NATURAL ANTIBIOTIC RESISTANCE GENES IN SOIL BACTERIA AND INFLUENCE OF ORGANIC FERTILISERS ON THEIR PREVALENCE AND HORIZONTAL TRANSFER

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Abstract

All natural antibiotics available to modern medicine are products of soil-dwelling bacteria and fungi. In addition, all resistance genes which are being detected in human pathogens existed in soil bacteria even before antibiotics were discovered and brought into use. However, the concentrations of natural antibiotics in soil are usually subinhibitory – insufficient for the selection of resistant subpopulations of microorganisms. The consumption of organic fertilisers for agricultural soil amendment increases proportionally to the consumers' growing demand for organically produced food. Manure originating from industrial pig, cattle and poultry farms is not only the source of nutrients which stimulate the vital functions of soil microorganisms, but also of antibiotics and bacteria harbouring various resistance mechanisms. The application of organic fertilizer leads to disruption of the natural balance between bacterial communities in the soil through several mechanisms, and influences the increase in the prevalence of resistance genes and promotes their horizontal transfer. Whether as-yet-unknown resistance genes in soil bacteria may pose threat to human health if transferred from commensal bacteria in the environment to pathogen species, or migrate to clinical settings via food chain or in some other possible route - remains an open question.

Key words: organic fertiliser, soil bacteria, antibiotic resistance genes

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GENI REZISTENCIJE NA ANTIBIOTIKE KOD BAKTERIJA U ZEMLJIŠTU I UTICAJ PRIMENE ORGANSKOG ĐUBRIVA NA NJIHOVU PREVALENCIJU I HORZONTALNI TRANSFER

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Kratak sadržaj

Svi prirodni antibiotici koji su na raspolaganju savremenoj medicini su produkti bakterija i glivica kojima je zemljište prirodno stanište, a svi geni rezistencije koji se danas ustanovljavaju kod humanih patogena postojali su kod bakterija u zemljištu i pre otkrića i upotrebe antibiotika. Međutim, koncentracije prirodnih antibiotika u zemljištu uobičajeno su na nivou subihnibitornih, a time i nedovoljne za selekciju rezistentnih subpopulacija mikroorganizama. Upotreba organskog đubriva za fertilizaciju poljoprivrednog zemljišta, povećava se srazmerno porastu zahteva potrošača za organski proizvednom hranom. Organsko đubrivo poreklom sa industrijalizovanih farmi svinja, goveda i živine, nije samo izvor nutrijenata koji podstiču životne funkcije mikroorganizama u zemljištu, već i antibiotika i bakterija sa raznovrsnim mehanizmima rezistencije. Dodavanje organskog đubriva preko više mehanizama remeti prirodnu ravnotežu zajednice bakterija u zemljištu, utiče na porast prevalencije gena rezistencije i podstiče njihov horizontalni transfer. Otvoreno je pitanje da li do sada neotkriveni geni rezistencije zemljišnih bakterija mogu biti nova pretnja ljudskom zdravlju ukoliko se prenesu sa komensalnih bakterija iz okruženja na patogene vrste, ili lancem ishrane i drugim mogućim putevima, migriraju u kliničke uslove.

Ključne reči: organsko đubrivo, bakterije u zemljištu, geni rezistencije na antibiotike

INTRODUCTION

Considered ecologically more acceptable than mineral, organic fertilisers have traditionally been used in agricultural areas throughout the world (Ding et al., 2014). The increasing consumer demands for organic products will very likely lead to the rise in their use in the future (Udikovic-Kolic et al., 2014).

Soil is fertilised mainly with manure or compost, derived from industrial, high-capacity poultry and pig farms. However, the sustainability of these branches of animal production has been based for decades on continual extensive use of antibiotics (Aarestrup, 2005; FAO, 2016). Tetracyclines, sulphonamides, aminoglycosides, β-lactams and macrolides are most frequently administered to animals and the majority of these, or some related, are in use in humans (Thiele-Bruhn, 2003; Allen et al., 2010). A dramatic development and spread of antibiotic resistance in bacteria rendered the maintenance of their efficacy a top priority in human healthcare, which can be achieved by their rational and justified use for therapeutic purposes only. Nonetheless, in food-producing animals antibiotics have increasingly been used in the absence of infection (for prophylaxis), and in many countries outside of the European Union, as growth promoters (Aarestrup, 2005). For instance, only in the US the sales and distribution of medically important antimicrobials approved for use in food-producing animals, in the period from 2009 to 2014 increased by 23% (FDA, 2015).

