Environmental Pollution 313 (2022) 120128

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Facultative pathogenic bacteria and antibiotic resistance genes in swine livestock manure and clinical wastewater: A molecular biology comparison☆

Norman Hembach [a], Gabriele Bierbaum [b], Christiane Schreiber [c], Thomas Schwartz [a,*]

[a] Karlsruhe Institute of Technology (KIT) Institute of Functional Interfaces, Hermann-von-Helmholtz-Platz 1, 76344, Eggenstein-Leopoldshafen, Germany
[b] Institute for Medical Microbiology, Immunology and Parasitology, University Hospital Bonn, Venusberg-Campus 1, 53127, Bonn, Germany
[c] Institute for Hygiene and Public Health, University Hospital Bonn, Sigmund-Freud-Str. 25, 53127, Bonn, Germany

ARTICLE INFO

Keywords:
Manure
Antibiotic resistance genes
Facultative pathogenic bacteria
Hospital wastewater
Swine livestock

ABSTRACT

Manure contains vast amounts of biological contaminants of veterinary origin. Only few studies analyse clinically critical resistance genes against reserve antibiotics in manure. In general, resistances against these high priority antibiotics involve a high potential health risk. Therefore, their spread in the soil as well as the aquatic environment has to be prevented.

Manures of 29 different swine livestock were analysed. Abundances of facultative pathogenic bacteria including representatives of the clinically critical ESKAPE-pathogens (*P. aeruginosa, K. pneumoniae, A. baumannii, E. faecium*) and *E. coli* were investigated via qPCR. Antibiotic resistance genes against commonly used veterinary antibiotics (*erm*B, *tet*M, *sul*1) as well as various resistance genes against important (*mec*A, *van*A) and reserve antibiotics (bla_{NDM}, bla_{KPC3}, *mcr*-1), which are identified by the WHO, were also obtained by qPCR analysis. The manures of all swine livestock contained facultative pathogenic bacteria and commonly known resistance genes against antibiotics used in veterinary therapies, but more important also a significant amount of clinically critical resistance genes against reserve antibiotics for human medicine. To illustrate the impact the occurrence of these clinically critical resistance genes, comparative measurements were taken of the total wastewater of a large tertiary care hospital (n = 8). Both manure as well as raw hospital wastewaters were contaminated with significant abundances of gene markers for facultative pathogens and with critical resistance genes of reserve antibiotics associated with genetic mobile elements for horizontal gene transfer. Hence, both compartments bear an exceptional potential risk for the dissemination of facultative pathogens and critical antibiotic resistance genes.

1. Introduction

The occurrence and spread of antibiotic resistance genes is a widely known and discussed problem of our today's society (Kaspar et al., 2016; Rossolini et al., 2014; Sib et al., 2020; WHO, 2019). One major point of these discussions is to locate different main sources of antibiotic resistance gene emissions into the environment. As already shown, wastewaters of either municipal, hospital or even industry origin can be major hotspots for clinically relevant resistance genes. The dissemination of antibiotic resistance genes can be prevented by advanced wastewater treatment at these hotspots in an either centralized (at wastewater treatment plants), or decentralized (at main discharge points) manner (Alexander et al., 2020; Hembach et al., 2017; Jäger et al., 2018; Savin et al., 2021). Other potential sources like stormwater overflow basins or the usage of manure for fertilization are often overlooked. In addition, most studies regarding these sources focus on widely used antibiotics that are no longer crucial for human therapy and whose resistance genes are ubiquitous (J. C. Chee-Sanford et al., 2001; Heuer et al., 2011; McKinney et al., 2018). Resistance genes against high priority or reserve antibiotics for human therapy are rarely part of studies involving food-producing sectors.

The use of antimicrobial agents in husbandry is considered the most important factor for the selection of antibiotic resistant bacteria in manure (Jayarao et al., 2019; Murphy et al., 2017), although the total sale of antimicrobial agents has decreased by 34.6% in the EU from 2001 to 2018 (More, 2020). Nevertheless, this decrease does not appear to be sufficient to control the burden of antibiotic resistance genes, due to their dissemination and environmental persistence (McKinney et al.,

☆ This paper has been recommended for acceptance by Prof. Dr. Klaus Kümmerer.

* Corresponding author.
E-mail address: thomas.schwartz@kit.edu (T. Schwartz).

https://doi.org/10.1016/j.envpol.2022.120128
Received 1 July 2022; Received in revised form 29 August 2022; Accepted 4 September 2022
Available online 8 September 2022

2018; Savin et al., 2021). However, antibiotic turnover only correlates directly with the antibiotic resistance burden in human populations, when high standards of sanitation infrastructure and hygiene practices are maintained, so that environmental factors do not have an impact on the transmission of resistant bacteria to humans and animals (Collignon et al., 2018). A relevant environmental factor that plays a role in the persistence and spread of antibiotic resistance into the environment is the increase in average temperatures associated with climate change. This factor alone may play a role in colonization with facultative pathogenic bacteria or even increase transfer of antibiotic resistance genes (Kaba et al., 2020). In addition, increased temperatures can also be considered as a stress situation for certain microorganisms and animals, which can be causative for an increased incidence of animal infection and consequently intensify the use of antibiotics with concomitant selection of resistance (Koutsoumanis et al., 2021).

The food-producing sectors have a direct or indirect role in the spread of antibiotic-resistant bacteria and antibiotic resistance genes in human settings, animal-related settings, and the environment (Hölzel et al., 2010; Sib et al., 2020). Antibiotic resistance genes, in bacteria or as extracellular DNA (i.e. eDNA), are released into the environment (soil surfaces, wash-off into surface waters, seepage, etc.) via faecal wastewater from food production (here, animal fattening) or via fertilization (Binh et al., 2008a; Su et al., 2014; Wichmann et al., 2014; Zhang et al., 2017).

Therefore, animal manure must be considered as a source in the transmission of antibiotic resistant bacteria and antibiotic resistance genes to the environment, and pig manure seems to be the highest contaminated source of resistance genes (Enne et al., 2008; Marti et al., 2014; McKinney et al., 2010; Pu et al., 2020). Fertilizers of faecal origin (such as manure) have already been identified as a route of entry of veterinary important antibiotic resistant bacteria and resistance genes into the environment and highlighted by WHO (WHO, 2020).

After reaching the environment, antibiotic resistant bacteria can colonize humans via serval routes by direct contact, e.g., by handling manure, by contact with contaminated water or by aerosol formation during manure spreading (Huijbers et al., 2015). The observation, that even vegetables which had been grown on manured fields contained increased abundances of resistance genes not only at harvest, but also on retail-level is a concerning issue (Rahube et al., 2014; Wang et al., 2015; Yang et al., 2018; Zhang et al., 2019; Zhu et al., 2017). Therefore, top fertilization of vegetables with liquid manure is forbidden in Germany. Besides direct health risks, the application of manure to fields has a major impact on the resistome and microbiome of the soil and the adjacent environment, increasing the persistent abundance of antibiotic resistance genes which in the long run also increases the overall risk potential (J. C. Chee-Sanford et al., 2001; Pu et al., 2020; Ruuskanen et al., 2016).

