAGATT	60	198	(Szczepanowski et al., 2009)
Kanamycin	*nptIII*	ATGGCTAAAATGAGAATATCACCG CTAAAACAATTCATCCAGTAAAATATA	60	795	(Woegerbauer et al., 2014)
MDR efflux pump, RND family	*acrD*	GGCAATCCTGTTGTGTCTGA ACATGAGATTATCGAGGCCG	62	185	(McArthur et al., 2013)
MDR efflux pump, RND family	*mexB*	GACCAAGGCGGTGAAGAAC AACACCTGGAAGTCACCGAC	62	147	(McArthur et al., 2013)

Metagenomic analysis

Metagenomic analysis is a modern approach that overcome the challenges and limitations of culture-dependent methods and amplification. By this approach direct genetic analysis of genomes contained with environmental samples is possible (Franzosa et al., 2015; Thomas, Gilbert, & Meyer, 2012). A metagenomics approach can be used alone or combined with a culture-based approach, which would provide more details about the bacteria present and the genes they carry. This would also allow detection of a greater number of resistance genes than in a culture-based approach, which until now has been the approach most often used in mapping of ARB and ARGs in environmental samples.

In this study total DNA extracted from ten whale faeces samples, animal 26, 30, 36, 37, 49, 50, 51, 86, 96 and 105 (see appendix 1), and were sent to the Norwegian Sequencing Centre (NSC) at the University of Oslo for metagenomic library preparation with the THRUplex kit (Rubicon) and sequencing on the Illumina HiSeq 3000/4000 platform.

The open source command line trimming tool Trimmomatic version 0.27 (http://www.usadellab.org/cms/index.php?page=trimmomatic) (Bolger et al., 2014) was used for processing of raw sequencing files. The recommended settings for Paired End sequences were used. Trimmed sequence files were then assembled using the open source assembler MEGAHIT v1.1.3 (http://www.metagenomics.wiki/tools/assembly/megahit) (Li et al., 2015).

Genes conferring antimicrobial resistance were identified using a local installation of the open source Resistance Gene Identifier (RGI) tool (v. 5.1.0) from the Comprehensive Antibiotic Resistance Database (CARD) version 3.0.4 (Jia et al., 2017, McArthur et al., 2013, McArthur and Wright, 2015). Homolog detection was performed using DIAMOND 0.8.36. “Loose” matches were allowed in addition to “strict” and “perfect” hits. The detected ARG matches were further categorized into groups according to antimicrobial resistance mechanism.

Results and Discussion

For results and discussion from the culture-based approach of phenotypic characterization (broth microdilution), and molecular characterization: DNA Isolation and PCR, see Venter 2019.

Molecular Characterization: DNA Isolation and PCR

In the previous study by Venter (2019) DNA was isolated from bacteria. We have included a brief presentation of the findings here. Venter carried out PCR with a selection of primers targeting various ARGs, including two genes targeting multidrug efflux pumps (*acrB* and *mexD*). In the case of the molecular characterization, in a total 600 selected isolates were screened.

Table 3 present a summary of the PCR results, listing amplicons which correspond to the length of the target sequence. Amplicons of incorrect size or multiple amplicons are not listed.

Table 3. Summary of PCR results after amplification with primers targeting selected ARGs (Venter, 2019).

Gene	Number of amplicons (Whale isolates)	Number of amplicons (Seal isolates)	Total
TetA	5	0	5
mecA	10	1	11
nptII	0	0	0
Erm(B)	2	0	2
qnrS	1	0	1
dfrA1	5	5	10
VanA	54	27	81
acrB	7	27	34
mexD	3	12	15

The highest number of amplicons were obtained for *vanA*, which confers resistance to vancomycin (Biavasco *et al*., 2007), which of all the genes tested also had the best association with high MIC values for the associated antibiotic. The genes coding for efflux pumps, *acrB* and *mexD*, which could potentially confer a degree of resistance to a number of antimicrobial compounds, also produced a high number of amplicons.

