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Effects of Veterinary Medicines Introduced via Manure into Soil on Microbial Communities

Holger HEUER, Chu Thi Thanh BINH, Christoph KOPMANN, Ute ZIMMERLING,
Ellen KRÖGERRECKLENFORT and Kornelia SMALLA

*Julius Kühn-Institut - Federal Research Centre for Cultivated Plants (JKI),
Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany*

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Abstract—Multiple antibiotic resistant bacterial pathogens pose a major threat for the successful therapeutic use of antibiotics in human and veterinary medicine. Veterinary antibiotics used in animal husbandry are assumed to contribute to an increased antibiotic resistance among bacteria in manure. Bacteria carrying transferable antibiotic resistances, antibiotics, metabolites and nutrients are inevitably brought into soil when manure is used as fertilizer. Some antibiotics such as sulfadiazine (SDZ) are almost completely excreted and persist during manure storage. Here we summarize the results of a study on 15 field-scale piggery manures by means of cultivation-dependent and -independent methods that clearly revealed that transferable antibiotic resistances are routinely introduced into soil via manure. In soil microcosms we showed that both manure spreading and SDZ spiking synergistically increased the abundance of SDZ resistance genes and their transferability in soil. Self-transferable plasmids carrying multiple antibiotic resistances could be easily captured into plasmid-free *E. coli* recipient strains and transfer frequencies were enhanced in soils treated with manure with SDZ added. Molecular characterization of the plasmids captured provided insights into the extant diversity of the soil mobilome.

Keywords: *sul* genes, plasmids, piggery manure, exogenous plasmid capture, qPCR

INTRODUCTION

Manure fertilization is one of the measures in agriculture to maintain soil fertility. However, the use of veterinary antibiotics in animal husbandry is suspected to affect the bacterial community structure in manure and increase the prevalence of antibiotic resistance of the bacteria in manure (Schauss *et al.*, 2009). Depending on the chemical structure of the antibiotic, a substantial part can be excreted and thus enter the soil via manure (Halling-Sørensen *et al.*, 1998; Heuer *et al.*, 2008). Sulfonamide and beta-lactam antibiotics are among the most frequently used antibiotics for animal husbandry (Mathew *et al.*, 2007) to control diarrhea and various other infectious diseases. Resistance towards these antibiotics can emerge in the gut micro-flora of treated animals. Three genes (*sul1*, *sul2*, *sul3*) were

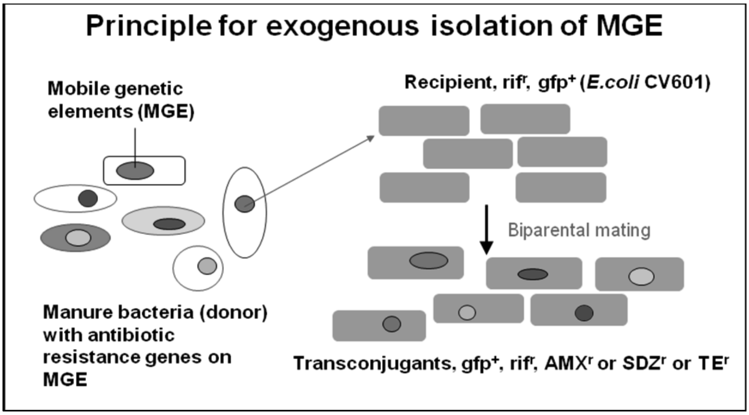

Fig. 1. Capturing mobile genetic elements (MGE) conferring resistance to ampicillin, sulfadiazine and tetracycline directly from manure bacteria into gfp-tagged rifampicin resistant *E. coli* recipient strains.

described to confer sulfonamide resistance by encoding dihydropteroate synthases which are sulfonamide-insensitive (Rådström and Swedberg, 1988; Sundström *et al*., 1988; Perreten and Boerlin, 2003). Many genes have been found to encode proteins involved in β-lactam antibiotic resistance (Bush, 1997) but *bla*-TEM genes are the most abundant β-lactamase encoding genes (Livermore, 1995). Only in a few studies, quantification of these genes in environmental flora was attempted (Pei *et al*., 2006; Bibbal *et al*., 2007; Heuer and Smalla, 2007).

