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Approaches to Assess the Effects and Risks of Veterinary Antibiotics Applied with Manure to Soil

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Abstract

In veterinary medicine, large quantities of antibiotic substances are administered each year for therapeutic and prophylactic purposes or to promote growth. As a consequence, the antibiotics and bacteria carrying transferable antibiotic resistance genes are excreted by the animals and reach the environment through run-off, leaching, and/or following manure application to agricultural fields, where they have been found to affect the structure and function of soil bacterial communities. However, we are only beginning to understand the global effects of environmental pollution with antibiotics and resistance determinants and the resulting risks for human health. For regulatory purposes, there is urgent need for criteria and methods that allow reliable and reproducible assessment of risks associated with release of realistic concentrations of antibiotics and resistance determinants into the environment following manure application. In this chapter, we will summarize recent advances, limitations, and research needed to optimize the methods to quantify and evaluate the effects and risks associated with these compounds. Approaches that are discussed focus on antibiotic resistance genes and include classical tools such as cultivation and PCR detection as well as quantitative real-time PCR and next-generation sequencing technologies used in combination with functional screening.

Keywords: Antibiotic resistance, resistome, manure, soil, mobilome

1. Introduction

Antibiotic-resistant pathogens are a growing public health threat challenging the achievements of modern medicine by making available treatment options for common infections ineffective.[1] It is widely accepted that this rise in antibiotic-resistant bacteria is due to the massive and worldwide use, misuse, and abuse of antibiotic agents in humans and animals. Additionally, antibiotics are applied prophylactically to control bacterial diseases of plants,

especially fire blight of pear and apple and bacterial spot of peach.[2] Besides clinical and agricultural use, large quantities of antibiotic substances are administered each year in veterinary medicine for therapeutic and prophylactic purposes or in different parts of the world still to promote growth.[3,4] Depending on pharmacokinetics and specific transformation processes in the animals, large proportions of antibiotics and antibiotic-resistant bacteria (often with resistance determinants located on mobile genetic elements) are typically excreted by the animals. Ultimately, these components reach the environment via run-off, leaching, and/or manure application, where they at least transiently affect the structure and function of soil bacterial communities.[5–8]

Recent studies have demonstrated the existence of a shared antibiotic resistome between clinical pathogens and the environmental bacteria.[9–11] Thus, although the observed increase in abundance and transferability of antibiotic resistance genes in manured soil is assumed to be only transient, it is very likely that the pollution of the environment with antibiotics and antibiotic resistance determinants influences the human microbiome and contributes to the rise of antibiotic resistance found in human pathogens.[12,13] Mobile genetic elements such as plasmids are considered to play an important role in the adaptation of bacterial communities facing selective pressure by antibiotics[14,15] and might be an important link between the environmental and human resistome. Co-selection processes by heavy metals such as copper and zinc[16] or by disinfectants such as quaternary ammonium compounds (QACs)[17,18] can further promote the spread and persistence of antibiotic resistance genes on similar genetic platforms. Moreover, the rhizosphere of plants is considered to increase the horizontal transfer of resistance determinants within bacterial communities and to modify the effects of antibiotics applied with manure by root exudates that affect the bacterial cell density, distribution, and metabolic activity (reviewed by Jechalke et al.[5]).

Nevertheless, little is known regarding the global effects of environmental pollution with antibiotics and resistance determinants by manure fertilization and the resulting risks for human health. To extend the time that antibiotics can be effectively used in human and veterinary medicine, agricultural management options are necessary to reduce the environmental release and spread of antibiotics and antibiotic resistance determinants. Policy makers are focusing on agricultural sources of antibiotic resistance as a result of recent reports that emphasize the importance of antibiotic resistance in environmental bacteria (pathogenic and non-pathogenic) as a point source for spread to environmental and human ecosystems.[6,19–24] These findings, coupled with the potential for spread of emergent antibiotic-resistant bacteria from livestock to human populations and the lack of new antibiotics entering the market, have placed pressure on the agricultural community, increasing the urgency for science-based studies that fill gaps in current knowledge about how antibiotic resistance spreads within environmental ecosystems. Furthermore, on a policy level, there is urgent need for criteria and methods that allow reliable and reproducible assessment of risks associated with realistic concentrations of different classes of antibiotics, resistance determinants, and mobile genetic elements applied to soil with organic fertilizers such as manure or digestates.

