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DOI
10.1093/femsle/fnw210

Publication date
2016

Document Version
Final published version

Published in
FEMS Microbiology Letters

FEMS Microbiology Letters, 363, 2016, fnw210

doi: 10.1093/femsle/fnw210
Advance Access Publication Date: 8 September 2016
Minireview

MINIREVIEW – Food Microbiology

The risk of low concentrations of antibiotics in agriculture for resistance in human health care

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One sentence summary: Antibiotic resistance selected for in agriculture can cause increased resistance in human pathogens due to transfer of resistance genes.
Editor: Hermann Heipieper

ABSTRACT

The contribution of antibiotic resistance originally selected for in the agricultural sector to resistance in human pathogens is not known exactly, but is unlikely to be negligible. It is estimated that 50% to 80% of all antibiotics used are applied in agriculture and the remainder for treating infections in humans. Since dosing regimens are less controlled in agriculture than in human health care, veterinary and environmental microbes are often exposed to sublethal levels of antibiotics. Exposure to sublethal drug concentrations must be considered a risk factor for *de novo* resistance, transfer of antimicrobial resistant (AMR) genes, and selection for already existing resistance. Resistant zoonotic agents and commensal strains carrying AMR genes reach the human population by a variety of routes, foodstuffs being only one of these. Based on the present knowledge, short treatments with the highest dose that does not cause unacceptable side-effects may be optimal for achieving therapeutic goals while minimizing development of resistance. Novel approaches such as combination or alternating therapy are promising, but need to be explored further before they can be implemented in daily practice.

Keywords: Antibiotic resistance; agriculture; transfer of resistance; dosing regime

INTRODUCTION

The contribution of antibiotic resistance selected for by the use of antimicrobial agents in the agricultural sector to the incidence of antimicrobial resistant (AMR) pathogens in human health care is under debate (Martinez 2011, 2012; Allen 2014; Paulson *et al.* 2015). It is still disputed whether clinical treatment is the most important driver for AMR development, in the sense of initiation, selection, propagation and spreading of resistance, in human pathogens. However, there is general consensus that AMR zoonotic agents and transfer of resistance genes via commensals that inhabit both sectors contribute as well (Holmes *et al.* 2016). The One Health concept (e.g. http://www.onehealthinitiative.com) emphasizes the continuity between the human and veterinary sectors. In that framework, selection for AMR taking place in agriculture is relevant for human health care. Estimates of the overall antibiotic usage vary widely, whereby roughly 50%–80% of the total antibiotic consumption is attributed to veterinary usage (Cully 2014). Consumption data are very hard to compare, veterinary data often being reported in tonnes of active compound and clinical usage in defined daily doses. In addition, the differences between

Received: 1 June 2016; **Accepted:** 31 August 2016

countries in policy regarding application of antibiotics are large (Coulter *et al.* 2015). Still, for overall reduction of AMR, antibiotic usage by agriculture undoubtedly plays a role.

Exactly how much of the resistance encountered in the human health care setting was originally selected for in agriculture is not clear. Resistant bacteria are encountered at all stages of the various food supply chains (Doyle 2015), providing at least one potential route for transfer of AMR bacteria and/or resistance genes. Antibiotics used on farms that end up in the environment play a role as well (Martinez 2009), illustrating one more reason to reduce antibiotic usage in agriculture. To successfully implement measures that will reduce the usage of antibiotics in agriculture, the public health gain must be clearly perceived by all stakeholders, including farmers (Visschers *et al.* 2015). Below, factors that drive the development of AMR in the agricultural sector and the subsequent transfer to human pathogens will be examined with the aim of identifying of implementable counter measures.