In poultry and pig production antibiotics are usually administered via food and/or water, which results in the fact that they are applied both to diseased and healthy animals. Orally administered antibiotics create favourable conditions for the selection, spread and persistence of antimicrobial-resistant bacteria in the digestive tract, including foodborne pathogens and other zoonotic bacteria (such as *Salmonella*, *Escherichia*, *Campylobacter* and *Enterococcus*). This effect concerns especially classes of antibiotics which are poorly or not at all absorbed from the gastrointestinal system, but are continually added to animal feed to stimulate growth. Antibiotics and their metabolic products, which may also be biologically active, are excreted from the body after a relatively short time of their action in the animal organisms (Thiele-Bruhn, 2003). For example, after oral application, 40-90% of sulphonamides consumed are excreted unchanged or as less active metabolites (Heuer and Smalla, 2007). Depending on their physical and chemical properties, antibiotics are eliminated predominantly via faeces or urine. Afterwards, they either do not undergo degradation, or degrade to a certain extent during manure processing (Heuer et al., 2010; Ambrožič Avguštin, 2012; Ding et al., 2014).

Manure provides soil microorganisms with nutrients which stimulate their vital functions, but is also a source of antibiotics and bacteria harbouring various mechanism of resistance (Heuer and Smalla, 2007; 2010). To assess the influence of organic fertilisers on the spread of resistance genes among soil bacteria is an especially difficult task. There is also an open question of potential transfer of resistance from soil bacteria to human opportunistic pathogens.

NATURAL ANTIBIOTIC RESISTANCE GENES IN SOIL BACTERIA

Real knowledge of the identity, natural diversity and patterns of distribution of antibiotics resistance genes (ARGs) in soil bacteria is limited, because it is virtually impossible to find a region without the influence of human activities (Riesenfeld et al., 2004; Allen et al., 2010; Forsberg et al., 2014; Wichmann et al., 2014). The period which preceded the beginning of chemotherapy (before 1936, when sulphonamides were first used) can be considered antibiotic-naive (Allen et al., 2010). Wide use of antibiotics in clinical and agricultural settings enormously contributed to the evolution and diversity of resistance genes in the environment. Antibiotic therapy began in 1941 when benzylpenicillin was first produced for clinical trials (EMEA, 1999). This was followed by the golden age of antibiotics, the period between the 1940s and the 1990s, when the majority of them, which are still available, were discovered and introduced into clinical practice (Nesme and Simonet, 2015). For centuries before the discovery of antibiotics, heavy metals were used to cure people. Metal contamination could have an important role in the maintenance od antibiotic resistance. These co-selection mechanisms include co-resistance: different resistance determinants present on the same genetic element) and cross-resistance (the same genetic determinant responsible for resistance to antibiotics and metals) (Baker-Austin et al., 2006).

It is especially difficult to conduct research on soil due to its physical and chemical heterogeneity and large genetic diversity, which is noticeable at distances of one metre, or even less (Allen et al., 2010; Nesme and Simonet, 2015). Additional hindrance results from the fact that sampling methods, storage time and conditions may strongly influence the results, and the absence of guidelines for resistance studies of environmental bacteria impede reaching the conclusions (Allen et al., 2010). Depending on the geographic location, a gram of soil contains from 10^3 to 10^6 different bacterial species, which varies with the methodology of investigations and the taxonomic units defined (Nesme and Simonet, 2015). Although the number of microorganisms per gram of soil is comparable with the human population on the earth, less than 1% can be iso-