In this chapter, we will summarize recent advances, limitations, and research needs regarding approaches to quantify and evaluate the effects and risks associated with veterinary antibiotics

and resistance determinants applied to soil through manure fertilization. One relevant endpoint for the environmental risk assessment (ERA) of antibiotics in the environment might represent the increase in the abundance of antibiotic-resistant bacteria and resistance genes, which can be caused by the application of resistant bacteria to the environment, the acquisition of resistance by environmental bacteria (e.g. by horizontal gene transfer), and the proliferation of indigenous resistant bacteria. For evaluation of changes in abundance of antibiotic-resistant bacteria and resistance genes, classical tools, such as quantitative real-time PCR (qPCR), on the one hand provide fast and reproducible results but are limited to known resistance gene sequences. On the other hand, rapidly advancing sequencing technologies in combination with functional screening led to the discovery of a vast diversity of novel antibiotic resistance genes and mobile genetic elements in environmental metagenomes and metamobilomes with and without human impact.[22,25] Hence, the quantification of marker genes that are widespread in the environment and affected by anthropogenic selective pressure, such as class 1 integrons, might represent a suitable approach for the evaluation of effects of antibiotics applied to soil via manure and other influencing factors. Currently, little is known about dose-response relationships and potential threshold concentrations of antibiotics applied to soil with manure.

2. Cultivation-dependent assessment of antibiotic resistance

Guidelines for ERA of pharmaceutically active compounds are available from different countries, e.g. from the European Medicines Agency or from the U.S. Food and Drug Administration. These risk assessments are typically based on traditional environmental toxicity measurements using standard tests. For example, Szatmári and colleagues[26] determined the degradation rate of doxycycline in manure-amended agricultural soil and provided ecotoxicological information regarding its effects on nitrification. In another study, Thiele-Bruhn[27] tested nine antibiotics for their effects on microbial iron(III) reduction in different soils, modeled dose-related effects, and calculated effective concentrations. However, it was demonstrated that bacteria can be affected by antibiotics even at sub-inhibitory concentrations, which not only can have considerable effects on gene expression and transcription but also can support the maintenance of resistance plasmids or select for resistant bacteria.[28–32] Furthermore, it is known that soil bacteria are a natural reservoir of antibiotic resistance determinants to both natural and synthetic antibiotics; the collection of genes that confer resistance to antibiotics is commonly referred to as the antibiotic resistome.[33–35] The application of antibiotics to soil with manure can have immediate effects on the composition of the soil bacterial community, e.g. by promoting the development, co-selection, spread, and transfer of antibiotic resistance determinants, which can indirectly affect human health if transferred to the human microbiome, e.g., by human contact with resistant bacteria in the agricultural environment or by the ingestion of vegetables from manured soil.[5] Different cultivation-dependent test methods for the assessment of resistance development and dissemination are available. One approach is to quantify bacteria resistant to a certain antibiotic within a number of isolates from an environmental sample to obtain the proportion of antibiotic-resistant bacteria within the cultivable population. These isolates can further be compared between treatments or environments by the determination of their antibiotic

susceptibility, as performed by Li et al.[36] for bacteria from wastewater produced at an oxytetracycline production plant. Antibiotic-resistant bacteria can also be quantified directly using selective plating or most probable number (MPN) plates and resistance quotients can be derived from comparison with results from unselective cultivation. For example, it was observed that a high proportion of bacteria added with manure to soil microcosms were resistant to the bacteriostatic sulfonamide antibiotic sulfadiazine (SDZ) (66%) and that, up to two months after application of manure containing SDZ, the MPN counts of resistant bacteria were still significantly higher.[37] These counts might have been biased by the growth of soil bacteria with naturally reduced susceptibility to sulfonamides. However, effects of manure and SDZ on the soil bacterial community were confirmed by the quantification of sulfonamide resistance genes *sul1* and *sul2*.