As mentioned above, most of the published research focuses on antibiotic resistances genes that are already very widespread and, despite playing a major role in medicine, are not clinically critical. The majority of already performed research focus on resistances against substances that are not used as reserve antibiotics like tetracyclines, sulfonamides. In consequence, the potential health risks and their distribution pathways are known, but have been put into the wrong perspective by focussing on old and widespread resistances, which have a limited risk potential for human healthcare. In addition, colistin, which has been adopted as a reserve antibiotic in human medicine after the rise of the carbapenems, has a long history of veterinary applications.

This work comparatively quantifies the abundances of antibiotic resistance genes with clinically importance in manures and clinical wastewater. Manure gets brought directly to the environment without major concerns, whilst clinical wastewater gets treated at conventional wastewater treatment plants. Our previous works suggest, that even conventional wastewater treatment is not sufficient for the retention of facultative pathogenic bacteria and antibiotic resistance genes (Jäger et al., 2018; Sib et al., 2020; Voigt et al., 2020).

2. Materials and methods

2.1. Sample collection and preparation

To observe the contamination of manure by antibiotic resistance genes and facultative pathogenic bacteria, 29 grab samples (100 mL) from different swine-producing facilities of different size were obtained (Table 1). Depending on the management of the farms, the manure had been stored for different lengths of time since the last manure spreading, which was between 2 and 10 months, as indicated in Table 1. It was unknown, if during the last withdrawal of manure, the complete storage tank had been emptied or whether only fractions of even older manure remained. Of each grab sample 6 mL liquid manure was centrifuged for 15 min and the DNA of the resulting pellet was extracted utilizing the FastDNA Spin Kit for Soil and FASTPREP® Instrument (MP Biomedicals, Santa Ana, CA). DNA concentrations were measured via an intercalating dye using a Qubit 3.0 system (Thermo scientific, Waltham, USA).

To compare the resistance situations of manures (n = 29) and raw hospital wastewater samples (n = 8), qPCR analyses were also performed. These hospital wastewater samples originated from the major sewer pipe containing sewage from a large, maximal care hospital with 38 independent clinics and more than 1200 beds. For each sampling 50 mL wastewater were filtrated through a polycarbonate membrane with a pore of diameter 0.2 μm (VWR, Germany) and DNA was extracted as mentioned above.

2.2. Molecular biology detection of antibiotic resistance genes and facultative pathogenic bacteria

For detection of both antibiotic resistance genes as well as facultative pathogenic bacteria, quantitative polymerase chain reaction (qPCR) was performed utilizing specific primers for a range of different microbial parameters (see SI Table 1). Derived from a list of facultative pathogens, which were defined by the WHO (World Health Organization, 2017) being responsible for 66% of all hospital acquired, nosocomial infections (National reference center for surveillance of nosokomial infections, 2016; Navidinia, 2016; Rice, 2008), the abundance of *Enterococcus faecium (cys)*, *Pseudomonas aeruginosa* (*ecfx*), *Klebsiella pneumoniae* (*glt*A), *Acinetobacter baumannii (secE)*, and *E. coli* (ycct) were analysed utilizing specific primers targeting the indicated taxonomic specific regions. Furthermore, the occurrence of three different veterinary associated antibiotic resistance genes (*erm*B, *tet*M, *sul*1) and five different clinically critical resistances genes (*mec*A, bla_{NDM}, *van*A, *mcr*-1, and bla_{KPC3}) were analysed. Resistance genes, that are associated with frequently employed veterinary antibiotics mediate resistances against tetracylines (*tet*M), sulfonamides (*sul*1), and marcolides (*erm*B) make up 42% (300 t) of the antibiotics used in veterinary medicine (BVL, 2019). The selected critical antibiotic resistance genes are directed against last line of resort as well as other clinically relevant antibiotics like glycopeptides (*van*A), carbapenems (bla_{NDM}, bla_{KPC3}), polymyxins (*mcr*-1), and methicillin in *S. aureus* (*mec*A) (Nordmann et al., 2011; Skov & Monnet, 2016). PCR assays contained specific primers for each resistance gene and taxonomic marker genes for facultative pathogenic bacteria. The qPCR-temperature profile consisted of an initial activation at 95 °C for 5 min followed by 40 cycles of 15 s denaturation at 95 °C and 1 min at 60 °C for annealing and elongation. The qPCR analyses were performed with 10 μL of Maxima SYBR Green Mastermix (Thermo Scientific, Waltham, USA), 0.8 μL of each 0.5 mM primer stock solution (Sigma Aldrich, Darmstadt, Germany), and 7.2 μL nuclease free water per reaction according to Hembach et al. (2019). A volume of 1 μL of extracted DNA solutions was added to each qPCR reaction. For additional quality control, qPCR amplicons were verified by electrophoresis and a melting curve analysis at the end of each run to assure both specificity and selectivity of each quantification. The detection limits of each primer system are also listed together with the other quality parameters in SI Table 1.

Table 1
Farm size, storage time, time since last spreading, sampling point, and yielded DNA concentration after DNA-extraction of all 29 sampled swine livestocks.

	Farm size	Average storage time [months]	time since last spreading [months]	sampling point	DNA concentration after extraction ng DNA/µL eluate
1	190 ha	9	2	elevated tank	253,4
2	4000 fattening places	6	3	elevated tank	419,5
3	455 sows	9	2	beneath slatted floor	430,5
4	1400 sows for breeding	10	5	elevated tank	150,9
5	unknown	unknown	unknown	unknown	324,6
6	1400 sows for breeding	10	5	elevated tank	324,2
7	unknown	unknown	unknown	unknown	247,8
8	2200 fattening places	6	3	elevated tank	216,8
9	unknown	unknown	unknown	unknown	288,7
10	1300 fattening places	6	6	elevated tank	223,55
11	560 sows, 3000 piglets	9	7	elevated tank	228,35
12	540 sows + piglets	10	8	elevated tank	432,6
13	50 ha	6	2	elevated tank	393,4
14	100 ha	9	4	elevated tank	184,4
15	220 sows; 1000 breeding places, 1000 fattening places	8	4	elevated tank	154,8
16	125 ha	12	3	elevated tank	226,3
17	185 ha	unknown	unknown	elevated tank	213,3
18	185 ha	9	2	preliminary tank	301,7
19	117 ha	3	2	preliminary tank	148,2
20	250 ha	3	4	preliminary tank	331,0
21	520 fattening sows	8	6	preliminary tank	179,85
22	1650 fattening sows	unknown	unknown	unknown	213
23	unknown	unknown	unknown	unknown	400
24	2600 fattening sows	unknown	unknown	unknown	140
25	2800 piglets, 500 sows	unknown	unknown	unknown	194,9
26	2200 fattening sows, 1200 piglets	unknown	unknown	unknown	90
27	1900 fattening sows	unknown	unknown	unknown	162
28	unknown	unknown	unknown	unknown	446
29	5700 fattening sows	unknown	unknown	unknown	232

2.3. Cultivation based detection

Supplementary methods of classical cultivation have also been performed. It became obvious, that commercially available selective media has limitations for liquid manure as unspecific growth was observed. The cultivation of such bacteria in environmental samples is also discussed in Schreiber et al. (2021).