Amplicons of the correct size were generated for all primer sets except *nptII* (neomycin phosphotransferase II), which codes for kanamycin resistance. This gene is frequently used as an antibiotic resistance marker during the development of genetically modified (GM) crops (Nordgård et

al, 2016). Though thought to be virtually ubiquitous in some environments, *nptII* was not detected in the present study, nor in Nordgård *et al.* (2017b) who screened for the presence of the gene in reindeer faeces.

Association of the presence of amplicons of the expected size with MICs which indicate a level of resistance to a given antibiotic was not a given. For example, none of the isolates which displayed MIC values of 8->32 mg/L for tetracycline produced an amplicon of the correct size when amplified with *tetA* primers. Of the 5 *tetA* amplicons of the correct size, three belonged to isolates which came from the same whale sample (W61), and were associated with MIC values of 2-4 mg/L. However, Venter (2019) only screened for *tetA*, and there is a plethora of other genes associated with tetracycline resistance (Roberts and Schwarz, 2016), including several multidrug efflux pumps such as *acrB* and *mexD* (McArthur et al., 2013). Neither *acrB* nor *mexD* amplicons were associated with high tetracycline MIC values either, however, suggesting that other genes were responsible. Ampicillin and cefotaxime MIC values were also not closely associated with positive results with any of the primer sets.

The limited association of the selected target genes with the MIC values of the antibiotics suggests that a wider net should be cast in the detection of ARGs in such samples. Based on the findings by Venter (2019), we decided to continue with a) molecular characterization: PCR on total DNA, and b) a metagenomics analysis. In a previous study by Nordgård et al. (2017a), it was demonstrated that such an untargeted examination of ARGs was very useful in analysing environmental samples. A non-culture based approach would also compliment the previous culture-based approach, since the limitations of cultivation in terms of community representation (particularly in cases where environmental samples had endured periods of freezing at non-ideal temperatures (such as in this project)) can be overcome.

Molecular Characterization: PCR on total DNA from whale faeces

The overall occurrence of the fourteen selected ARGs was determined by ARG specific primers using DNA extracted directly from ten of the whale faeces samples. Given the difficulties in cultivating a substantial part of the bacteria from any environment, DNA based techniques, especially PCR, are the preferred techniques for examination of resistance genes present in total DNA.

The ARG targeted PCR showed that all ten whale faeces samples were negative for the majority of selected ARGs (*mecA, tetA, tetB, tetM, qnrS, erm(B), dfrA1*). All ten samples were, however, positive for one or more of the selected ARGs coding for the RND family of efflux pumps (*mexB, mexB, acrB, acrD*) as shown in table 4 (negative results indicated by the – sign, while positive results are noted with

a + sign). In total, seven of the samples were positive for *mexB* (W49, W37, W50, W51, W86, W96 and W105), and *acrD* (W26, W30, W50, W51, W86, W96 and W105). The bacterial DNA from W50 and W51 showed similar patterns of resistance, as the PCR detected four of the ARGs targeted in this screening. The three faeces samples from W26, W30 and W36 were only positive for one of the fourteen ARGs in this assay. These results indicate a low prevalence of enzymatic resistance in the bacteria present in the ten selected faces samples from minke whales. These results are in accordance with the MIC values measured on the collected isolates in this study. Unfortunately, *vanA* was not included in this PCR screening, although it showed to be detected in 54 isolates cultivated from whale faeces (table 3). The overall low MIC values for the tested antibiotics, illustrating a general low level of resistance of the whale gut microbiota, can be described by the presence of the detected genes coding for RND efflux pumps. The presence of efflux pumps have been reported as the major mechanism of resistance in previous screening of environmental samples in Norway (Nordgård et al, 2016).

