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# Deep sequence analysis reveals the ovine rumen as a reservoir of antibiotic resistance genes☆

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## ABSTRACT

Antibiotic resistance is an increasingly important environmental pollutant with direct consequences for human health. Identification of environmental sources of antibiotic resistance genes (ARGs) makes it possible to follow their evolution and prevent their entry into the clinical setting. ARGs have been found in environmental sources exogenous to the original source and previous studies have shown that these genes are capable of being transferred from livestock to humans. Due to the nature of farming and the slaughter of ruminants for food, humans interact with these animals in close proximity, and for this reason it is important to consider the risks to human health. In this study, we characterised the ARG populations in the ovine rumen, termed the resistome. This was done using the Comprehensive Antibiotic Resistance Database (CARD) to identify the presence of genes conferring resistance to antibiotics within the rumen. Genes were successfully mapped to those that confer resistance to a total of 30 different antibiotics. Daptomycin was identified as the most common antibiotic for which resistance is present, suggesting that ruminants may be a source of daptomycin ARGs. Colistin resistance, conferred by the gene *pmrE*, was also found to be present within all samples, with an average abundance of 800 counts. Due to the high abundance of some ARGs (against daptomycin) and the presence of rare ARGs (against colistin), we suggest further study and monitoring of the rumen resistome as a possible source of clinically relevant ARGs.

## 1. Introduction

The increasingly widespread presence of antibiotic resistance has made it imperative to consider diverse environments as sources of emerging resistance. This has led to genes or genetic elements that confer antibiotic resistance being considered to be environmental pollutants (Lekunberri et al., 2016). Resistance was identified as being present in microbial communities before the widespread clinical and agricultural use of antibiotics (Wright, 2007). These resistances are likely to have developed in complex microbial communities where the production of antibiotics conferred an evolutionary advantage in terms of survival. The presence of such compounds would in turn put evolutionary pressure on competing organisms to evolve resistance to these antibiotics in order to ensure their own survival. The development of resistance can be further exacerbated by anthropogenic intervention, such as the overuse of antibiotics or their use in agriculture (Kanwar et al., 2014).

Analysis of resistance in important environments could facilitate the prediction of future mechanisms of antibiotic and antimicrobial resistance that arise through transfer to clinically important microorganisms (Costa, 2012). Due to the close contact that occurs between humans and ruminants both on farms and in abattoirs, the rumen resistome may be regarded as an important source of clinically relevant antibiotic resistance genes (ARGs) with opportunities to transfer to human pathogens. Since the microbial ecosystem in the rumen is so diverse, the likelihood of novel antimicrobial compounds being produced is high, as is the resulting development of resistance to these compounds. Because of this, the rumen has already been a target for the mining of novel antimicrobial substances (Azevedo et al., 2015). The presence and the abundance of specific ARGs within the bovine digestive tract are known to change during treatment with antibiotics (Kanwar et al.,

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2014), and ARGs have previously been identified within the rumen as well as in ruminant faeces (Flint and Stewart, 1987).

The advancement of technology has allowed metagenomic sequencing techniques to be used to provide a more complete insight into the ARGs present within the microbiome (Reddy et al., 2014). This type of study looks at the ‘resistome’, which is comprised of all those genes that confer resistance (Berendonk et al., 2015). These genes may underlie known methods of resistance, such as the modification of an antibiotic target, or the production of an enzyme capable of disabling active compounds or allowing the efflux of antibiotic compounds from the cell (Gomez-Alvarez et al., 2012). Other genes, those considered to be putative or precursor resistance genes with the potential for development into full resistance-conferring genes, also form part of the resistome (Wright, 2007).

In the present study, the ovine rumen resistome is characterised by searching for ARGs in the deep sequenced metagenomic dataset of Shi et al. (2014). Any ARGs that were annotated as being conferred by point mutation according to the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) were excluded. This represents the first comprehensive analysis of the presence and abundance of antibiotic resistance within the ovine rumen.

## 2. Methods

### 2.1. Data collection and sequencing

The Shi et al. (2014) dataset consists of 10 Rams from the Woodlands Research Station progeny flock, fed a lucerne pellet diet. Samples were collected by stomach intubation 4 h after morning feeding and immediately snap frozen in liquid nitrogen. Samples were taken at two time points (total of 20 samples). An Illumina HiSeq 2000 (2 × 150bp) was used to sequence a total of 1 Tb of metagenomic data and 120 Gb of metatranscriptomic data from the 20 samples. The reads were then passed through the JGI-developed filtering program (Shi et al., 2014) to provide quality checked reads which were then joined using FLASH (Magoč and Salzberg, 2011). Details of the sampling and sequencing procedures are given in Shi et al. (2014). The sequence files were downloaded from the JGI website (jgi.doe.gov).