2.4. Statistical analysis and data presentation

Each qPCR analysis was performed in technical triplicates and gene copies were calculated from the mean ct-values with the target specific calibration curve and normalized to 100 mL sample volume and 100 ng DNA, respectively. For data presentation for each parameter the mean of all 29 manure samples, as well as the mean of the 8 hospital wastewater samples were calculated. In Fig. 1 each individual measurement is represented by a dot, whilst a bar represents the calculated mean of all samples. Gene abundances of individual swine livestock and hospital samples are listed in SI Tables 2–5

3. Results and discussion

The average value of extracted DNA from 1 mL of manure was 7357 ng DNA (±46%) whilst 1 mL clinical wastewater contained on average 1331 ng DNA (±59%). The high variation in DNA yields was caused by the wide natural variety of different samples. As no discrimination between living bacteria, dead bacteria or bacteria which were viable but not cultivable (VBNC) was performed, the DNA yields represent the entirety of all physically intact bacteria. DNA from lysed bacteria and free DNA, which would be available for horizontal gene transfer, was not analysed since free DNA is removed by the DNA extraction method. Hence, the utilization of molecular biology techniques like qPCR allows capturing data from both aerobic and anaerobic bacteria, injured and dead but morphologically intact bacteria of the whole community.

3.1. Contamination of manure with clinically relevant antibiotic resistance genes and bacteria

Analysis of the manure samples showed, that besides resistance genes against antibiotics that are frequently used in veterinary medicine (*tet*M, *erm*B), both facultative pathogenic bacteria that belong to the ESKAPE group as well as clinically critical resistance genes were present in manure originating from swine farming. Resistance genes against the veterinary antibiotics were present in the highest abundances from 1.17×10^{10} gene copies/100 mL (*erm*B) to 1.2×10^{11} gene copies/100 mL (*tet*M). The sulfonamide resistance gene (*sul*1) was found in similar high abundance. The taxonomic gene markers specific for facultative pathogenic bacteria could be detected over a broad range of concentrations, from 6.0×10^{3} (*P. aeruginosa*) to 2.3×10^{7} (*E. faecium*) gene copies/100 mL. Here species of the intestinal microbiota (*E. coli* and *E. faecium*) were more abundant than the facultative pathogenic bacteria *P. aeruginosa, K. pneumoniae,* and *A. baumannii,* which are also associated with municipal or hospital wastewaters (Alexander et al., 2020). The clinically critical antibiotic resistance genes were present in significant abundances between 5.2×10^{4} (*van*A) to 1.2×10^{6} (bla_{NDM}) gene copies/100 mL (see Fig. 1A) in manure samples. No correlation between farm size and resistance gene contamination could be established, neither by total hectare sizes nor by livestock. Fig. 1 shows the mean abundance of facultative pathogenic bacteria as well as antibiotic resistance genes, which were present in all 29 analysed manure grab samples.

In addition to the volume-based evaluation, abundances of facultative pathogenic bacteria were referred to 100 ng DNA extracted from manure samples (Table 1 and Fig. 1C). These values were about 3–4 $\log_{10}$ units fold lower, when compared to the abundances per 100 mL manure (Fig. 1A), indicating that the high loads with resistance genes and facultative pathogens per 100 mL was caused by the overall high bacterial density in the manure. The composition of the observed ESKAPE-pathogens favoured intestinal flora (3.3×10^{2} gene copies/100 ng DNA for *E. coli* and 3.6×10^{3} gene copies/100 ng for *E. faecium*)

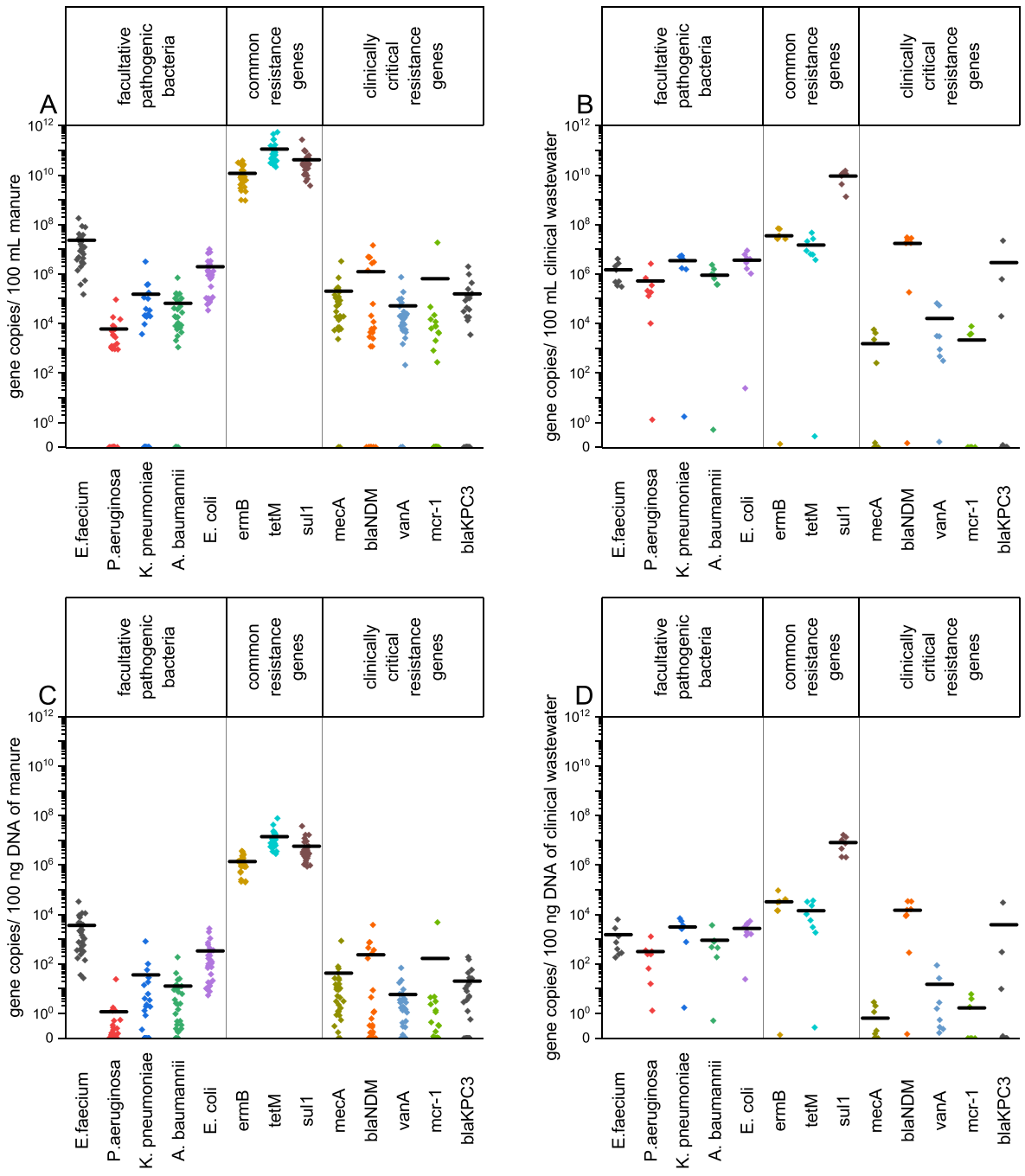

Fig. 1. Abundance of facultative pathogenic bacteria and antibiotic resistance genes in 100 mL (A&B) and per 100 ng DNA (C&D) in liquid manure (A&C) and clinic wastewater (B&D).