lated in vitro on standard laboratory media (Allen et al., 2009; Wang and Yu, 2012; Schmieder and Edwards, 2012). Thus, the investigation into the diversity of resistance genes in soil bacteria requires the use of culture-independent methods (Schmieder and Edwards, 2012; Wang and Yu, 2012). The polymerase chain reaction (PCR) technique enables the detection of resistance genes which are already known or some closely related ones and, thus, has limited capability (Riesenfeld et al., 2004; Allen et al., 2009). Additional information has been obtained from functional metagenomics, which is based on the extraction and DNA cloning directly from environmental samples, such as soil (Riesenfeld et al., 2004). The analyses of metagenomic clones are performed with random sequencing or PCR amplification of target genes (Allen et al., 2009), and the expression of metagenomic DNA sequence in a heterologous, surrogate host (most frequently *Escherichia coli*) and activity–based screening (Demanèche et al., 2008; Allen et al., 2009, 2010; Udikovic-Kolic et al., 2014; Forsberg et al., 2014; Nesme and Simonet, 2015).

More than 80% of antibiotics available to modern medicine are produced by soil-dwelling bacteria and fungi or are their semi-synthetic pharmaceutical derivatives (D'Costa et al., 2006; Schmieder and Edwards, 2012). For example, about 30% to 50% soil actinomycetes of the genus *Streptomyces* can synthesise antibiotics (Thiele-Bruhn, 2003). The numbers of natural antibiotics, which are secondary metabolic products of soil bacteria, are far beyond those available to human and veterinary medicine. Given that actinomycetes alone synthesise approximately a hundred substances with antimicrobial activities (out of which only 30 has been purified and in use), it is clear why soil bacteria are a huge natural reservoir of resistance genes even in completely intact regions. ARGs, which are being detected nowadays in human pathogens, are common in soil community (which are designated as the soil resistome) and existed in the environment before the discovery and use of antibiotics in medicine (D'Costa et al., 2006; Allen et al., 2009; 2010; Knapp et al., 2011; FAO, 2016).

The first genes confering resistance to antibiotics were developed in antibiotic-producing bacterial species, with the aim of protection against self-inhibition (Baquero et al. 1998; D'Costa et al., 2006). To survive, bacteria living in the close vicinity of antibiotic producers had to develop defence mechanisms (Demanèche et al., 2008; FAO, 2016). For instance, soil is rich in microorganisms that produce β-lactam antibiotics, which is why resistance genes to a wide spectrum of β-lactam antibiotics, including third-generation cephalosporins, are naturally found in soil (Demanèche et al., 2008; Allen et al., 2009; Forsberg et al., 2014). The diversity of genes which code for enzymes β-lactamases in soil bacteria is wider than in clinical environments (Nesme and Simonet,

2015). Moreover, in bacteria from soil samples collected in Alaska, far from anthropogenic influence, 13 diverse β-lactamases belonging to all four structural classes of these enzymes were found, among which one was even bifunctional, wich in inusual in bacteria (Allen et al., 2009).

In agricultural and grassland soil samples 2895 ARGs were detected, which represent all the main resistance mechanisms to 18 types of antibiotics (Forsberg et al., 2014). In every cultivable isolate of actinomycetes originating from the soil collected in the woods, urban surroundings and agricultural areas the resistance to several natural antibiotics (from 7 to 8 on average), semisynthetic derivates and synthetic preparations was detected (D'Costa et al., 2006). Furthermore, some highly variable profiles of resistance, as well as resistotypes (antibiotic resistance profiles) unknown until then were discovered (D'Costa et al., 2006). From a 30,000-year-old Alaskan permafrost soil sample, DNA molecules containing *vanHAX* vancomycin resistance operon were extracted, whose expression in a heterologous host - *Escherichia coli* - results in a vancomycin-resistant phenotype (D'Costa et al., 2011).