TRANSFERABLE ANTIBIOTIC RESISTANCES IN PIGGERY MANURE

The effect of antibiotics introduced via manure into soils on soil microbial communities is presently investigated in the frame of a Deutsche Forschungsgemeinschaft (DFG: German Research Foundation) research group (Binh *et al*., 2007; Heuer and Smalla, 2007; Heuer *et al*., 2008; Kotzerke *et al*., 2008; Schauss *et al*., 2009). In order to provide baseline data on bacterial communities typical of manure used for soil fertilization, a survey of a unique set of 16 manures collected from 15 different pig producing facilities in Germany (so-called field-scale manure) was done. The farms varied in numbers of pigs, production type, and antibiotic usage. Cultivation-dependent methods were used to determine the proportion of bacteria resistant to SDZ, amoxicillin and tetracycline. Between 2 to 69% of cultured bacteria were resistant to the sulfonamide antibiotic SDZ, while less than 0.3% showed resistance to the much less stable β-lactam antibiotic amoxicillin. The abundance of *sul1*, *sul2* and *bla*-TEM resistance genes in total community DNA was quantified by real-time PCR. In all manures, a high proportion of bacteria carried *sul* genes, especially *sul2*, while the relative abundance of *bla*-TEM was at least two log units lower. Integron gene cassette diversity in manure and manured soils was studied by

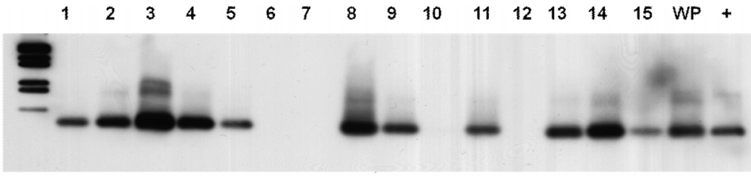

Fig. 2. Southern blot hybridization of IncQ *oriV* PCR products amplified from total community DNA of manure.

cloning PCR products amplified with primers targeting the conserved segments of class1 integrons (Heuer and Smalla, 2007) from total community DNA, restriction fragment length polymorphism (RFLP) analysis and sequencing. The data showed that *aadA* gene cassettes encoding resistance to streptomycin and spectinomycin were frequently detected in DNA from all manures and from manure-treated soils (Binh *et al.*, 2009). Furthermore, transfer frequencies for capturing mobile genetic elements conferring resistance to SDZ, tetracycline and amoxicillin in *E. coli* CV601 were determined, and a representative subset of plasmids was characterized (Binh *et al.*, 2008; see Fig. 1). A total of 228 transconjugants were isolated from selective media supplemented with amoxicillin, SDZ or tetracycline. Transfer frequencies varied between 10^{-4} and 10^{-8}. Most of the transconjugants captured plasmids conferring multi-drug resistance, four of them acquired even resistance towards seven of the eight antibiotics tested, and 40 transconjugants were resistant towards six of the eight antibiotics tested (Binh *et al.*, 2008). Based on their restriction patterns and on the origin of the sample, eighty-one plasmids were selected and characterized by PCR and hybridization with primers and/or probes specific for different plasmid groups and antibiotic resistance genes. Replicon probing showed that the majority of the plasmids captured belonged to the IncN group, one plasmid was assigned to IncW, 12 to IncP-1, none to IncQ. Nineteen transconjugants carried plasmids related to the recently discovered group of pHHV216-like plasmids (Heuer *et al.*, 2009). The *bla*-TEM gene was detected on 44 plasmids, and 68 plasmids carried sulfonamide resistance genes. Thus our data overwhelmingly demonstrated that manure is also a reservoir of bacteria carrying transferable antibiotic resistance genes (Binh *et al.*, 2008). Southern blot analysis of PCR products amplified from total community DNA of different manures by means of primers targeting backbone genes of broad-host range plasmids (Götz *et al.*, 1996) revealed a high abundance of plasmids belonging to IncP-1, and IncQ (see Fig. 2) in many of the manure samples while hybridization signals for IncN and IncW were much less strong and detected in fewer manures (Binh *et al.*, 2008).