AGRICULTURE AS SOURCE OF AMR

The exact routes resistance genes follow into the human gut and commensal microbiota is still a point of discussion, but transfer of AMR bacteria and resistance genes is believed to result from direct contact with animals (Levy, FitzGerald and Macone 1976; Graveland *et al.* 2010; Frana *et al.* 2013; Rinsky *et al.* 2013), spreading of environmental bacteria (Martinez 2014) and the consumption of contaminated food or water (CDC 2000; Sorensen *et al.* 2001). A curated set of 40 AMR genes in human fecal samples from Spain, Denmark and the United States had the highest abundance when the corresponding antibiotic was approved for animal use by the national governments (Forslund *et al.* 2013). These resistance genes persisted more than a year in same person samples from the human gut, supporting the 'farm-to-fork hypothesis' that suggests that part of the AMR in human microbiota originates from the agricultural sector. The alternative explanation that AMR in human microbiota is caused by residues in the food that would provide a constant selection pressure is improbable, because antibiotic residues are barely ever detected in foodstuffs due to strict legislation on this matter.

Multidrug resistance (MDR) is commonly found in food and animal microbes, such as various *Escherichia coli, Salmonella* and *Campylobacter* strains, *Enterococcus faecium* and *Enter. faecalis* (Giraffa 2002; Mevius *et al.* 2010). These organisms can efficiently transfer AMR genes to strains encountered in human health care by a variety of mechanisms, underlining the importance of food isolates as a reservoir of AMR genes and the potential for their transfer to the human microbiota (Vignaroli *et al.* 2011; Jahan *et al.* 2015). In addition to direct, food-related routes, there may also be indirect ways for AMR bacteria from farms to reach the human population through the environment (Blaak *et al.* 2015; Schijven *et al.* 2015; Evers *et al.* 2016), resulting in considerable additional exposure to resistant pathogens and AMR genes. Surface water, wind transport, flies or other pathways may be more important quantitatively than direct transfer through food (Andersson and Hughes 2014), but regardless of the route, the source and destination are the same.

DEVELOPMENT OF RESISTANCE

Exposure of bacteria to antibiotics in any environment leads to resistance through three main mechanisms: (i) physiological adaptation (Handel *et al.* 2013); (ii) mutations at the DNA level (Handel *et al.* 2014) and (iii) transfer of genes that confer resistance (Davies and Davies 2010; Handel *et al.* 2015). Each of these events occurs under different environmental conditions and selection pressures. The correlation between antibiotic concentration and the rise of AMR clones has been characterized extensively (Drlica 2003; Andersson and Hughes 2012). Sub-MIC levels not only select for existing AMR but also allow the susceptible microbes to survive and adapt, acquiring enough resistance to survive subsequent therapeutic doses. Sublethal concentrations enable bacteria to induce protection mechanisms, such as the SOS response that increases the mutation rate and thus the chance that specific resistance mutations will occur (Baharoglu and Mazel 2014; Long *et al.* 2016).

The mutant selection concentration is dependent on the antibiotic–microbe combination and on the particular resistance mutation (Gullberg *et al.* 2011). In some cases, even extremely low concentrations can have a selective effect. For example, exposure to a concentration, 230-fold lower than the minimal inhibitory concentration (MIC), caused enrichment of a ciprofloxacin-resistant *Escherichia coli* mutant. Tetracycline concentrations, 150-fold lower than the MIC, stimulated the transfer of resistance plasmids and low levels of antibiotics that may also stimulate conjugative transposons to transfer to unrelated genera (Jutkina *et al.* 2016). As a rule, higher concentrations are needed for AMR development, but sub-MIC concentrations already have a selective effect (Andersson and Hughes 2014). In that same line, *E. coli* cells with a reduced susceptibility to amoxicillin due to short-term exposure outcompeted the ancestor *in vitro,* whenever antibiotics were present in the growth medium (Feng *et al.* 2014). In agreement with obtained data *in vitro*, selection of AMR variants *in vivo* was shown to occur already at low drug dosages of only 2.5% of the normal therapeutic dose in the microbiota of chicken guts (van der Horst *et al.* 2013).