Table 4. Summary of PCR results after amplification from total DNA extraction with primers targeting selected ARGs.

	beta-lactam	Tetracycline			Fluoro-quinolones	Macrolide	Trimetoprim	MDR-Efflux				Aminoglycosides	
Whale sample	*mecA*	*tetA*	*tetB*	*tetM*	*qnrS*	*erm(B)*	*dfrA1*	*MexB*	*MexD*	*acrB*	*acrD*	*nptII*	*nptIII*
W26	-	-	-	-	-	-	-	-	-	-	+	-	-
W30	-	-	-	-	-	-	-	-	-	-	+	-	-
W36	-	-	-	-	-	-	-	-	+	-	-	-	-
W37	-	-	-	-	-	-	-	+	-	+	-	-	-
W49	-	-	-	-	-	-	-	+	-	+	-	-	-
W50	-	-	-	-	-	-	-	+	+	+	+	-	-
W51	-	-	-	-	-	-	-	+	+	+	+	-	-
W86	-	-	-	-	-	-	-	+	+	-	+	-	-
W96	-	-	-	-	-	-	-	+	-	-	+	-	-
W105	-	-	-	-	-	-	-	+	+	-	+	-	-

It is important to keep in mind when interpreting these results, that when screening for ARG in the natural environment there are numerous variants of any given gene, and the primer specificity is important in this regard. The potential detection of specific gene variants in a metagenomics approach could prove important for primer design for further analysis with quantitative PCR.

As with the positive PCRs of the total DNA isolated from bacterial isolates, the positive results need to be verified by sequencing. Our results do not say anything about the quantities of the different ARGs. This can be followed up by a quantitative PCR analysis. A quantitative PCR would add more information about the extent of the total ARG reservoir in the different whale samples.

Detection of ARG using a metagenomics approach

Metagenomics is a modern approach that overcome the challenges and limitations of culture dependent methods and amplification (Schmieder & Edwards, 2012). By this approach, direct genetic analysis of genomes contained with environmental samples is possible (Franzosa et al., 2015; T. Thomas, Gilbert, & Meyer, 2012).

The RGI algorithm is a freely-available open-source algorithm designed to search the Comprehensive Antibiotic Resistance Database (CARD) antibiotic resistance ontology (ARO) for the presence of antimicrobial resistance genes in DNA sequences (McArthur et al., 2013). In this report, a local installation of the RGI (v. 5.1.0) and accompanying CARD (v.3.0.4) were utilized to search the metagenomes derived from total DNA isolated from eight of the whale faeces samples (W26, W30, W36, W49, W51, W86, W96, W105) for the presence of AMR genes. Assembled metagenomes (contigs) were input as query sequences into the RGI tool for two reasons: 1) assembly reduces the size of metagenome datasets through contig formation, making AMR gene identification less computationally intensive, and 2) assembly decreases sequence errors through increased coverage of individual nucleotide positions.

In total, the RGI was able to identify 237 matches with a cut-off at 75% identity to the CARD (v.3.0.4, February 2020) reference, among the eight assembled whale metagenome datasets. Of these 237 matches, only three hits had an identity percentage above 90% to the homologues available in CARD. Two of these matches indicated that the identified resistance was associated with mutations; one of the 23S rRNA subunit and the other of the *rpoB* gene, and should be addressed with caution. Moreover, resistance as a result of mutations were the case for a high number of the hits detected in several of the metagenomes analyzed. To verify these mutations and that they in fact result in resistance to a given antibiotic, requires further analysis. The third hit, with a percentage identity above 90%, was the gene identified as *mupA,* detected in the metagenome of W30. This gene is a gene conferring resistance to mupirocin, an antibiotic used in the treatment of skin infections caused by *Staphylococcus haemolyticus* (do Carmo Ferreira et al., 2011).

Figure 3 shows a summary of the genes with a percentage identity above 75%. It does not include resistance caused by mutations. The metagenome of W51 showed to be the most diverse and numerous, with several genes coding for tetracycline (*tet36, tetS, tetW*) and vancomycin (*vanRG, vanRI, vanUG*) resistance genes. Overall, variants of *tet* genes, resulting in resistance to tetracycline, were detected in three of the metagenomes (W26, W30, W51), while variants of *van* genes, conferring

resistance to vancomycin, were detected in all six metagenomes reported in Figure 3. This is in accordance with the results presented in table 3, and the identification of vancomycin resistance in cultivated isolates from whale faeces.