Pollution-induced community tolerance (PICT) is another approach to measure changes in community tolerance after exposure to a pollutant such as an antibiotic substance.[38] For example, using Biolog® multiwell plates, Schmitt et al.[39] showed that soil treatment with the sulfonamide sulfachloropyridazine led to an increased community tolerance in bacterial soil extracts compared with the untreated control, which was enhanced upon additional soil amendment with nutrients such as pig slurry and alfalfa meal.

In another study by Brandt et al.[40], PICT was used to compare the development of tolerance to SDZ between bulk soil and nutrient-amended soil hotspots. They demonstrated that bacterial growth rates ([³H]leucine incorporation) were reduced 24 h after SDZ amendment to a concentration of 0.1 µg SDZ/g dry weight of soil, while soil respiration remained unaffected even at 100 µg SDZ/g dry weight of soil. Carbon substrate amendment *per se* led to an increased PICT response. The presence and enrichment of soil bacteria able to degrade the applied antibiotics might also have strong impacts on the evaluation of antibiotic effects on soil bacterial communities. Tappe et al.[41] could isolate a SDZ-degrading *Microbacterium* strain from soil previously manured with slurry from SDZ-medicated pigs. Topp et al.[42] showed that sulfamethazine was rapidly mineralized in soils repeatedly treated with swine manure over a period of six years. They could also culture a sulfamethazine-degrading *Microbacterium* sp. from the soil and suggested that microbial populations repeatedly exposed to livestock manures may attenuate environmental exposure to antibiotics.

Heuer and Smalla[37] compared soil treated with manure containing SDZ with untreated soil over a two-month period. Cultivation-dependent determination of SDZ-resistant bacteria, transfer frequencies, and PCR quantification of the resistance gene *sul1* revealed a transient effect of manure alone and a synergistic effect of SDZ and manure. However, the cultivation of antibiotic-resistant bacteria from soil with the subsequent physiological and genetic characterization of the isolates is limited by the extraction efficiency from soil and their culturability, which is considered to be low.[43–45]

3. PCR-based approaches

PCR-based methods allow simple and rapid cultivation-independent detection and quantification of antibiotic resistance genes in DNA extracted directly from environmental samples

such as soil or manure. Classical PCR assays can only be used to determine the presence or absence of a gene in the sample, while bacterial hosts and concentrations remain unknown. Reported detection limits for PCR depend on the extraction method used and range from 10^3 to 10^8 gene copies/genomic equivalents per gram soil and 10^3 gene copies per gram soil for hybridization with digoxigenin-labeled probes.[46–48] Depending on the primers used, additional information regarding the location of genes and their association with mobile genetic elements can be obtained. These elements including integrons, transposons, and insertion sequence common region elements can be transferred by conjugative elements among soil bacteria.[6] By amplifying, for example, the variable region of class 1 integrons from community DNA and subsequent Southern blot hybridization, Binh et al.[49] demonstrated that *aadA* gene cassettes were introduced via manure into agricultural soils. Ponce-Rivas et al.[50] used PCR to evaluate the prevalence and origin of class 1 integrons and Qnr determinants in fluoroquinolone-resistant *Escherichia coli* isolates from poultry litter. They showed that resistance determinants within *E. coli* of poultry origin are genetically diverse and suggested the need for surveillance programs focused on the detection of genetic elements related to horizontal transfer genes. Despite the fact that the majority of poultry litter is applied to agricultural land, limited data are available on the ability of antibiotic-resistant bacteria and antibiotic resistance genes to persist and/or be mobilized within agricultural soils with applied poultry litters.