The concentrations of natural antibiotics in soil are considered subinhibitory and, for this reason, insufficient to prompt the multiplication of species with resistance genes (Götz and Smalla, 1997). Thus, an interesting hypothesis has been proposed: antibiotics in the nature do not act as 'weapons' but those subminimum inhibitory concentrations modulate bacterial gene expression and play the role of signalling molecules (Davies et al., 2006; Allen et al., 2009). Indeed, multiple studies have confirmed the effects of subinhibitory concentrations of antibiotics on global changes in gene transcription (Davies et al., 2006). Moreover, the synthesis of β-lactamases as a response to the presence of β-lactams in soil is aimed at the intercellular signalisation, not unlike the enzymes in proteobacteria which hydrolyse acylhomoserine lactone signalling molecules (Allen et al., 2009). It has also been hypothesised that the plasmid-transferable resistance in the indigenous soil microflora in natural soil is a rare and virtually immeasurable event (Götz and Smalla, 1997). Soil is predominantly an oligotrophic environment in which bacteria have limited growth parameters and activity, including horizontal transfer of genetic material. Very little evidence exists for horizontal gene transfer of ARGs across soil communities (Forsberg et al., 2014). Amending soil with organic fertilisers influences the equilibrium in soil resistome and the horizontal transfer of mobile genetic elements containing ARGs.

EFFECTS OF ORGANIC FERTILISERS ON THE SOIL RESISTOME

A clear, strong connection between the use of antibiotics in animal farming and the increase in resistance genes in soil treated with manure was confirmed by the results of multiple investigations (Binh et al., 2008; Knap et al., 2010; 2011; Heuer et al., 2011; Zhu et al., 2013; Wichmann et al., 2014; Graham et al., 2016). The extensive research on resistance genes in DNA of soil samples collected from 1923 to 2010 and stored in the soil archive took place in Denmark, at Askov Experimental Station, when the emergence of β-lactam-resistance genes - bla_{TEM}, bla_{SHV}, bla_{OXA} and bla_{CTX-M}, and class 1 integron (int1) - was monitored over time (Graham et al., 2016). Using qPCR it was proven that after 1940 the total levels of ARGs were significantly higher in soil treated with manure than in soil fertilised with inorganic fertilisers. The results revealed that the emergence of genes coding for β-lactam resistance found in manured soil coincided with the one in clinical bacterial isolates, as well as that the increase in int1 in soil was proportional to the usage of manure (Graham et al., 2016). Similarly, in soil samples from five locations collected in the Netherlands, from 1940 to 2008, the increase in the concentrations of genes coding for resistance towards tetracyclines and β-lactam antibiotics was detected: the levels of β-lactam ARG (bla_{TEM} and bla_{SHV}) increased by 15 fold in the monitored period (Knap et al., 2011).

In veterinary medicine, especially in poultry and pig farming, tetracyclines and sulphonamides are widely used. Extensive investigations confirmed that pig manure influences the increase in the tetracycline and sulphonamide resistance genes in soil bacteria (Schmitt et al., 2006; Heuer and Smalla, 2007). In bacteria isolated from soil which was not treated with manure, tet(T), tet(W) and tet(Z) resistance genes were detected (Schmitt et al., 2006). However, the treatment with pig manure resulted in direct transfer of tetracycline resistance genes tet(Y), tet(S), tet(C), tet(Q), and tet(H) and their appearance in soil bacteria (Schmitt et al., 2006). It was also experimentally proven that the use of manure originating from pigs treated with sulfadiazine (SDZ) leads to the increase in the total number of bacteria in soil and cultivable strains resistant to SDZ (Heuer and Smalla, 2007). Given that sulphonamide resistance is usually mediated by the $sul1$, $sul2$ and $sul3$ genes (Heuer et al., 2007; 2010), it is not surprising that in pig manure and manured soil samples a high prevalence of $sul1$ resistance genes was detected, which are normally found in class 1 integrons (Heuer and Smalla, 2007). In addition, it was proven that SDZ can strongly influence the soil bacteria population: on the one hand it exerts a stimulating effect on the multiplication of species affiliated to the genera *Devo-*

sia, *Shinella*, *Stenotrophomonas*, *Clostridium*, *Peptostreptococcus*, *Leifsonia* and *Gemmatimonas* (among which there are human pathogens), but on the other, caused a decrease in the relative numbers of bacteria which are normally found in quality soils (*Pseudomonas*, *Lysobacter*, *Hydrogenophaga* and *Adhaeribacter)* (Ding et al., 2014). It has experimentally been proven that pig manure facilitates the horizontal transfer of IncQ pIE723 plasmide from donor *Escherichia coli* strains to recipient *Pseudomonas putida* UWC1 strains (Götz and Smalla, 1997). Plasmid transfer was detected even in non-manured soil, but the number of transconjugantss which had pIE723 in manured was a thousand times higher. Moreover, pig manure had a stimulating effect on the growth and survival of the recipient *P. putida* UWC1 in soil (Götz and Smalla, 1997).