### 2.2. Assembly

The metagenomic dataset from Shi et al. (2014) was combined into a single file and digitally normalised by kmer abundance using khmer (Crusoe et al., 2015) with a kmer size of 20, four hash tables of size 32e9 and an ideal median of 20. The normalised dataset was then assembled using Spherical (https://github.com/thh32/Spherical) with a subset size of 50 Gb for 6 iterations (base assembler = Velvet 1.2.10 (Namiki et al., 2012), kmer = 51). Assembly statistics provided in Supplementary Table 1.

### 2.3. Annotation

#### 2.3.1. Taxonomic annotation

The assembly was annotated against the UNIPROT database (downloaded October 2015) (Bateman et al., 2015) using DIAMOND v0.7.0.49 (Buchfink et al., 2015) with default settings. The annotation output was converted into a GFF file and filtered by overlap and by requiring a bitscore $\geq$ 40.0 using MGKIT (Rubino and Creevey, 2014). The UNIPROT mapping files were downloaded and used to provide taxonomic and functional groupings for each gene.

#### 2.3.2. CARD annotation

To identify antibiotic resistance genes within the assembly, the Comprehensive Antibiotic Resistance Database (CARD) (McArthur et al., 2013) was used. In order to ensure accurate identification of antibiotic resistance genes, open reading frames (ORFs) within the assembly were selected for study using the “longest-orf” option in TransDecoder (Haas et al., 2014), Supplementary Fig. 1 and Table 2. The ORFs were then annotated against the CARD database (downloaded January 2016) using DIAMOND v0.7.0.49. The annotation output was converted into a GFF file and filtered by overlap and by requiring a bitscore $\geq$ 60.0 and percentage identity of 75%, using MGKIT (Rubino and Creevey, 2014), Supplementary Table 3 and Table 4. Any annotations to resistance conferred by mutations were removed to prevent the false identification of non-mutated genes within the results.

### 2.4. Abundance measurements

HTSeq-count (v0.6) (Anders et al., 2015) was used to count the number of reads from each sample aligning to each CARD annotated ORF. The settings used were a minimum alignment quality of 8 and the intersection-nonempty overlap resolution mode. The read counts within each sample were scaled to the minimum sample size to prevent sample size bias.

## 3. Results

### 3.1. CARD categories

The abundance of each CARD category was determined using the CARD ORF annotations and the count information from HTSeq-count, Fig. 1. Only categories with a total count across samples $\geq$ 100 were included. This resulted in a final list of 28 CARD categories, where a single CARD category can represent resistance to multiple antibiotics present within the ovine rumen, Fig. 1. The use of 20 metagenomic samples allowed for confidence intervals for the abundance of each CARD ontology term to be calculated.

### 3.2. Antibiotic resistances

The CARD database provided a list of the antibiotics against which each of the CARD categories confers resistance. This information was utilised to identify the presence and abundance of genes for resistance to specific antibiotics within the ovine rumen. Antibiotics were filtered based on resistance occurring more than 100 times across the samples. This yielded a final list of 30 antibiotics for which resistance was identified and occurrence quantified within the ovine rumen, Fig. 2. As shown in Figs. 1 and 2, the antibiotic with the highest abundance of antibiotic resistant genes is daptomycin.

Within the assembly, seven intact antibiotic resistance genes were recovered. These included APH(3')-IIIa, *cpxR* and five copies of ANT(6)-Ib, each recovered within a different contig.

### 3.3. Resistome diversity

The diversity of ARGs within each sample was calculated using the Shannon Diversity Index (SDI). The abundances of genes for resistance to different antibiotics were used to calculate the SDI for each sample. The average SDI value was 2.54, with a standard deviation of 0.1.