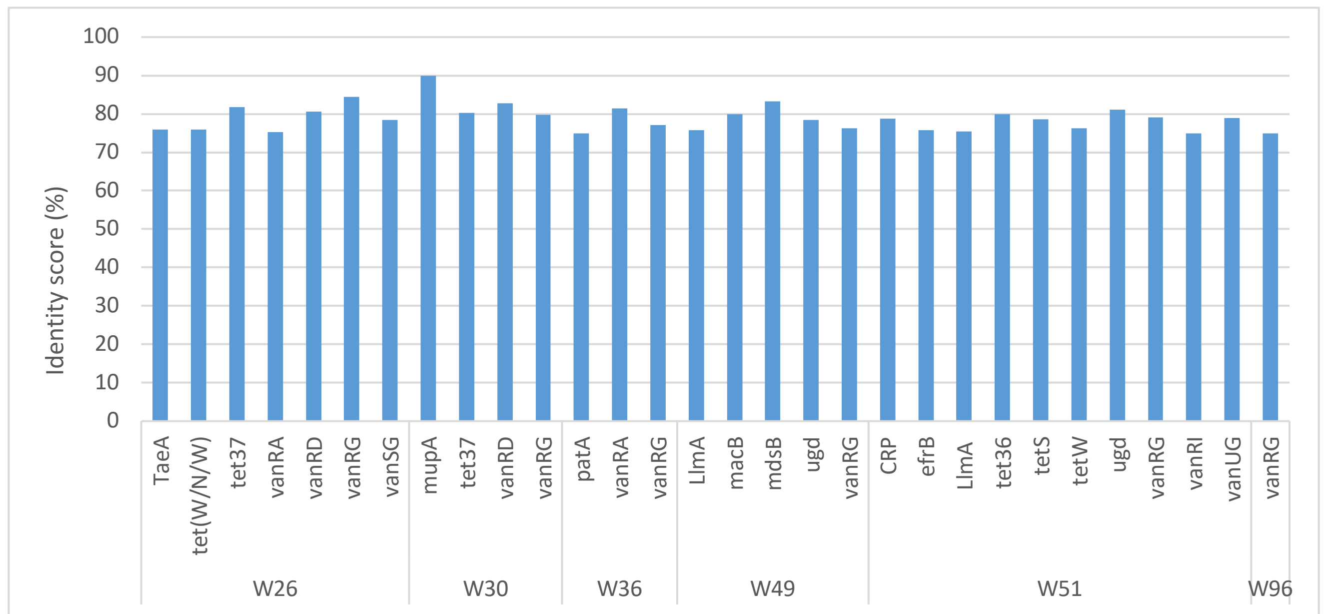

Figure 3. Overview the ARG (excluding mutations) with an identity score above 75% compared to homologue in CARD from six of the whale metagenomes.

As illustrated in Figure 4, antibiotic target alterations were found to be the most abundant mechanism of resistance in the analyzed metagenomes. The high number of *van* genes present in all datasets is the probable explanation for this. Additionally, antibiotic efflux, were also one of the more prevalent modes of antibiotic resistance. Interestingly, several genes related to efflux were detected in the targeted PCR screening of total DNA from whale faeces. One of the genes associated with antibiotic efflux identified in the metagenome of W49, namely *mdsB*, belong to the same gene family as the *mexB* gene.

The PCR screening of total DNA from whale faeces showed that the W49 microbiome were positive for *mexB*. It would be interesting to investigate the different variants of the antibiotic MDR efflux genes, as it has been previously noted to play an important part in antibiotic resistance of environmental bacteria.