In contrast to “conventional” PCR, qPCR can be used to quantify genes permitting correlations to be made between the abundance of antibiotic resistance genes and the application of selective pressure over time or to evaluate concentration-dependent associations, such as the occurrence of antibiotic resistance genes and the application of manure containing antibiotics.[5,51] Typically, besides absolute quantification, the genes are quantified relative to 16S rRNA gene copies to correct for differences in amplification efficiency between samples and differences in DNA extraction or using the $2^{-\Delta\Delta C_T}$ method to compare relative changes or fluctuations in gene concentration.[52] Heuer et al.,[53] for example, observed an accumulation of sulfonamide resistance genes *sul1* and *sul2* in soil repeatedly treated with manure containing SDZ, compared with soil treated with antibiotic-free manure. In another study, Zhu et al.[7] used high-capacity qPCR-arrays to correlate the abundance of antibiotic resistance genes with antibiotic and metal concentrations in samples from commercial swine farms in China and nearby agronomic fields to which manure-based compost had been applied. However, little is known about effects of antibiotics applied to the soil over a long period of time. Knapp et al.[54] found evidence for an increase in resistance gene abundances in soils from the Netherlands between 1940 and 2008, although this increase could not be correlated directly with manure application due to the lack of quantitative data in the historic documentation.

Besides antibiotic compounds, high concentrations of antibiotic-resistant bacteria, resistance genes, and the associated mobile genetic elements such as broad-host-range plasmids are applied to soil with manure.[5,6,55–57] Manure bacteria might not be well adapted to the soil environment, and therefore the horizontal transfer of genes from manure-associated bacteria to soil-associated bacteria plays an important role in the dissemination of antibiotic resistance.[6,58] By quantifying mobile genetic elements such as broad-host-range plasmids, their role in

the spread of antibiotic resistance in the environment can be assessed. IncP-1 plasmids, for example, are known to carry genes that often code for resistance to antibiotics, heavy metals, and disinfectants such as QACs.[15] The relative abundance of plasmids of the IncP-1 ϵ subgroup in samples from pig farms was found to be positively correlated with antibiotic usage, indicating their importance for the dissemination of antibiotic resistance genes in agricultural systems.[59] It was shown that manure exposure can further increase the transferability of antibiotic resistance genes and the permissiveness of the soil bacterial community for plasmid uptake and maintenance and therefore contributes to the spread of antibiotic resistance genes.[37,60] You et al.[61] demonstrated the persistence of antibiotic resistance genes such as the tetracycline resistance gene *tet(L)* in chicken litter-impacted soil two years after the farm ceased operation. The high prevalence of *tet(L)* was explained by a group of *tet(L)*-carrying mobilizable broad-host-range plasmids, which might have contributed to the persistence of *tet(L)* in the soil bacterial community.

However, PCR-based methods are always limited by our knowledge of resistance mechanisms, resistance gene databases, and primer specificity. Moreover, these tools rely on the quality and purity of extracted DNA, which can be influenced by the soil type and extraction protocol,[62] and the sole detection of a resistance gene does not provide evidence for its activity in the respective host. Alternatively, RNA-based assays allow the analysis of gene expression but are challenging due to the short lifetime of RNA caused by the ubiquitous prevalence of ribonucleases.[63]

4. Quantification of marker genes and plasmids

The specificity of PCR in combination with the vast diversity of antibiotic resistance genes makes the general assessment of effects of antibiotics applied with manure on the abundance of resistance genes by “conventional” PCR and qPCR methods challenging. For tetracycline resistance, for example, to date, four resistance mechanisms were identified including 47 distinct classes of efflux pumps, ribosomal protection proteins, degradation enzymes, and 16S rRNA mutations that reduce the binding affinity of the drug to the ribosome.[64] This diversity of resistance genes and mechanisms might dilute the effect of selective pressure on each single resistance gene below the limit of detection and therefore might lead to an underestimation of antibiotic effects in the environment. An alternative to the direct quantification of antibiotic resistance genes might be the usage of marker genes as a proxy for the selective pressure exerted by antibiotics.