### 3.4. Phylum-specific antibiotic resistances

Utilising the taxonomic annotations, CARD annotated ORFs were

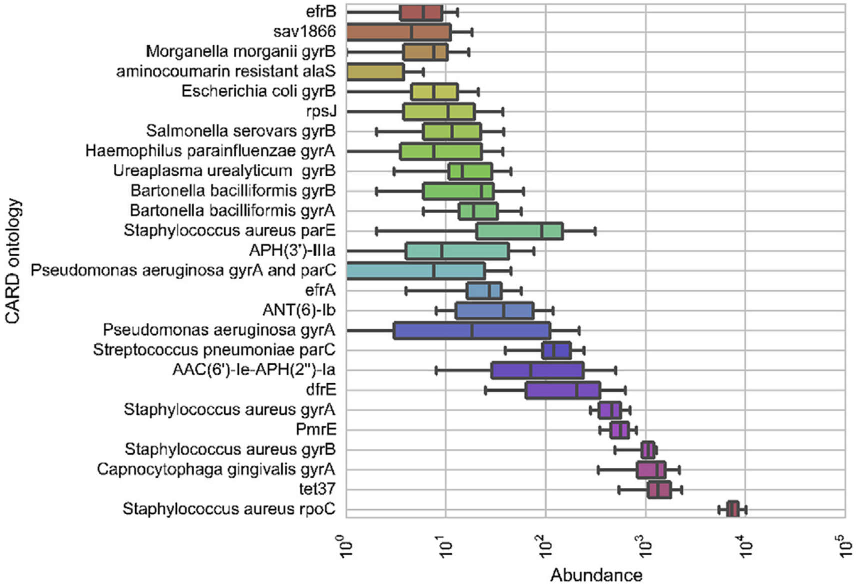

**Fig. 1.** Abundances of CARD ontological terms within the ovine rumen. For each CARD ontology, the number of reads aligning to it within each of the 20 samples was identified and these values were plotted to create the abundance boxplots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

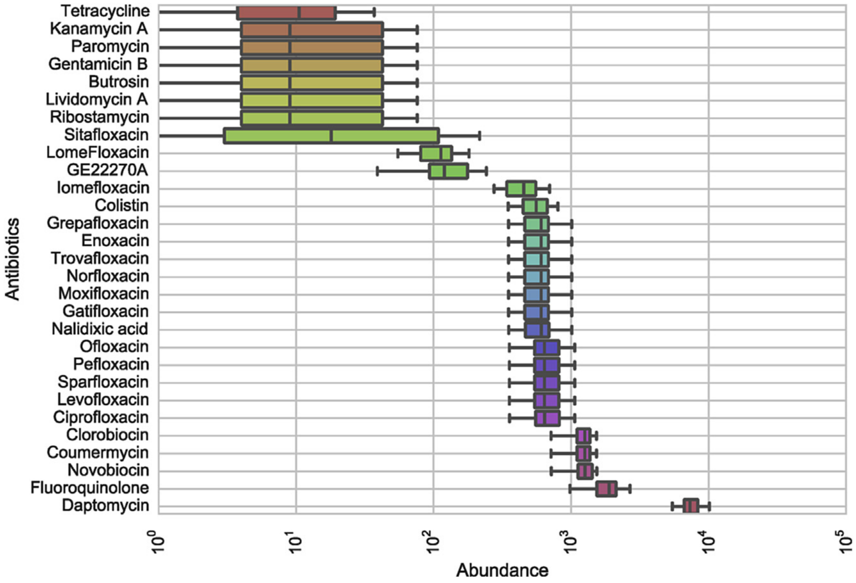

**Fig. 2.** Abundance of resistance genes to specific antibiotics within the ovine rumen. For each antibiotic, the number of reads aligning to it within each of the 20 samples was identified and these values were plotted to create the abundance boxplots. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

linked to their taxonomic groups of origin. ARGs for specific antibiotics were identified within both *Firmicutes* and *Proteobacteria*. Both groups were found to contain resistance to coumermycin and clorobiocin. *Firmicutes* were shown also to contain resistance to novobiocin and GE2270A. *Proteobacteria* contained resistance to 19 antibiotics in total: pefloxacin, ciprofloxacin, moxifloxacin, fluoroquinolone, daptomycin, trovafloxacin, norfloxacin, novobiocin, gatifloxacin, coumermycin, levofloxacin, enoxacin, sparfloxacin, colistin, nalidixic acid, grepafloxacin, lomefloxacin, ofloxacin, clorobiocin. Antibiotic resistances with abundance levels greater than 100 were identified within *Proteobacteria* (Supplementary Fig. 2) and *Firmicutes* (Supplementary Fig. 3). The abundance of each antibiotic-specific resistance was highly variable between samples.

## 4. Discussion

The ovine rumen has been shown in this study to contain genes for resistance to at least 30 antibiotics, including daptomycin and colistin. Due to the use of 20 ovine samples, confidence intervals could be provided for each of these instead of for a single example.

Daptomycin is a cyclic lipopeptide used to treat a range of Gram positive bacterial skin infections, especially those that already show resistance traits (Arbeit et al., 2004). Fortunately, resistance to daptomycin has been deemed to be extremely low globally (Montera et al., 2008; Sader et al., 2014). Daptomycin was identified as the antibiotic for which resistance within the ovine rumen was the most common, with an average of 90,000 counts per sample.

Since daptomycin is used to treat skin infections, there is a possibility that resistance genes may be transferred from the rumen to the human skin microbiome of workers in close proximity to livestock or contaminated retail meat (White et al., 2001).