whilst the clinically more relevant facultative pathogenic bacteria (*P. aeruginosa, K. pneumoniae,* and *A. baumannii*) were with 1.1–36.0 gene copies/100 ng DNA less frequently detected. The same trend can be seen within the spectrum of the different antibiotic resistance genes. Veterinary resistance genes were with 1.4×10^6 (*erm*B) to 1.4×10^7 (*tet*M) gene copies/100 ng DNA present in a greater relative abundance of the population than the clinically critical antibiotic resistance genes with 5.8 to 2.3×10^2 gene copies/100 ng DNA. In conclusion, clinically critical pathogens made up a quite small part of the overall microbiome in contrast to the different categories of antibiotic resistance genes. However, through the overall high bacterial load of manure, the amount of facultative pathogenic bacteria and antibiotic resistance genes spread to the field without further treatment display a critical situation. In general, manure contributes to the microbial pollution with antibiotic resistance genes and clinically relevant bacteria are disseminated via the food producing chains into the environment (Heuer et al., 2011; Savin et al., 2021; Venglovsky et al., 2009; Zhu et al., 2017).

For an improved comparability with other wastewater compartments the complex texture of manures containing uncharacterised animal derived liquids, solid fractions, but also waters from farming operations have to be considered. Therefore, the DNA concentration-based evaluation delivers a more realistic state of contamination.

In most reports only resistance genes of veterinary commonly used antibiotics are analysed and therefore solely detected (Chee-Sanford et al., 2009; McKinney et al., 2018). It is noteworthy that in our study also critical resistance genes, which mediate resistances against reserve

antibiotics, were detected in significant abundances in manure samples. These critical genes were present in the majority of the manure samples without any information about the carrier microorganisms. Nevertheless, the first clinical isolates harbouring are facultative pathogenic bacteria of the ESKAPE group. Most of these antibiotic resistance genes are located on mobile genetic elements which participate in horizontal gene transfer. Such transfer events may happen during manure processing or storage time from pathogenic bacteria to manure inherent microorganisms or *vice versa*.

3.2. Antibiotic resistance genes and facultative pathogenic bacteria in hospital raw wastewater in comparison to manure results

To compare and assess the urgency of the problem regarding clinically critical antibiotic resistance genes in manure, Fig. 1 displays the contamination of clinical wastewater from a large hospital (n = 8) in both volume-based (B) and DNA-based (D) abundances. The values for bla_{NDM}, *sul1,* and *mcr1* have been published previously by Sib et al. (2020).

In clinical wastewater, facultative pathogenic bacteria could be detected with 5.5×10^5 (*P. aeruginosa*) and 3.6×10^6 (*K. pneumoniae*) gene copies/100 mL at a higher abundance than in manure from swine livestock. As mentioned above, the selected biological parameters were adapted to monitor hospital associated targets. Therefore, the higher loads in hospital wastewater were expected. Their share of the microbiome is conclusive. On one hand, the abundances of intestinal *E. faecium* decreased in comparison to the manure. On the other hand, the proportion of nosocomial facultative pathogens rose up to 3.2×10^3 gene copies/100 ng DNA for *K. pneumoniae* (36 gene copies per 100 ng DNA in manure) and for *A. baumannii* to 9.2×10^2 gene copies per 100 ng DNA (12 gene copies per 100 ng DNA in manure). Antibiotic resistance genes of older antibiotics, which are also used in veterinary settings, were determined with 1.5×10^4 (*tet*M) and 3.2×10^4 (*ermB*) gene copies/100 mL, i.e. about 3 $\log_{10}$ units less abundant in the hospital wastewater than in manure. The resistance gene *sul1* was with 8.1×10^6 gene copies/100 mL more abundant and determined in a comparable range in both hospital wastewater and manure. This higher frequency might be caused by the class 1 integron (*int*I1), a hospital associated mobile genetic element, which contains a *sul*1 gene in its genetic backbone (Hall, 1997). Fig. 1D illustrates the impact of frequently used antibiotics in infectious therapy on the resistome of the bacterial wastewater population. The carbapenemase resistance genes bla_{NDM} and bla_{KPC3} made up the majority of the clinically critical antibiotic resistance genes. A dominant facultative pathogenic bacterial species could not be observed, as all taxonomic gene markers were present within one $\log_{10}$ unit range between 3.0×10^2 and 2.0×10^3 gene copies/100 ng DNA.

Interestingly, the clinically critical resistance genes (*mecA*, *vanA*, *mcr*-1) were 2 $\log_{10}$ units less frequent in hospital wastewater than in manure. Only the resistance genes bla_{NDM} and bla_{KPC} were more abundant in clinical wastewater, with 1.7×10^7 (bla_{NDM}) and 2.8×10^6 (bla_{KPC}) gene copies/100 mL (1.2×10^6 resp. 1.6×10^5 gene copies/100 mL in manure).

3.3. Consequences of clinically relevant resistances in manure

Manure is not only highly contaminated with veterinary associated and commonly found antibiotic resistance genes, but it also contains high loads of clinically relevant resistance genes, like bla_{NDM} and bla_{KPC3}, and in a lower abundance with *van*A and *mcr*-1. Thus, manure poses a high potential risk, especially when applied to fields. It is as strongly contaminated as untreated clinical wastewater. But in contrast to hospitals, food producing facilities, and wastewater treatment plants, manure is not in the main focus of interest for dissemination of antibiotic resistance genes to the environment (Alexander et al., 2022; Hembach et al., 2017; Jäger et al., 2018; Savin et al., 2021). As a consequence, the dissemination of clinically critical antibiotic resistance genes through manure is not noticed sufficiently by the regulatory authorities and political societies.

Highly contaminated wastewaters from hospitals are treated at wastewater treatment plants before they are released to the environment. Conventional wastewater treatment plants do not remove all antibiotic resistant bacteria, but additional treatment technologies exist and their application is discussed (Alexander et al., 2016; Hembach et al., 2019; Sib et al., 2020). In contrast, manure is spread on the fields mostly without further treatment beside storage, only relying on the filter and degradation properties of the soil to protect the water resources from contamination.