EFFECTS OF ANTIBIOTICS USAGE IN AGRICULTURE

The lowest concentrations encountered in agriculture are brought about by the so-called carry-over of antibiotics when regular feed is produced on a production line that was used for medicated feed immediately before and not cleaned according to the prescribed standards. Though the effects vary quantitatively, resistance can develop (Stolker *et al.* 2013; van der Horst *et al.* 2013; Scherz *et al.* 2014). Veterinary antibiotics are excreted through animal urine and feces and often remain to some extent after sewage treatment or end up in the environment near the farm (Watkinson, Murby and Costanzo 2007). When these residues end up on agricultural lands, they cause selection for resistance genes in microbiota of the soil (Thiele-Bruhn and Beck 2005; Gullberg *et al.* 2011; Jechalke *et al.* 2014). Antibiotics are also produced by naturally occurring environmental microorganisms (Taylor, Verner-Jeffreys and Baker-Austin 2011). The effects of this low-level exposure are not documented quantitatively. Therefore, a quantitative comparison of the contribution of low-level exposure to selection for resistance with consequences for therapeutic applications is not in reach. It has been well described though, that the regular usage of antibiotics in agriculture for therapeutic and prophylactic purposes and in particular as growth promoters has caused widespread resistance (European Food Safety Authority (EFSA) 2016).

For years antibiotics were commonly administered to food-animals as prophylactics and growth promoters (Barton 2014). Subtherapeutic doses of antibiotics are still administered in some countries to healthy livestock as this practice is thought to enhance growth rate and feed-to-weight ratio for poultry, swine

and beef cattle (Marshall and Levy 2011; Barton 2014). Usage of antibiotics as growth promoters has already been controversial for a long time, since resistance was reported shortly after the introduction of antibiotic use in livestock (Bates, Jordens and Griffiths 1994). The European Union (EU) has banned the use of antibiotics as growth promoter and many countries outside the EU, including major producers as the USA and Australia, restrict antibiotic usage in agriculture in a comparable manner (Cogliani, Goossens and Greko 2011; Maron, Smith and Nachman 2013). The Swedish experience shows that such policies are likely to be successful, as the reduction of antibiotic usage for livestock was not accompanied by animal disease and did succeed to prevent build-up of resistance (Wierup 2001).

TRANSFER OF RESISTANCE GENES TO HUMAN PATHOGENS

Exposure to antibiotics not only selects for *de novo* acquired resistance, but just as much for transmissible AMR. In this latter case, it is conceptually important, but in practice very hard, to separate gene transfer from subsequent selection. Gene transfer can occur by a wide variety of mechanisms, such as plasmids, transposons, phages, etc. (Davies and Davies 2010). The minimal selective concentration for clinically important MDR plasmids was shown to be lower than the MIC of the plasmid-free susceptible organism (Gullberg *et al.* 2014). The combined effects of several compounds, such as antibiotics and heavy metals, resulted in an even more decreased selective concentration. This affects the selection of MDR plasmids or resistant clones in natural habitats and on farms where various activities take place simultaneously, resulting in complex environments.

The rate of transfer of plasmids containing genes that code for antibiotic resistance depends on a variety of factors, the intrinsic properties of the plasmid being only one of them (Frost and Koraimann 2010). Rates of plasmid transfer between donor and acceptor seem to be strongly reduced in the presence of high antibiotic concentrations (Schuurmans *et al.* 2014; Handel *et al.* 2015), though this effect may depend on plasmid size and differ for other transfer mechanisms, such as phages and transposons. As availability of energy and growth rate also affect rates of transfer (MacDonald, Smets and Rittmann 1992; Schuurmans *et al.* 2014), it is possible that additional metabolic stress due to exposure to the antibiotic reduces transfer rates. Transfer of conjugative transposons can be enhanced by exposure to low concentrations of antibiotics (Toleman and Walsh 2011). After transfer of AMR genes has occurred, the selective advantage compared to the ancestral microbes determines how fast the AMR variant will take over in the population. When the advantage is large, the takeover will be so rapid that the rate of transfer of the AMR genes exerts less influence over the final outcome than in cases where the advantage is only minor (Kivisaar 2003).

SELECTION AND SPREAD OF RESISTANCE ACQUIRED BY HGT

Plasmids carrying AMR genes are ubiquitously present in the environment, both on agricultural lands and in aquatic ecosystems (Andersson and Hughes 2014; Marti, Variatza and Balcazar 2014). Heavy metals and sublethal concentrations of antibiotics provide enough selection to maintain the presence of MDR plasmids (Gullberg *et al.* 2014). Some evidence suggests that AMR genes detected in human pathogens at least partly originate from commensal and environmental microorganisms (Martinez 2014). Resistance to cephalosporins was transferred between *Escherichia coli* strains from farms to human isolates by specific plasmid lineages (de Been *et al.* 2014). In addition, in the environment bacteriophages may function as vehicles for AMR genes (Balcazar 2014). For example, a set of extended spectrum beta-lactamase (ESBL) genes and fluoroquinolone-resistant genes had phages as reservoir (Marti *et al.* 2014). These observations suggest that there are several pathways for AMR genes that were enriched in the agricultural setting to spread to human pathogens.