Colistin is a polymyxin antibiotic originally discovered in 1949 in the bacterium *Bacillus polymyxa* (Falagas and Kasiakou, 2005). Because colistin is highly active against Gram positive bacteria, it has been utilised to treat lung infections in cystic fibrosis patients (Falagas and Kasiakou, 2005). The infrequent use of colistin since its discovery has led to it being called one of the “last hope” antibiotics, as resistance has yet to become common or widespread (Fernández et al., 2010). Within the ovine rumen, we identified high levels of the *pmrE* gene. This gene confers resistance via modification of the lipopolysaccharides that colistin targets, thereby rendering colistin ineffective. The high levels of *pmrE* in the ovine rumen would represent a risk to human health if the antibiotic resistances present within the rumen microbiome were to cross over into the human microbiome or human pathogens.

There was a total of seven resistance genes that were fully reconstructed: APH(3′)-IIIa, *cpxR* and five copies of ANT(6)-Ib. APH(3')-IIIa is an aminoglycoside phosphotransferase enzyme commonly found in resistant *Bacillus* strains (Thompson et al., 2002). The *cpxR* gene plays a role in the stress response of *Escherichia coli* to hydroxyurea and aminoglycosides (Mahoney and Silhavy, 2013), but abundance of this gene in the samples was low. ANT(6)-Ib is a nucleotidyltransferase, which confers resistance to streptomycin, also acting by modifying aminoglycosides (Abril et al., 2010). The ability to reconstruct intact resistance genes may, in the future, play an important role in the identification and characterisation of novel resistance genes.

Using Shannon diversity index (SDI) values, it was found that the ovine rumen resistome is consistent across members from the same flock. This suggests that as long as individuals receive similar treatment then the development of resistance to new antibiotics is unlikely. However, the antibiotic-specific resistance genes identified within certain phyla (*Proteobacteria* and *Firmicutes*) were highly variable across samples. This information, combined with the SDI values, indicates that although the presence/abundance of antibiotic resistance genes within specific phyla is highly variable, their total abundance within the rumen as a whole remains consistent.

*Proteobacteria* were found to contain resistance to the highest number of antibiotics (19 in total) of all of the phyla analysed. This highlights *Proteobacteria* as a group that is either regularly assaulted by antibiotics, leading it to evolve multiple ARGs as a survival mechanism, or able to acquire or evolve resistance to antibiotics more quickly than other groups within the rumen. The first of these explanations appears the more likely, since among the *Proteobacteria* there are many pathogens of humans and livestock (Batut et al., 2004), causing diseases including respiratory tract, urinary tract and gastrointestinal infections, which are frequently treated with antibiotics in the clinical and veterinary settings. Such exposure will be in addition to that resulting from antibiotics produced by competing organisms in the environment.

Our findings suggest that the rumen resistome should be regarded as a potentially important source of antibiotic resistance and that transfer of resistance genes from the rumen to the human microbiome is a topic meriting further investigation. The potential exists for resistance against two last-line-of-defence antibiotics (daptomycin and colistin) to enter the clinical setting via this transfer route. Forsberg et al. (2012) showed that there is extensive overlap between the soil resistome and that of human pathogens, and provided evidence for two-way transfer of resistance genes between these pathogens and soil microorganisms. It can be hypothesised that the potential for genetic transfer between the rumen microbiome and human commensal and pathogenic species is as great as that in the case of soil. This potential is evident by the frequency with which humans are infected with animal intestinal microbes such as *Cryptosporidium* (Richardson et al. 1991; Ramirez et al. 2004) or the occurrence of faecal matter contamination on meat products (Ghafir et al. 2007).

## 5. Conclusions

We have shown that the ovine rumen contains bacterial species with resistance to a diverse range of antibiotics. We suggest that the ovine rumen may become a source of ARGs for clinically important antibiotics. We identified resistances to 30 known antibiotics in this study, including high abundance of daptomycin and colistin resistance genes. Daptomycin and colistin are both considered “last hope antibiotics” as resistance to them is not yet commonplace within the clinical setting (Montera et al., 2008). It is therefore important to acknowledge that the rumen contains ARGs that may play a role in the dissemination of antibiotic resistance genes to bacteria pathogenic to humans. These ARGs could be passed on to human-associated bacteria through close contact with ruminants, such as occurs on farms, in abattoirs or through the consumption of contaminated meat products.

## Author contributions

**Thomas C.A. Hitch;** assembly, analysis, writing.
**Ben J. Thomas**; CARD analysis.
**Jessica C. A**. Friedersdorff; writing.
**Helen J. Ougham**; writing and editing.
**Christopher J. Creevey**; TCAH, BJT and JCAF supervisor.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.067.

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