Class 1 integrons are widespread in the environment and have been proposed as a surrogate marker for anthropogenic pollution.[17,65] Class 1 integrons are genetic elements that are able to acquire, exchange, and express genes embedded in gene cassettes; these gene cassettes can contain resistance genes for almost all antibiotic families and may also contain genes encoding disinfectant and heavy metal resistance.[65,66] Class 1 integrons are not self-transferable but are often carried by mobile genetic elements such as plasmids and transposons, which facilitate their rapid transfer and spread within bacterial communities.[67] Furthermore, they are

widespread in environmental compartments, observed in pathogenic and commensal bacteria of humans and animals as well as in the clinic, where all recovered *intI1* genes had essentially identical DNA sequences pointing to a common ancestor.[17,65,66] It is estimated that up to 80% of enterobacteria in humans and farm animals carry these “clinical” class 1 integrons.[65] After the application of pig slurry containing realistic concentrations of sulfachloropyridazine and oxytetracycline to soil, quantitative PCR revealed an increased relative abundance of *intI1* integrase genes, that was still detectable 10 months after application.[68] In another study, an increased abundance of *intI1* was detected in bulk soil and rhizosphere treated with manure from difloxacin-treated pigs compared with soil treated with manure from unmedicated pigs, while no effect was observed for the quinolone resistance genes tested.[69] These results suggest that concentrations of *intI1* could be used as an indicator of the general selective pressure exerted as a result of the presence of antibiotics with a higher sensitivity than could the quantification of antibiotic resistance genes alone. However, it has to be kept in mind that, in contrast to “clinical” *intI1* genes, environmental *intI1* genes exhibit a considerable but not fully surveyed sequence diversity,[70] which might limit the universality of the designed primers and hence the precise quantitative analysis. Furthermore, Jechalke et al.[17] observed an enrichment of *intI1* genes in the rhizosphere of lettuce grown in soil that did not receive manure for at least 10 years, suggesting that also other factors such as root exudation might select for bacterial populations carrying *intI1* genes.

Additionally, antibiotic resistance genes and class 1 integrons can be co-selected by other factors such as the presence of heavy metals, QACs, or stress situations in general. For example, integrase over-expression and a concomitant increase in recombination events of gene cassettes were observed in the presence of antibiotics that lead to direct or indirect DNA damage, including the antibiotic classes of fluoroquinolones, beta-lactams, trimethoprim, and aminoglycosides.[66] Besides antibiotics, co-selection of antibiotic resistance was observed, e.g. for the heavy metals copper and zinc,[16] which are regularly found in pig manure. QACs are used as disinfectants in pig farms, hospitals, and the food-processing industry and also in household products, shampoos, and cosmetics.[71–73] Resistance against QACs is mediated by *qac* resistance genes, and particularly the *qacE* and *qacEΔ1* gene variants are frequently associated with class 1 integrons.[17] Accordingly, selection with QACs could be linked to an increase in class 1 integron incidence in bacterial isolates, and the prevalence of class 1 integrons and *qac* genes in the environment was correlated with exposure to detergents and/or antibiotic residues.[18,74] Hence, co-selection is an important factor, which can influence the abundance of not only antibiotic resistance genes but also class 1 integrons in the environment. Therefore, co-selection has to be considered when using, e.g. *intI1* as a marker gene for selection by antibiotics.

Another approach to determine the concentration of antibiotics in soil that exert a selective pressure on bacterial communities is to perform competition experiments using inocula of resistance plasmid-carrying and plasmid-free bacterial populations. In a study by Jechalke et al.[75] it was demonstrated in a microcosm experiment that SDZ introduced via manure into soil provided a fitness advantage for the population of *Acinetobacter baylyi* BD413 carrying a plasmid conferring SDZ resistance, while the plasmid conferred a fitness disadvantage

without selective pressure by SDZ. The authors suggest that this approach might be used in future studies for the assessment of bioavailability of antibiotic compounds in soil.