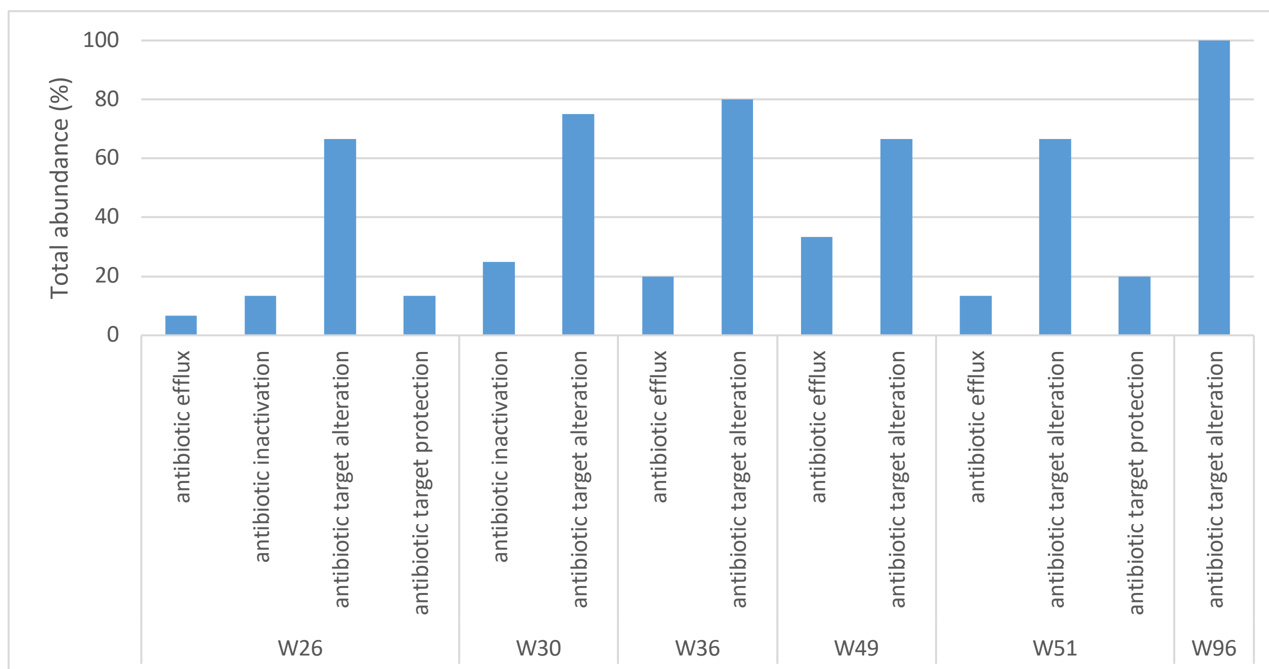

Figure 4. Overview of the abundancy of the resistance mechanisms detected at six of the whale metagenomes identified by RGI compared to CARD.

The metagenome results presented show large differences when using respectively 90% and 75% identity scores as cut-offs. When analyzing clinical data, the 80% cut-off is recommended. Most of the ARGs identified in this study, however, lie between 75-85% in identity scores to the homologue in CARD. It has previously been shown that using a threshold of 90% for identification of resistance genes in environmental bacteria, may create a large number of false negatives (Arango-Argoty et al., 2018). The algorithms used to create CARD and similar databases are biased towards clinical isolates, and thus important data may be lost or not identified when using them to examine environmental microbiomes. As a result of this, the cut-off was set to 75%, and this needs to be taken into account when interpreting the results. Additionally, many of the identified hits in CARD referred to mutations leading to resistance. The data provided here are not sufficient to validate these mutations and needs to be investigated in more detail. These results have thus been excluded from this report. The data from metagenomes of W86 and W105 contained resistance genes solely associated with mutations with identity scores above 75%, explaining the lack of data from these metagenomes in Figures 3 and 4.