China is the world's largest producer and consumer of antibiotics, which means that in its industrial pig farms all major classes of antibiotics are used as feed supplements or for therapeutic reasons. Thus, the annual swine manure production of 618 billion kilograms is highly likely to increase substantially the resistance gene concentrations in the environment (Zhu et al., 2013). In contrast to control soil samples, in soil treated with pig manure the genes coding for resistance to antibiotics critically important for human healthcare, such as macrolides, cephalosporins, aminoglycosides and tetracycline were detected. There was a 192-fold median increase in the top 63 ARGs, but also a maximum of 28,000-fold increase in one single gene (Zhu et al., 2013).

Although it is clear that the use of manure originating from animals treated with antibiotics contribute to the increase in the resistance towards antibiotics in soil bacteria, research revealed that a similar effect is exerted by the manure from animals which were untreated. A team led by Jo Handelsman, a microbiologist at Yale University in New Haven, Connecticut, investigated the influence of manure from cows which were free of antibiotics, and a nitrogen-based fertiliser, on the resistance of soil bacteria (Reardin, 2014). Two weeks after the treatment with manure significantly higher numbers of bacteria producing β-lactamases in soil were detected in comparison to those in soil treated with nitrogen-based fertilisers. By tracing genetic markers, it was discovered that these bacteria did not originate from the fertiliser but from soil: manure induced a bloom of resistant bacteria already present in soil, particularly of *Pseudomonas* species which is a human opportunistic pathogen.

Cows' manure is less explored in comparison to that of pigs and chickens, although it is commonly used in crop production. Only in the USA cows generate daily between 1.9 and 14.2 billion pounds of manure which is used to fertilize fields (Wichmann et al., 2014). Wichmann et al., (2014) applied functional metagenomics and identified 80 different antibiotic-resistance genes,

among which some chloramphenicol-resistance genes, unknown until then (coding for a novel clade of acetyltransferases), were specific to the cow microbiome, which are only distantly related to previously known genes. Udikovic-Kolic et al. (2014) claimed that soil treated with cow's manure originating from animals which had not been treated with antibiotics contain a higher abundance of β-lactam-resistant bacteria in comparison with the one treated with inorganic fertiliser. By means of identification of β-lactam-resistance gens with functional metagenomics and quantitative PCR analysis, it was proven that cows' manure influenced the enrichment of resident soil bacteria that harbour β-lactamases, such as *Pseudomonas* spp., *Janthinobacterium* sp. and *Psychrobacter pulmonis* (Udikovic-Kolic et al., 2014). It is assumed that the growth and multiplication of resistant soil bacteria are facilitated by either some nutritional component of the manure or by heavy metals (Knapp et al., 2011). Indeed, genes which make it possible for bacteria to resist widely used metals (copper, arsenic, zinc, silver and mercury), as well as those usually present in the environment (cadmium, lead, cobalt, nickel and tin) are frequently detected along with antimicrobial-resistance genes on mobile genetic elements (Summers, 2002; Knapp et al., 2011). Copper, arsenic and zinc, which are added to animal feed supplements, may also pose long-term pressure for antibiotic resistance (Zhu et al., 2013). Knapp et al. (2011) tested soil samples from the early phase of antibiotic era (between 1940 and 1970) and those collected in 2008 from fields containing sewage sludge amended with copper at 0, 50, 100 and 200 mg-copper/kg. In the extracted DNA materials the presence of genes coding resistance to tetracycline (*tet*), extended-spectrum beta-lactamases (*bla*) and erythromycin resistant methylases (*erm*) were detected. It was revealed that copper exerted the major influence on ARG abundances, and that the one of chromium and nickel were less strong (Knapp et al. 2011).