Once a plasmid is present in a population, it has a natural tendency to spread, but factors such as plasmid stability or fitness costs can interfere with successful dissemination. For example, the global spreading of plasmid pCT that confers β-lactam resistance is explained by plasmid stability and a lack of fitness burden rather than the presence of particular genes (Cottell *et al.* 2014). The gut microbiota forms an invigorating environment for conjugation and *in vivo* transfer of AMR conferring genes, both within species (Lester, Frimodt-Moller and Hammerum 2004; Karami *et al.* 2007; Trobos *et al.* 2009) and between species (Duval-Iflah *et al.* 1980; Yong *et al.* 2009; Goren *et al.* 2010; Cremet *et al.* 2012). The biofilms in the human intestinal tract provide physical protection in addition to optimal conditions for cell-to-cell contact and hence horizontal gene transfer (HGT) by means of plasmids and conjugative transposons (Huddleston 2014).

TRANSFER OF AMR WITHIN THE FOOD CHAIN

AMR food-borne commensal bacteria and pathogens both end up on meat products during slaughter or subsequent processing. Consumers of meat are exposed to these microbes through cross-contamination in the kitchen or consumption of raw or insufficiently cooked meat. On the one hand, the food chain might therefore be envisioned to substantially contribute to the transmission of AMR strains and genes to the human intestinal bacterial flora (Oloya, Doetkott and Khaitsa 2009). On the other hand, consumption of meat might not be the most important exposure route for humans, as vegetarians are not less colonized with ESBL-containing bacteria than meat eaters (Koniger *et al.* 2014). Possibly, fruits and vegetables are contaminated with AMR bacteria due to irrigation with untreated surface water, even though this practice is forbidden in many countries. Direct transfer of AMR bacteria from chickens to humans is relevant for workers on broiler farms; as such workers have been shown to carry ESBL producing *Escherichia coli* far more often than the general population (Huijbers *et al.* 2014). Otherwise, direct clonal transmission is not a quantitatively important route. Instead, AMR genes are most often disseminated between animals and humans via HGT (de Been *et al.* 2014).

Once AMR variants have reached a certain location within the food chain, e.g. farm holding pen or slaughterhouse, or within the human population, they often persist there, contrary to the expectation that they will be outcompeted once the pressure of the antibiotic is removed (Wang *et al.* 2012). Fitness costs of resistance, defined as reduced growth rate or a larger proportion of the energy source devoted to purposes other than growth, are reduced over time both when resistance develops *de novo* (Handel *et al.* 2013, 2014) and also when cells acquire AMR conferring plasmids by HGT (Bouma and Lenski 1988; Dahlberg and Chao 2003; Dionisio *et al.* 2005). The success of mutations among a population depends on the balance of benefits and costs. Multiple genetic changes that result in a highly AMR

phenotype are not necessarily linked to severely decreased bacterial fitness. The costs can also consist of a reduced ecological range (Handel *et al.* 2013), which under the right conditions does not have to be detrimental, but it might, for example, limit the number of foodstuffs in which a particular resistant strain can maintain itself. Moreover, success of paired genetic changes is strongly affected by the genetic interaction itself, so-called epistasis (Phillips 2008). The genetic interaction of two or more beneficial mutations can cause positive epistasis and hence improve bacterial fitness over the single mutation variant (Weinreich, Watson and Chao 2005). The majority of allelic combinations conferring AMR were found to exhibit positive epistasis (Trindade *et al.* 2009; Baker *et al.* 2013). Fitness benefits of mutations in the absence of antibiotic pressure (Baker *et al.* 2013) preserve mutants even when the antibiotic exposure is discontinued.