They key objective of previous actions, to minimize the usage of antibiotics in veterinary did obviously not reached the expected results so far and further actions have to been taken to protect water resources (Durso & Cook, 2014; Pruden et al., 2013). Frequently, the storage is mentioned as an additional step to reduce antibiotic resistances and facultative pathogenic bacteria loads in manure. But as the manure samples were stored up to 7 months before sampling, the effect of storage times under realistic conditions is depicted in the 29 samples (Table 1). Indeed, it has already been documented, that bacterial pathogens are able to persist in animal manure under typical farming-conditions (Venglovsky et al., 2009). Typical storage conditions did not result in an significant contamination reduction during this research. This finding confirmed already mentioned doubt about the ability of digestion or composting to reduce the occurrence of antibiotic resistances in manure (Tien et al., 2017; Tran et al., 2021). Former studies have demonstrated that storage over a certain time is not sufficient to eliminate bacteria, but that specific fermentation is necessary for an efficient removal of unwanted bacteria and resistance genes (Tien et al., 2017). Earlier research showed that fermentation or even anaerobic composting significantly reduces the load of resistance genes (Anjum et al., 2017; Gou et al., 2018). Although removal via anaerobic digestion shows species selection processes towards gram positive bacteria (Tran et al., 2021).

It is known that manure supports horizontal gene transfer, and furthermore that antibiotic resistance genes accumulate on manured fields (Gotz & Smalla, 1997; Heuer et al., 2007; Knapp et al., 2010). In addition, the application of manure to the fields leads to an unwanted mixing of resistomes from different origins. Hence the manure resistome containing both, resistance genes against veterinary antibiotics, facultative pathogenic bacteria, and resistance genes critical for human health (e.g. against reserve antibiotics like *mcr*-1, bla_{NDM}), comes in contact with the biggest and most concerning origin of antibiotic resistance, the environment. Such a blending of factors leads to an enormous potential health risk. On one hand an emerging environmental pathogen might acquire an additional healthcare relevant resistance gene (HGT from the human related to the environment bacteria), on the other hand a new resistance mechanism might be transferred to a well-known facultative pathogen (HGT from the environment to a human pathogen) or the combination of the two (Knapp et al., 2010). Especially the latter situation is not limited to resistance genes, but also to other beneficial traits for the bacterium. In addition to the introduction of clinically critical resistance genes, manure has been shown to promote horizontal gene transfer (e.g. via elevated phosphorous concentration), which further speeds up the merging of the resistomes (Alegbeleye & Sant'Ana, 2020; Binh et al., 2008b; Von Wintersdorff et al., 2016). The focus of public interest lies nevertheless upon hospital wastewater or even wastewater in general, where discussions about additional treatment steps for reduction of the dissemination of resistant bacteria are already under way (Alexander et al., 2022). As there is a strong emphasis on preventing critical antibiotic resistance genes reaching the environment, the blending of resistomes caused by the application of manure to the field could stand for a significant drawback in the approach to fight against the spread of antimicrobial resistances (Levy & Bonnie, 2004; Organization, 2001).

As all the farms investigated in this research regularly bring their manure to the field, there is a relevant health risk of spreading clinically critical resistances, which has been ignored so far. Beside the analysed resistance genes, it is assumed that a broader spectrum of resistance genes reaches the resistome of the environment, where their persistence might lead to an increasing health risk (Heuer & Smalla, 2007; Knapp et al., 2010; Wright, 2010). Thus, the risk potential is shown to be (1) the occurrence of genetically mobile resistance genes in an overall microbial population that may be available for horizontal gene transfer, (2) the simultaneous occurrence of clinically relevant bacteria in the overall bacterial population that may act as donors or recipients of the resistance genes, and 3) people including farmers become colonized with such bacteria. Another step contributing to the assessment of manures' risk potential would be (4) to document the presence or absence of the combination of both pathogenic bacteria harbouring resistance genes via cultivation-based methods. It has been shown, that crops or even commercially available vegetables grown on manured fields have an high intracellular load of antibiotic resistance genes previously originating from field soils (Zhang et al., 2019; Zhu et al., 2017). These examples ultimately demonstrate the dynamics of resistance genes of clinically relevant antibiotics in the food chain. Additional risks are posed by the infiltration of high loads of biological contaminations into the environment, where even critical organisms or genes reach adjacent water sources like rivers, groundwaters, and reservoirs for drinking waters (Cole et al., 1999).

4. Conclusion

Based on the findings of this research, it is suspected that manure as direct animal waste not only plays a major role in the spread of antibiotic resistance genes in general, but more specifically also contributes substantially to the spread of antibiotic resistance genes against critical reserve antibiotics for human medicine. Manure is collected from livestock, stored and applied to agricultural land without further inactivation measures. In case of other hotspots of dissemination of facultative pathogenic bacteria and antibiotic resistance genes (like urban wastewater), there is an ongoing discussion about an implementation of advanced purification technology aiming at removal of micropollutants and antibiotic resistances. Actually, the application of manure for fertilization of fields is not intensively discussed for the spread antibiotic resistances and is in consequence not regulated at all. The highly contaminated material, which is comparable to raw hospital wastewater, but without any downstream treatment at wastewater treatment plants, is brought into the environment without further thought about consequences or subsequent health risks. After reaching the environment, antibiotic resistance genes are able to persist and spread via horizontal gene transfer to the endogenous bacterial population, thus incorporating the resistance genes into the environmental resistome, and resulting in unnecessary additional health risks for humans upon colonization either via diverse activities, groundwater or agricultural goods.

The use of manure as a natural soil fertilizer thereby creates a reservoir for transmissible antibiotic resistance genes and facultative pathogenic bacteria as possible recipients of antibiotic genes. This dissemination path of critical genes needs to be further assessed and even more importantly regulated.

Author statement

Norman Hembach: Writing- original Draft, Investigation, Formal analysis, Project administration, Gabriele Bierbaum: Writing-review & editing, Investigation, Christiane Schreiber: Writing-review & editing, Investigation, Thomas Schwartz: Founding acquisition, Writing- original draft, Conceptualization, Project administration

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

We thank the BMBF for financial support within the framework of the HyReKA-project as part of the RiSKWa-program. The HyReKA research project is also headed by Prof. Dr. Dr. Martin Exner and Dr. Dr. Ricarda Schmithausen (University Clinics Bonn, Germany). We acknowledge support by Deutsche Forschungsgemeinschaft. We also thank Dr. Chistophe Merlin (University of Lorraine, Nancy) providing the *E. coli* reference strain carrying the pNORM plasmid.Further thanks goes to all project partners and farms which were involved the acquiring of the analysed samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120128.

References

Alegbeleye, O.O., Sant'Ana, A.S., 2020. Manure-borne pathogens as an important source of water contamination: an update on the dynamics of pathogen survival/transport as well as practical risk mitigation strategies. Int. J. Hyg Environ. Health 227, 113524. https://doi.org/10.1016/j.ijheh.2020.113524.