5. Next-generation sequencing approaches

Recent advances in the development of high-throughput sequencing of DNA allow for the cultivation-independent analysis of environmental community structures and functions. By correlation with environmental parameters, these approaches can be used to unravel complex ecosystem interactions and help identify responders to a specific treatment, such as the application of antibiotics with manure. In a study by Ding et al.,[76] the effect of repeated application of manure and manure containing SDZ on the soil bacterial community was explored by barcoded pyrosequencing of 16S rRNA gene fragments. It revealed bacterial taxa that were significantly enriched or decreased compared with soil treated with manure alone. Although these changes in relative abundance of taxa were in the low percentage range, which might suggest a high sensitivity of this approach, soil bacterial communities are extremely diverse and contain a large “rare biosphere” with an enormous number of low-abundance and unique taxa, which can have important ecological roles and serve as reservoirs of genetic and functional diversity.[77]

Furthermore, bacterial phylogenetic and taxonomic information alone is only able to give indications about community functions. Besides effects on bacterial community structures, metagenomic approaches combined with bioinformatic tools can provide additional functional information, e.g. on the diversity and abundance of antibiotic resistance genes. In a holistic approach, Huang et al.[78] investigated antibiotic resistance genes in activated sludge using Illumina® high-throughput sequencing in combination with 16S rRNA gene pyrosequencing and qPCR of *tet(A)*, *tet(C)*, and *tet(G)* resistance genes. Effects of tetracycline treatment on the bacterial community structure in sludge were observed and potentially tetracycline-resistant bacteria were identified. Furthermore, they showed by qPCR, molecular cloning and metagenomic analysis that tetracycline treatment increased the abundance and diversity of *tet* genes but decreased the occurrence and diversity of other antibiotic resistance genes.

However, similar to the case of PCR approaches discussed above, the identification of antibiotic resistance genes is limited by sequences available in the databases, and the mere detection of a gene does not prove its functionality or activity. In addition, the characterization of the genetic context of putative antibiotic resistance genes is limited by the short read length of many novel sequencing platforms.[79] In contrast, functional metagenomic selections from environmental resistomes can be used to directly link genotypes with the respective resistance phenotypes. This culture- and sequence-independent approach allows for the identification of antibiotic resistance genes in complex metagenomes by shotgun cloning of total community DNA into an expression vector and transforming the library into an indicator host.[80] Using this approach, Forsberg et al.[22] discovered approximate-

ly 3,000 antibiotic resistance genes from agricultural and grassland soils, which were mostly novel; the average amino acid identity to their closest homologue in the NCBI database was only 61.1%, emphasizing the vast diversity of known and still unknown antibiotic resistance genes within the soil resistome. Furthermore, the authors were able to correlate the resistome composition with the soil microbial phylogenetic and taxonomic structure and found indications that bacterial community composition is the primary determinant of the antibiotic resistance gene content in soil.

By functional screening of fosmid and small-insert libraries obtained from dairy cow manure, 80 different antibiotic resistance genes were identified with deduced protein sequences, which were on average only 50–60% identical to sequences deposited in GenBank.[79] Combining functional metagenomics and PacBio sequencing, the authors could analyze the genomic context and taxonomic affiliation of the antibiotic resistance genes. They found that many of the antibiotic resistance genes were affiliated to a diverse set of phyla and were flanked by mobile genetic elements, which indicates that they can be horizontally transferred between bacterial species in the cow microbiome but probably also to the environmental microbiome when applied with manure as fertilizer.

By using a combination of PCR, qPCR, and functional metagenomics, Udikovic-Kolic et al.[8] assessed the impact that manure from cows not treated with antibiotics has on the composition and resistance profiles of soil bacterial communities. They found that a higher frequency of β -lactam-resistant bacteria existed in soil amended with manure, compared with soil treated with inorganic fertilizer, which could be attributed to an enrichment of resident soil bacteria that harbor β -lactamases. However, they suggest that the lack of mobile elements in regions flanking these resistance genes may prevent their spread from soil bacteria to clinical settings.

6. Conclusions

These examples demonstrate the complexity and diversity of the soil resistome, its transferability, associated microbial taxa, and influencing factors, making it a challenge to assess the risks associated with the application of manure containing realistic concentrations of antibiotics and resistance determinants. Holistic approaches using the combination of cultivation-dependent and -independent methods may therefore be the most promising procedure for the determination of dose-response relationships and potential threshold concentrations.

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