HOW TO CONTROL EXPANSION OF RESISTANCE

Taken together, all available information indicates that sublethal drug concentrations can induce *de novo* resistance, stimulate horizontal transfer of AMR genes, as well as select for already-existing antibiotic resistance. The widespread dissemination of AMR and the lack of new antibiotics focus attention towards efforts to limit the problem and evade the effects, as solving it appears a remote possibility. Even though a considerable reduction seems possible, completely eliminating all antibiotic applications in agriculture is incompatible with the demands of animal health and welfare (Littmann, Buyx and Cars 2015). Adapting dosing protocols is one potential avenue. *In vitro* experiments using chemostats to simulate treatment suggested that the highest concentrations that are safe for the patient, human or animal, for the shortest time possible to achieve elimination of the infection is the best way to prevent development of resistance (Feng *et al.* 2016). Whether this holds in reality should be tested extensively in animals before considering implementation in human clinical trials. For livestock, strictly controlled trials will be needed before standing practices can be changed.

Possibly the dogma of always completing the prescribed treatment, even when the infection has been eliminated, should be reconsidered, partly because a much larger part of the microbiota consists of AMR bacteria today than in the times this principle was formulated. Instead, treatment at the highest levels the patient can tolerate may be applied to control the infection and support the host immune system during the acute stages. Once the infection is under control, antibiotic treatment could be discontinued in order to avoid selection for resistant strains. The danger of stopping too early is that if the infection would recur, AMR variants are bound to dominate. Therefore, terminating an antibiotic cure early can only be done after examination by a qualified physician or veterinarian and even then professional error could cause considerable risks.

Since antibiotics act on growing cells, new approaches have been proposed to stimulate bacterial metabolism through the addition of compounds that activate the central bacterial metabolism and thereby increase drug uptake during treatment (Bhargava and Collins 2015). In fact the effectiveness of this procedure has been demonstrated (Allison, Brynildsen and Collins 2011; Peng *et al.* 2015). Lately, epistatic interactions between various drugs have been investigated regarding their potential to enhance or decrease the development of antibiotic resistance (Yeh *et al.* 2009). Combination therapy is becoming commonplace practice for complex infections (Falagas *et al.* 2015). On the one hand, certain combinations of drugs that show synergistic growth inhibition compared to each individual drug, were found to increase selection of resistance (Chait, Craney and Kishony 2007). On the other hand, the use of drug cycling or alternating antibiotic treatments can slow down the evolution of resistance (Imamovic and Sommer 2013; Kim, Lieberman and Kishony 2014). Thus, in addition to reduction of usage, optimization of treatment strategies still harbors considerable potential to slow down and gain control over the development and spread of antibiotic resistance in microbes both in agriculture and in human health care.

IMPLEMENTATION

Competent authorities of national governments have several responsibilities concerning the usage of antibiotics for agricultural purposes (Turnidge 2004). These include approving new drugs, monitoring resistance and usage, supervision of good agricultural practices on farms and performing risk assessments relevant to the problems caused by antibiotic resistance. Both EFSA and FDA and international organizations, such as FAO, OIE and WHO, have published a large number of documents and guidelines on this subject over the years, as have national authorities in many countries. Professional veterinary organizations promote prudent use of antibiotics by directly informing their members of best practices laid down in guidelines for prescription (Earnshaw *et al.* 2009). Prudent use guidelines are not only to be strictly adhered to, but also to be updated when new insights arise. At present, one crucial measure is to reserve classes of antibiotics that are essential for human health care solely for that purpose (Miller, McNamara and Singer 2006). Sweeping measures simply aiming to reduce antibiotic usage by restricting the amount that can be used could be counterproductive if the dosage is lowered, because, as discussed above, low concentrations select for resistance while lethal levels eliminate the target organism. This example illustrates that reducing the total burden for human health caused by antibiotic usage in agriculture requires very well thought out measures. The lines of research performed in the framework of the 'One Health' concept that approaches human and animal health from an environmental point of view are crucial for achieving this aim.

ACKNOWLEDGEMENTS

The authors thank John Thelfall for suggestions and comments on an earlier version of the manuscript.

Conflict of interest. None declared.

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