Alexander, J., Hembach, N., Schwartz, T., 2020. Evaluation of antibiotic resistance dissemination by wastewater treatment plant effluents with different catchment areas in Germany. Sci. Rep. 10 (1), 1–9. https://doi.org/10.1038/s41598-020-65635-4, 2020 10:1.

Alexander, J., Hembach, N., Schwartz, T., 2022. Identification of critical control points for antibiotic resistance discharge in sewers. Sci. Total Environ. 820, 153186 https://doi.org/10.1016/J.SCITOTENV.2022.153186.

Alexander, J., Knopp, G., Dötsch, A., Wieland, A., Schwartz, T., 2016. Ozone treatment of conditioned wastewater selects antibiotic resistance genes, opportunistic bacteria, and induce strong population shifts. Sci. Total Environ. 559, 103–112. https://doi.org/10.1016/j.scitotenv.2016.03.154.

Anjum, R., Grohmann, E., Krakat, N., 2017. Anaerobic digestion of nitrogen rich poultry manure: impact of thermophilic biogas process on metal release and microbial resistances. Chemosphere 168, 1637–1647. https://doi.org/10.1016/j.chemosphere.2016.11.132.

Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008a. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 66 (1), 25–37. https://doi.org/10.1111/j.1574-6941.2008.00526.x.

Binh, C.T.T., Heuer, H., Kaupenjohann, M., Smalla, K., 2008b. Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 66 (1), 25–37. https://doi.org/10.1111/j.1574-6941.2008.00526.x.

BVL, 2019. *Erneut weniger Antibiotika in der Tiermedizin abgegeben.* Pressemitteilungen. https://www.bvl.bund.de/SharedDocs/Pressemitteilungen/05_tierarzneimittel/2019/2019_07_25_PI_Antibiotikaabgabe.html.

Chee-Sanford, J.C., Aminov, R.I., Krapac, I.J., Garrigues-Jeanjean, N., Mackie, R.I., 2001. Occurrence and diversity of tetracycline resistance genes in lagoons and groundwater underlying two swine production facilities. Appl. Environ. Microbiol. 67 (4), 1494–1502. https://doi.org/10.1128/AEM.67.4.1494-1502.2001.

Chee-Sanford, J.C., Mackie, R.I., Koike, S., Krapac, I.G., Lin, Y.-F., Yannarell, A.C., Maxwell, S., Aminov, R.I., 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. J. Environ. Qual. 38 (3), 1086–1108. https://doi.org/10.2134/jeq2008.0128.

Cole, D.J., Hill, V.R., Humenik, F.J., Sobsey, M.D., 1999. Health, safety, and environmental concerns of farm animal waste. In: Occupational Medicine (Philadelphia, Pa.), vol. 14, pp. 423–448. Issue 2. https://europepmc.org/article/med/10329913.

Collignon, P., Beggs, J.J., Walsh, T.R., Gandra, S., Laxminarayan, R., 2018. Anthropological and socioeconomic factors contributing to global antimicrobial resistance: a univariate and multivariable analysis. Lancet Planet. Health 2 (9), e398–e405. https://doi.org/10.1016/S2542-5196(18)30186-4.

Durso, L.M., Cook, K.L., 2014. Impacts of antibiotic use in agriculture: what are the benefits and risks? Curr. Opin. Microbiol. 19 (1), 37–44. https://doi.org/10.1016/J.MIB.2014.05.019.

Enne, V.I., Cassar, C., Sprigings, K., Woodward, M.J., Bennett, P.M., 2008. A high prevalence of antimicrobial resistant Escherichia coli isolated from pigs and a low prevalence of antimicrobial resistant E. coli from cattle and sheep in Great Britain at slaughter. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett. 278 (2), 193–199. https://doi.org/10.1111/j.1574-6968.2007.00991.x.

Gotz, A., Smalla, K., 1997. Manure enhances plasmid mobilization and survival of Pseudomonas putida introduced into field soil. Appl. Environ. Microbiol. 63 (5), 1980–1986. https://doi.org/10.1128/aem.63.5.1980-1986.1997.

Gou, M., Hu, H.W., Zhang, Y.J., Wang, J.T., Hayden, H., Tang, Y.Q., He, J.Z., 2018. Aerobic composting reduces antibiotic resistance genes in cattle manure and the resistome dissemination in agricultural soils. Sci. Total Environ. 612, 1300–1310. https://doi.org/10.1016/j.scitotenv.2017.09.028.

Hall, R.M., 1997. Mobile gene cassettes and integrons: moving antibiotic resistance genes in Gram-negative bacteria. Ciba Found. Symp. 207 (207), 192–205. https://doi.org/10.1002/9780470515358.ch12.

Hembach, N., Alexander, J., Hiller, C., Wieland, A., Schwartz, T., 2019. Dissemination prevention of antibiotic resistant and facultative pathogenic bacteria by ultrafiltration and ozone treatment at an urban wastewater treatment plant. Sci. Rep. 9 (1), 1–12. https://doi.org/10.1038/s41598-019-49263-1.

Hembach, N., Schmid, F., Alexander, J., Hiller, C., Rogall, E.T., Schwartz, T., 2017. Occurrence of the mcr-1 colistin resistance gene and other clinically relevant antibiotic resistance genes in microbial populations at different municipal wastewater treatment plants in Germany. Front. Microbiol. 8 (JUL), 1–11. https://doi.org/10.3389/fmicb.2017.01282.

Heuer, H., Fox, R.E., Top, E.M., 2007. Frequent conjugative transfer accelerates adaptation of a broad-host-range plasmid to an unfavorable Pseudomonas putida host. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 59 (3), 738–748. https://doi.org/10.1111/j.1574-6941.2006.00223.x.

Heuer, H., Schmitt, H., Smalla, K., 2011. Antibiotic resistance gene spread due to manure application on agricultural fields. Curr. Opin. Microbiol. 14 (3), 236–243. https://doi.org/10.1016/J.MIB.2011.04.009.

Heuer, H., Smalla, K., 2007. Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months. Environ. Microbiol. 9 (3), 657–666. https://doi.org/10.1111/j.1462-2920.2006.01185.x.

Hölzel, C.S., Schwaiger, K., Harms, K., Küchenhoff, H., Kunz, A., Meyer, K., Müller, C., Bauer, J., 2010. Sewage sludge and liquid pig manure as possible sources of antibiotic resistant bacteria. Environ. Res. 110 (4), 318–326. https://doi.org/10.1016/j.envres.2010.02.009.

Huijbers, P.M.C., Blaak, H., De Jong, M.C.M., Graat, E.A.M., Vandenbroucke-Grauls, C. M.J.E., De Roda Husman, A.M., 2015. Role of the environment in the transmission of antimicrobial resistance to humans: a review. Environ. Sci. Technol. 49 (20), 11993–12004. https://doi.org/10.1021/acs.est.5b02566.