SPREADING ANTIBIOTIC RESISTANCES IN SOIL BACTERIA VIA SPREAD MANURE?

Based on the survey of 15 field-scale manures it was assumed that fertilization by means of manure might stimulate the spread of antibiotic resistance among soil bacteria that might end as plant-associated bacteria in uncooked food or feed. The capacity of soil bacteria to sample the horizontal gene pool provides them with the ability to rapidly adapt to environmental stresses such as veterinary antibiotics introduced into soil via manure. Manure treatments of arable soils might stimulate horizontal gene transfer and the spread of resistance genes (Götz and Smalla, 1997; Smalla *et al.*, 2000). The effects of pig manure and SDZ on the abundance, diversity and transferability of resistance genes in soil bacterial communities were studied in soil microcosm experiments (Heuer and Smalla, 2007). A silt loam and a loamy sand were mixed with manure containing SDZ (10 or 100 mg/kg soil), and compared to untreated soil and manure-treated soil without SDZ over a two-month period. In both soils, manure positively affected the quotients of total and SDZ resistant cultivable bacteria. Also transfer frequencies of plasmids conferring SDZ resistance in filter mating of soil bacteria and an *E. coli* recipient were significantly increased by manure. Both effects were enhanced by SDZ (Heuer and Smalla, 2007). Most captured plasmids conferred multiple antibiotic resistances to the *E. coli* recipient. The plasmids were analyzed for transferability, host range, replicon type and *sul* genes. By PCR quantification of *sul1*, *sul2* and bacterial *rrn* (ribosomal RNA) genes, a transient effect of manure alone and a long-term effect of SDZ plus manure on absolute and relative *sul1* and *sul2* abundance in soil was shown (Heuer and Smalla, 2007; Heuer *et al.*, 2009). The synergistic effects of the manure and SDZ were still detectable after two months. The results suggest that manure from treated pigs enhances spread of antibiotic resistances in soil bacterial communities. Presently, we investigate the effects of repeated applications of manure from pigs treated with SDZ or untreated, and of the rhizosphere on the abundance and transferability of *sul* resistance in bacterial communities of bulk and rhizosphere samples. The experiments are being performed under mesocosm and field conditions. The unpublished data indicate that both the repeated application of manure to soils and the rhizosphere amplify the effect of manure from antibiotic-treated pigs on soil bacterial communities.

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REFERENCES

Bibbal, D., V. Dupouy, J. P. Ferré, P. L. Toutain, O. Fayet, M. F. Prère and A. Bousquet-Mélou (2007): Impact of three ampicillin dosage regimens on selection of ampicillin resistance in *Enterobacteriaceae* and excretion of bla_{TEM} genes in swine feces. *Appl. Environ. Microbiol.*, **73**, 4785–4790.

Binh, C. T. T., H. Heuer, N. C. M. Gomes, A. Kotzerke, M. Fulle, B. M. Wilke, M. Schloter and K. Smalla (2007): Short-term effects of amoxicillin on bacterial communities in manured soil. *FEMS Microbiol. Ecol.*, **62**, 290–302.

Binh, C. T. T., H. Heuer, M. Kaupenjohann and K. Smalla (2008): Piggery manure used for soil fertilization is a reservoir for transferable antibiotic resistance plasmids. *FEMS Microbiol. Ecol.*, **66**, 25–37.

Binh, C. T. T., H. Heuer, M. Kaupenjohann and K. Smalla (2009): Diverse *aadA* gene cassettes on class 1 integrons introduced into soil via spread manure. *Res. Microbiol.*, **160**, 427–433.