The benefits from combining different methodological approaches

The results in this study from the targeted PCR and metagenomic analysis on total microbial DNA extracted from whale faeces illustrates the importance of combining different methodological approaches when working with microbiomes of the natural environments.

In the targeted PCR of total microbial DNA, several of the analyzed samples were positive for one or more genes coding for antibiotic efflux (*mexB, mexD, acrB, acrD*), while negative for the *tet* gene variants (*tetA, tetB*). The results of the metagenomics data, however, identified the presence of four *tet* variants in three of the analyzed samples. These were *tetW*, *tetS, tet37* and *tet36*. It would be interesting to perform sequence analysis of these variants. In regards to genes coding for antibiotic efflux, these were less numerous in the metagenomics data compared to both PCR assays in this study. Further analysis investigating the role of MDR efflux pumps in the isolated samples with observed antibiotic resistance patterns, should be further assessed.

The results from PCR on DNA from isolates showed a high number of *vanA* present in bacteria cultivated from whale faeces. This is in consistency with the metagenomics analysis, were variants of *van* genes were present in the six metagenomes presented in Figure 3. A follow-up study using qPCR to measure the abundance of the *van* variants in the whale microbiome would prove valuable.

Limitations

There are many limitations/uncertainties related to environmental studies and antimicrobial resistance. Many of the uncertainties arise due to technical limitations, data limitations and lack of standardized protocols. In this study, some of the listed limitations are also due to the limited project period and can easily be followed up, while others are connected to sampling procedures and lead rather to recommendations for future studies. This study represents a snapshot of the samples tested, and cannot provide all details.

- Sampling and sample preservation: there are practical aspects connected to sampling in the environment which can mean that sample preservation methods are not ideal. In this case, the whale samples were collected as part of a larger field study, and could not be returned to the lab for analysis immediately. See Venter (2019) for further discussion of this limitation.
- Cultivation based methods themselves introduce biases and limitations to a study, and are only capable of recovering a small percentage of the total community in such diverse samples. In the part of the study published by Venter (2019) we used a variety of culture media to try to recover a good representation of the community present.
- Isolation of total DNA for PCR and metagenomic sequencing may need optimization. Low yields, and issues with amplification suggest the presence of inhibitors which may have reduced the probability of detecting genes present in those samples. Both the methods for DNA isolation and for PCR conditions require more optimization.

- The amplicons obtained through PCR screening for the selected ARGs have not been sequenced to confirm their identities, this could easily be achieved in follow-up.
- Our results do not say anything about the detection frequencies of the different ARGs. This can be followed up by a quantitative PCR. A quantitative PCR would add more information about the total ARG reservoir in the different samples.
- Lack of knowledge about the concentration of antibiotics, biocides and heavy metals in the environment the whales migrate in. Such information can give us insight into the type of selective pressure the microorganisms may be facing.
- It is difficult to compare the outcome of this study with other published studies on ARGs in the environment because there is very little standardization within the field (Huibers et al., 2019). Cultivation conditions, DNA extraction methods, targeted resistance phenotypes and genotypes or primers sets used in different environmental studies varies and make it difficult to compare data. More standardized guidelines for resistance testing in different environments would enable comparison between different environmental studies worldwide more easily.

Recommendations for further studies

This study has provided a foundation of results relating to the antimicrobial capabilities and presence of resistance genes in samples from whales. The whale faeces samples in particular are rare and not easily obtained, so it is strongly recommended to get the most out of these samples, since they provide a view into the ARG profiles of highly mobile marine mammals.

Recommendations:

- We have analysed 2 years' worth of whale samples (this study and the study by Venter (2019)). More data, provided over a longer time period, would help to fill knowledge gaps about environmental antimicrobial resistance reservoirs and the directionality of the spread of ARGs.
- The metagenomics data can be used for the development of more accurate primer sequences and thus allow for more in-depth analysis of selected genes of interest, such as the various *van* and *tet* genes or genes coding for antibiotic efflux pumps.
- Targeted PCR with more specific primers, followed by amplicon sequencing could provide new valuable information on the presence and evolution of ARGs in the microbiomes of minke whales, moreover, in marine environments. Furthermore, the development of more accurate

primers could make quantitative PCR possible, which would provide information on the abundancy of ARGs in these environments. This is especially relevant since the targeted PCR of isolated DNA show low level of resistance.