Jäger, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G., Schwartz, T., Jager, T., Hembach, N., Elpers, C., Wieland, A., Alexander, J., Hiller, C., Krauter, G., Schwartz, T., 2018. Reduction of antibiotic resistant bacteria during conventional and advanced wastewater treatment, and the disseminated loads released to the environment. Front. Microbiol. 9 (2599), 2599. https://doi.org/10.3389/fmicb.2018.02599.

Jayarao, B., Almeida, R., Oliver, S.P., 2019. Antimicrobial resistance on dairy farms, pp. 1–4. https://doi.org/10.1089/FPD.2019.29011.EDI, 16(1). https://Home.Liebertpub.Com/Fpd.

Kaba, H.E.J., Kuhlmann, E., Scheithauer, S., 2020. Thinking outside the box: association of antimicrobial resistance with climate warming in Europe – a 30 country observational study. Int. J. Hyg Environ. Health 223 (1), 151–158. https://doi.org/10.1016/J.IJHEH.2019.09.008.

Kaspar, H., Wallmann, J., Kern, W., Greif, G., Peters, G., Rösler, U., 2016. Germap 2015 – report on the consumption of antimicrobials and the spread of antimicrobial resistance in human and veterinary medicine in Germany. In: Bundesamtes für Verbraucherschutz und Lebensmittelsicherheit (BVL). https://www.p-e-g.org/files/content/Ueber uns/GERMAP/GERMAP2012englisch.pdf.

Knapp, C.W., Dolfing, J., Ehlert, P.A.I., Graham, D.W., 2010. Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. Environ. Sci. Technol. 44 (2), 580–587. https://doi.org/10.1021/ES901221X.

Koutsoumanis, K., Allende, A., Álvarez-Ordóñez, A., Bolton, D., Bover-Cid, S., Chemaly, M., Davies, R., De Cesare, A., Herman, L., Hilbert, F., Lindqvist, R., Nauta, M., Ru, G., Simmons, M., Skandamis, P., Suffredini, E., Argüello, H., Berendonk, T., Cavaco, L.M., et al., 2021. Role played by the environment in the emergence and spread of antimicrobial resistance (AMR) through the food chain. EFSA J. 19 (6) https://doi.org/10.2903/j.efsa.2021.6651.

Levy, S.B., Bonnie, M., 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10 (12S), S122–S129. https://doi.org/10.1038/nm1145.

Marti, E., Variatza, E., Balcazar, J.L., 2014. The role of aquatic ecosystems as reservoirs of antibiotic resistance. Trends Microbiol. 22 (1), 36–41. https://doi.org/10.1016/J.TIM.2013.11.001.

McKinney, C.W., Dungan, R.S., Moore, A., Leytem, A.B., 2018. Occurrence and abundance of antibiotic resistance genes in agricultural soil receiving dairy manure. FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Ecol. 94 (3), 10. https://doi.org/10.1093/FEMSEC/FIY010.

McKinney, C.W., Loftin, K.A., Meyer, M.T., Davis, J.G., Pruden, A., 2010. Tet and sul antibiotic resistance genes in livestock lagoons of various operation type, configuration, and antibiotic occurrence. Environ. Sci. Technol. 44 (16), 6102–6109. https://doi.org/10.1021/es9038165.

More, S.J., 2020. European perspectives on efforts to reduce antimicrobial usage in food animal production. Ir. Vet. J. 73 (1), 1–12. https://doi.org/10.1186/S13620-019-0154-4/FIGURES/3.

Murphy, D., Ricci, A., Auce, Z., Beechinor, J.G., Bergendahl, H., Breathnach, R., Bureš, J., Duarte Da Silva, J.P., Hederová, J., Hekman, P., Ibrahim, C., Kozhuharov, E., Kulcsár, G., Lander Persson, E., Lenhardsson, J.M., Mačiulskis, P., Malemis, I., Markus-Cizelj, L., Michaelidou-Patsia, A., et al., 2017. EMA and EFSA Joint Scientific Opinion on measures to reduce the need to use antimicrobial agents in animal husbandry in the European Union, and the resulting impacts on food safety (RONAFA). EFSA J. 15 (1) https://doi.org/10.2903/J.EFSA.2017.4666.

National reference center for surveillance of nosokomial infections, 2016. Deutsche nationale Punkt-Prävalenzerhebung zu nosokomialen Infektionen und Antibiotika-Anwendung 2016. In: *NRZ-National reference center for surveillance of nosokomial infections*. Nationales Referenzzentrum für Surveillance von nosokomialen Infektionen. http://www.nrz-hygiene.de/fileadmin/nrz/download/pps2016/PPS_2016_Abschlussbericht_20.07.2017.pdf.

Navidinia, M., 2016. The clinical importance of emerging ESKAPE pathogens in nosocomial infections. J. Paramed. Sci. 7 (3), 43–57.

Nordmann, P., Poirel, L., Walsh, T.R., Livermore, D.M., 2011. The emerging NDM carbapenemases. Trends Microbiol. 19 (12), 588–595. https://doi.org/10.1016/j.tim.2011.09.005.

Organization, W.H., 2001. WHO Global Strategy for Containment of Antimicrobial Resistance.

Pruden, A., Joakim Larsson, D.G., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D. W., Lazorchak, J.M., Suzuki, S., Silley, P., Snape, J.R., Topp, E., Zhang, T., Zhu, Y.G., 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. Environ. Health Perspect. 121 (8), 878–885. https://doi.org/10.1289/EHP.1206446.

Pu, Q., Zhao, L.X., Li, Y.T., Su, J.Q., 2020. Manure fertilization increase antibiotic resistance in soils from typical greenhouse vegetable production bases, China. J. Hazard Mater. 391, 122267 https://doi.org/10.1016/j.jhazmat.2020.122267.

Rahube, T.O., Marti, R., Scott, A., Tien, Y.C., Murray, R., Sabourin, L., Zhang, Y., Duenk, P., Lapen, D.R., Topp, E., 2014. Impact of fertilizing with raw or anaerobically digested sewage sludge on the abundance of antibiotic-resistant coliforms, antibiotic resistance genes, and pathogenic bacteria in soil and on vegetables at harvest. Appl. Environ. Microbiol. 80 (22), 6898–6907. https://doi.org/10.1128/AEM.02389-14.

Rice, L.B., 2008. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: No ESKAPE. In: Journal of Infectious Diseases, vol. 197. The University of Chicago Press, pp. 1079–1081. https://doi.org/10.1086/533452. Issue 8.

Rossolini, G.M., Arena, F., Pecile, P., Pollini, S., 2014. Update on the antibiotic resistance crisis. Curr. Opin. Pharmacol. 18, 56–60. https://doi.org/10.1016/j.coph.2014.09.006.

Ruuskanen, M., Muurinen, J., Meierjohan, A., Pärnänen, K., Tamminen, M., Lyra, C., Kronberg, L., Virta, M., 2016. Fertilizing with animal manure disseminates antibiotic resistance genes to the farm environment. J. Environ. Qual. 45 (2), 488–493. https://doi.org/10.2134/jeq2015.05.0250.