Bush, K. (1997): The evolution of β-lactamases. p. 152–163. In *Antibiotic Resistance: Origins, Evolution, Selection and Spread*, ed. by D. J. Chadwick and J. Goode, John Wiley & Sons, Chichester.

Götz, A. and K. Smalla (1997): Manure enhances plasmid mobilization and survival of *Pseudomonas putida* introduced into field soil. *Appl. Environ. Microbiol.*, **63**, 1980–1986.

Götz, A., R. Pukall, E. Smit, E. Tietze, R. Prager, H. Tschäpe, J. D. van Elsas and K. Smalla (1996): Detection and characterization of broad-host range plasmids in environmental bacteria by PCR. *Appl. Environ. Microbiol.*, **62**, 2621–2628.

Halling-Sørensen, B., N. S. Nors, P. F. Lanzky, F. Ingerslev, H. C. H. Lutzhøft and S. E. Jørgensen (1998): Occurrence, fate and effects of pharmaceutical substances in the environment—a review. *Chemosphere*, **36**, 357–393.

Heuer, H. and K. Smalla (2007): Manure and sulfadiazine synergistically increased bacterial antibiotic resistance in soil over at least two months.. *Environ. Microbiol.*, **9**, 657–666.

Heuer, H., A. Focks, M. Lamshöft, K. Smalla, M. Matthies and M. Spiteller (2008): Fate of sulfadiazine administered to pigs and its quantitative effect on the dynamics of bacterial resistance genes in manure and manured soil. *Soil Biol. Biochem.*, **40**, 1892–1900.

Heuer, H., C. Kopmann, C. T. T. Binh, E. M. Top and K. Smalla (2009): Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. *Environ. Microbiol.*, **11**, 937–949.

Kotzerke, A., S. Sharma, K. Schauss, H. Heuer, S. Thiele-Bruhn, K. Smalla, B. M. Wilke and M. Schloter (2008): Alterations in soil microbial activity and *N*-transformation processes due to sulfadiazine loads in pig-manure. *Environ. Pollut.*, **153**, 315–322.

Livermore, D. M. (1995): β-lactamases in laboratory and clinical resistance. *Clin. Microbiol. Rev.*, **8**, 557–584.

Mathew, A. G., R. Cissell and S. Liamthong (2007): Antibiotic resistance in bacteria associated with food animals: a United States perspective of livestock production. *Foodborne Pathog. Dis.*, **4**, 115–133.

Pei, R., S. C. Kim, K. H. Carlson and A. Pruden (2006): Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.*, **40**, 2427–2435.

Perreten, V. and P. Boerlin (2003): A new sulfonamide resistance gene (*sul3*) in *Escherichia coli* is widespread in the pig population of Switzerland. *Antimicrob. Agents Chemother.*, **47**, 1169–1172.

Rådström, P. and G. Swedberg (1988): RSF1010 and a conjugative plasmid contain *sul2*, one of the two known genes for plasmid-borne sulfonamide resistance dihydropteroate synthase. *Antimicrob. Agents Chemother.*, **32**, 1684–1692.

Schauss, K., A. Focks, H. Heuer, A. Kotzerke, H. Schmitt, S. Thiele-Bruhn, K. Smalla, B.-M. Wilke, M. Matthies, W. Amelung, J. Klasmeier and M. Schloter (2009): Analysis, fate and effects of antibiotic sulfadiazine in soil ecosystems. *Trends Anal. Chem.*, **28**, 612–618.

Smalla, K., H. Heuer, A. Götz, D. Niemeyer, E. Krögerrecklenfort and E. Tietze (2000): Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl. Environ. Microbiol.*, **66**, 4854–4864.

Sundström, L., P. Rådström, G. Swedberg and O. Sköld (1988): Site-specific recombination promotes linkage between trimethoprim- and sulfonamide-resistance genes: sequence characterization of *dfr5* and *sul1* and a recombination active locus of Tn*21*. *Mol. Gen. Genet.*, **213**, 191–201.

K. Smalla (e-mail: kornelia.smalla@jki.bund.de)