Bacteria are extremely capable of adjusting to unfavourable environmental conditions and in short time acquire or develop mechanisms which will enable their survival.Antibiotic residues which are excreted from animal organisms and reach agricultural land via organic fertilisers influence directly the resistance of soil bacteria. The selection of antibiotic-resistant bacteria strains is proportional to the concentration of antibiotics which they are exposed to and to the duration of this exposure. An antimicrobial agent can multiply the population of bacteria resistant to some other antibiotics, if their resistance genes are related to each other (Summers, 2002; Binh et al., 2008; Wang and Yu, 2012). If more than one antibiotic is present in the environment the resulting multiple pressure selectively influences those bacteria which use multiple or multipurpose mechanisms (Baquero et al., 1998).

Repeated exposure of bacteria to a certain antibiotic is of outstanding importance to the selection of resistant strains and horizontal transfer of resistance genes, which has clearly been proven in clinical practice. This is the reason why only few, but largely disseminated resistance genes can be found in clinical environment in comparison to soil (Nesme and Simonet, 2015). However, direct exposure of bacteria to antibiotics is only one of the factors collectively termed "selective pressure". In broader sense it comprises a set of factors which create favourable environment for the emergence of *de novo* mutations or the acquisition of properties which contribute to the survival and, what is of crucial importance, to the selection of resistant microorganism (Baquero et al., 1998). Although all mechanisms of resistance already exist in bacteria in the nature, the multiplication of resistant populations will occur only in multifactorial conditions which act as selective pressure.

POTENTIAL RISK FOR HUMAN HEALTH

Many bacterial ARG are located on mobile and horizontally transferable genetic elements such as plasmids, transposons or integrons (Allen et al., 2010). Their horizontal transfer enables efficacious spread of resistance among related and distant bacterial species, including both commensals and even species pathogenic to humans (Allen et al., 2010; Udikovic-Kolic et al., 2014). The exchange of ARG between soil bacteria and those of clinical importance is possible in both directions (Wang and Yu, 2012). Resistance genes in natural reservoirs (such as soil and ocean water) are the likely source of ARG in clinical environment due to various means of horizontal transfer (Nesme and Simonet, 2015).

Soil is the environment in which various bacterium species, including those important for public health, may acquire resistance determinants to known and possibly those classes of antibiotics which have yet to be synthesised (Demanèche et al., 2008). Little is known about natural reservoirs of resistance genes in the environment, as well as how much they can influence the resistance detected in clinical settings (Allen et al., 2009). Even currently unknown ARGs in soil bacteria may present a grave threat to human health if transferred to pathogen species and migrate into clinical environment. However, there still remains an open question: are all resistance genes which are being discovered nowadays in soil bacteria transferable to clinically important species, and how will this reflect on the efficacy of antibiotics in the future? There is considerable void in the understanding of mechanisms by which microorganisms acquire resistance to antibiotics, as well as on the interactions in microbial ecosystems which contribute to their transfer. The transfer of resistance genes between

bacteria in the soil is not as easy as it is between pathogenic species. The limited mobility of the soil resistome may explain why ARGs are rarely shared between soil and human pathogens (Forsberg et al., 2014).

Not only are vast quantities of antibiotics used in animal farming being deposited into soil, but are also resistant populations of gastrointestinal microbiota (including opportunistic human pathogens), both via organic fertilisers. From there bacteria carrying genes coding for resistance towards antibiotics may enter the food chain via contaminated crops or groundwater (Thiele-Bruhn, 2003; Wang and Yu, 2012; Udikovic-Kolic et al., 2014) and via vegetables which are consumed raw (Binh et al., 2008; Marti et al., 2013). Antibiotic use in intensive animal and genetically modified crop farming additionally increases the selection pressure and the risk from the distribution of resistance genes. The release of antibiotics and antibiotic-resistant bacteria into the environment poses a serious problem to the control of antimicrobial resistance, which is why animal health care has to be based on high hygiene and biosecurity, accompanied with prudent antibiotic deployment.

ACKNOWLEDGMENT

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