Savin, M., Alexander, J., Bierbaum, G., Hammerl, J.A., Hembach, N., Schwartz, T., Schmithausen, R.M., Sib, E., Voigt, A., Kreyenschmidt, J., 2021. Antibiotic-resistant bacteria, antibiotic resistance genes, and antibiotic residues in wastewater from a poultry slaughterhouse after conventional and advanced treatments. Sci. Rep. 11 (1), 1–11. https://doi.org/10.1038/s41598-021-96169-y, 2021 11:1.

Schreiber, C., Zacharias, N., Essert, S.M., Wasser, F., Müller, H., Sib, E., Precht, T., Parcina, M., Bierbaum, G., Schmithausen, R.M., Kistemann, T., Exner, M., 2021. Clinically Relevant Antibiotic-Resistant Bacteria in Aquatic Environments - an Optimized Culture-Based Approach, vol. 750. The Science of the Total Environment. https://doi.org/10.1016/J.SCITOTENV.2020.142265.

Sib, E., Lenz-Plet, F., Barabasch, V., Klanke, U., Savin, M., Hembach, N., Schallenberg, A., Kehl, K., Albert, C., Gajdiss, M., Zacharias, N., Müller, H., Schmithausen, R.M., Exner, M., Kreyenschmidt, J., Schreiber, C., Schwartz, T., Parčina, M., Bierbaum, G., 2020. Bacteria isolated from hospital, municipal and slaughterhouse wastewaters show characteristic, different resistance profiles. Sci. Total Environ. 746, 140894 https://doi.org/10.1016/j.scitotenv.2020.140894.

Skov, R.L., Monnet, D.L., 2016. Plasmid-mediated colistin resistance (mcr-1 gene): three months later, the story unfolds. Euro Surveill. 21 (9), 30155 https://doi.org/10.2807/1560-7917.ES.2016.21.9.30155.

Su, J.Q., Wei, B., Xu, C.Y., Qiao, M., Zhu, Y.G., 2014. Functional metagenomic characterization of antibiotic resistance genes in agricultural soils from China. Environ. Int. 65, 9–15. https://doi.org/10.1016/J.ENVINT.2013.12.010.

Tien, Y.C., Li, B., Zhang, T., Scott, A., Murray, R., Sabourin, L., Marti, R., Topp, E., 2017. Impact of dairy manure pre-application treatment on manure composition, soil dynamics of antibiotic resistance genes, and abundance of antibiotic-resistance genes on vegetables at harvest. Sci. Total Environ. 32–39. https://doi.org/10.1016/J.SCITOTENV.2016.12.138. *581–582*.

Tran, T.T., Scott, A., Tien, Y.C., Murray, R., Boerlin, P., Pearl, D.L., Liu, K., Robertson, J., Nash, J.H.E., Topp, E., 2021. On-farm anaerobic digestion of dairy manure reduces the abundance of antibiotic resistance-associated gene targets and the potential for plasmid transfer. Appl. Environ. Microbiol. 87 (14), 1–20. https://doi.org/10.1128/AEM.02980-20/SUPPL_FILE/AEM02980-20_SUPP_1_SEQ4.PDF.

Venglovsky, J., Sasakova, N., Placha, I., 2009. Pathogens and antibiotic residues in animal manures and hygienic and ecological risks related to subsequent land application. Bioresour. Technol. 100 (22), 5386–5391. https://doi.org/10.1016/J.BIORTECH.2009.03.068.

Voigt, A.M., Zacharias, N., Timm, C., Wasser, F., Sib, E., Skutlarek, D., Parcina, M., Schmithausen, R.M., Schwartz, T., Hembach, N., Tiehm, A., Stange, C., Engelhart, S., Bierbaum, G., Kistemann, T., Exner, M., Faerber, H.A., Schreiber, C., 2020.

Association between antibiotic residues, antibiotic resistant bacteria and antibiotic resistance genes in anthropogenic wastewater – an evaluation of clinical influences. Chemosphere 241, 125032. https://doi.org/10.1016/J.CHEMOSPHERE.2019.125032.

Von Wintersdorff, C.J.H., Penders, J., Van Niekerk, J.M., Mills, N.D., Majumder, S., Van Alphen, L.B., Savelkoul, P.H.M., Wolffs, P.F.G., 2016. Dissemination of antimicrobial resistance in microbial ecosystems through horizontal gene transfer. Front. Microbiol. 7 (FEB), 173. https://doi.org/10.3389/FMICB.2016.00173/BIBTEX.

Wang, F.H., Qiao, M., Chen, Z., Su, J.Q., Zhu, Y.G., 2015. Antibiotic resistance genes in manure-amended soil and vegetables at harvest. J. Hazard Mater. 299, 215–221. https://doi.org/10.1016/J.JHAZMAT.2015.05.028.

Who, 2019. No time to wait: securing the future from drug-resistant infections. Artforum Int. 54 (April), 113–114.

Who, 2020. Technical Brief on Water, Sanitation, Hygiene and Wastewater Management to Prevent Infections and Reduce the Spread of Antimicrobial Resistance. https://www.who.int/water_sanitation_health/publications/wash-wastewater-management-to-prevent-infections-and-reduce-amr/en/.

Wichmann, F., Udikovic-Kolic, N., Andrew, S., Handelsman, J., 2014. Diverse antibiotic resistance genes in dairy cow manure. mBio 5 (2). https://doi.org/10.1128/MBIO.01017-13/SUPPL_FILE/MBO002141797ST3.DOCX.

World Health Organization, 2017. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. Cadernos Pesquisa 43 (148), 348–365.

Wright, G.D., 2010. Antibiotic resistance in the environment: a link to the clinic? Curr. Opin. Microbiol. 13 (5), 589–594. https://doi.org/10.1016/j.mib.2010.08.005.

Yang, L., Liu, W., Zhu, D., Hou, J., Ma, T., Wu, L., Zhu, Y., Christie, P., 2018. Application of biosolids drives the diversity of antibiotic resistance genes in soil and lettuce at harvest. Soil Biol. Biochem. 122, 131–140. https://doi.org/10.1016/J.SOILBIO.2018.04.017.

Zhang, Y.J., Hu, H.W., Chen, Q.L., Singh, B.K., Yan, H., Chen, D., He, J.Z., 2019. Transfer of antibiotic resistance from manure-amended soils to vegetable microbiomes. Environ. Int. 130, 104912 https://doi.org/10.1016/J.ENVINT.2019.104912.

Zhang, Y.J., Hu, H.W., Gou, M., Wang, J.T., Chen, D., He, J.Z., 2017. Temporal succession of soil antibiotic resistance genes following application of swine, cattle and poultry manures spiked with or without antibiotics. Environ. Pollut. 231, 1621–1632. https://doi.org/10.1016/J.ENVPOL.2017.09.074.

Zhu, B., Chen, Q., Chen, S., Zhu, Y.G., 2017. Does organically produced lettuce harbor higher abundance of antibiotic resistance genes than conventionally produced? Environ. Int. 98, 152–159. https://doi.org/10.1016/J.ENVINT.2016.11.001.