- A majority of the antibiotic resistance identified by RGI in the analysed metagenomes, were reported as mutations in CARD. The data provided in this study is not sufficient to draw any conclusions on these results, and thus more in-depth analysis of mutations could be of interest.

Conclusion

The results of this study reveal a snapshot of the antimicrobial susceptibility and resistance genes present in isolates collected from whale samples. Venter (2019) found in first part of this study that for each antibiotic, there were some isolates which were capable of growing at concentrations of ≥ 32 mg/L, the maximum concentration tested in this study.

In this part of the study we found that DNA extracted from whale faeces samples gives insight to the ARG present in the microbiome of the whale gut through targeted PCR and metagenomics analysis. One of the main conclusions that can be withdrawn from our data is that different ARGs are prevalent in samples from marine mammals. By using both culture-dependent and culture-independent methods the results indicate that the bacteria in the samples are resistant to different antibiotics. The other conclusion is that the majority of ARG and microbial resistance functional types were encoding antimicrobial efflux pumps. It was also found that a high number of *vanA,* possibly encoding resistance to the antibiotic vancomycin*,* present in bacteria cultivated from whale faeces. This is in consistency with the metagenomics analysis, were variants of *van* genes were present in six metagenomes. In addition, several variants of *tet* genes were found. The results might hence indicate that environments outside the clinical settings also possibly play an important role in the dissemination and spread of antibiotic resistance.

It is also important to highlight that the present study is limited in terms of scale, and continued research is required to fully understand the role of marine mammals in the context of maintenance and spread of antimicrobial resistance.

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Appendix 1

Supplementary table 5: information regarding whales from which samples were taken

Whale number	Sampling year	Length	Diameter	Sex	F-length	Comments
20	2017	760	360			
22	2017	790	380	1	37	
23	2017	820	420	2	51	
24	2017	880	460	2	52	
25	2017	830	400	2	42	
26	2017	790	400	1	63	
28	2017	740	400	1	32	
29	2017	900		1	90	
30	2017	840	410	2	53	
32	2017	860	400	2	60	
33	2017	820	400	2	65	
35	2017	930	420	1	25	
36	2017	800	410	1	57	
37	2017	910	450	2	65	
38	2017	830	430	2	44	
39	2017	850	450	2	78	
40	2017	810	430	1	89	
41	2017	840	410	2	86	
44	2017	920	490	2	86	
45	2017	840	450	2	43	
46	2017	840	450	1	65	
47	2017	820	400			
48	2017	710	350			
49	2017	840	410	1	19	
50	2017	690	340			
51	2017	740	400	2	76	
52	2017	840	400	1	67	
54	2017	910	420	1	71	
55	2017	790	400	2	59	
56	2017	820	430	1	65	
57	2017	810	410	2	76	
58	2017	650	340			
59	2017	770	410	1	62	
60	2017	820	420			Pregnant, but no foetus yet
61	2017	650	350			
62	2017	800	360	1	34	
63	2017	730	320			
64	2017	740	370			
65	2017	750	360			
86	2018	670	270			

88	2018	800	420	1	78	Liver parasite
90	2018	690	300			
92	2018	650	350			
93	2018	810	450	2	48	
94	2018	850	500	2	139	External abdominal parasite
96	2018	780	440	2	124	
97	2018	800	440	1	117	
98	2018	820	440	1	120	Liver parasite
99	2018	870	440	2	60	
100	2018	810	460	1	131	
102	2018	860	460	1	120	
103	2018	860	480	2	151	
104	2018	820	460	2	149	
105	2018	810	470	2	173	
107	2018	810	470	2	160	