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Antimicrobial metal ion resistance and its impact on co-selection of antibiotic resistance

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Antimicrobial Resistance

The World Health Organization has declared antimicrobial resistance (AMR) "a major global threat to public health"[1]. At the United Nations General Assembly on 21 September 2016 Heads of State committed to taking a "broad, coordinated approach to address the root causes of AMR across multiple sectors, especially human health, animal health and agriculture"[2]. Although AMR is a broad term used to describe resistance to a wide range of antimicrobials, there are particular global concerns about: antibiotic resistance in bacterial pathogens, multi-drug resistant tuberculosis, and antimicrobial resistance in influenza, HIV, systemic candidiasis, and malaria.

In particular, antibiotic resistance in bacteria is now considered in the United Kingdom to be of sufficient importance that it has been included in the UK National Risk Register of Civil Emergencies 2015[3]. The O'Neill Review on antimicrobial resistance, commissioned by the UK Government (2014-2016), has predicted that, if current trends continue, by 2050 approximately 10 million people a year world-wide will die of untreatable infections (more than will die from cancer), and that AMR will have economic costs of 100 trillion US Dollars[4,5]. The increase in antibiotic resistance in key pathogens is an example of microbial evolution, due to selective pressure exerted by human use of antibiotics. The conditions which have encouraged this selection of antibiotic resistant bacteria are exacerbated by overuse, and inappropriate use, of antibiotics in medicine and agriculture; the environmental release of antibiotics during manufacture and usage; and substandard or counterfeit antibiotic use[4,5]. The problem is compounded by the difficulties of developing new antibiotic classes[5,6].

Although there has been a focus on 'antibiotic' resistance in bacteria as the major AMR concern, antimicrobials are a much wider group of chemicals than antibiotics, and include: antivirals, antifungals, antiparasitics, and antibacterials, and can include antimicrobial metals/metalloids, and biocides. Less attention has been given to antimicrobial metals and biocides—which are used widely in healthcare, agriculture, public health and in consumer items, but are less important in clinical medicine than antibiotics. There is a growing body of evidence that biocide and antimicrobial metal use may be contributing to the antibiotic resistance problem[7,8,9,10,11].

Antibiotics and antibiotic resistance

Antibiotics are naturally produced by soil bacteria and fungi. Antibiotic production and resistance genes are now believed to be ancient genes (possibly millions of years old), which have moved from the 'environmental resistome' (the antibiotic resistance genes present in environmental bacteria) into pathogens[12,13,14]. There is evidence that crude antibiotic preparations were sporadically used by humans in ancient history[15,16], but by the turn of the twentieth century, in conventional Western medicine at least, the weapons in the antimicrobial armoury for treating infections were largely limited to plant extracts, and inorganic chemicals which included mercury, copper, silver, bismuth and arsenic/antimony compounds[17].

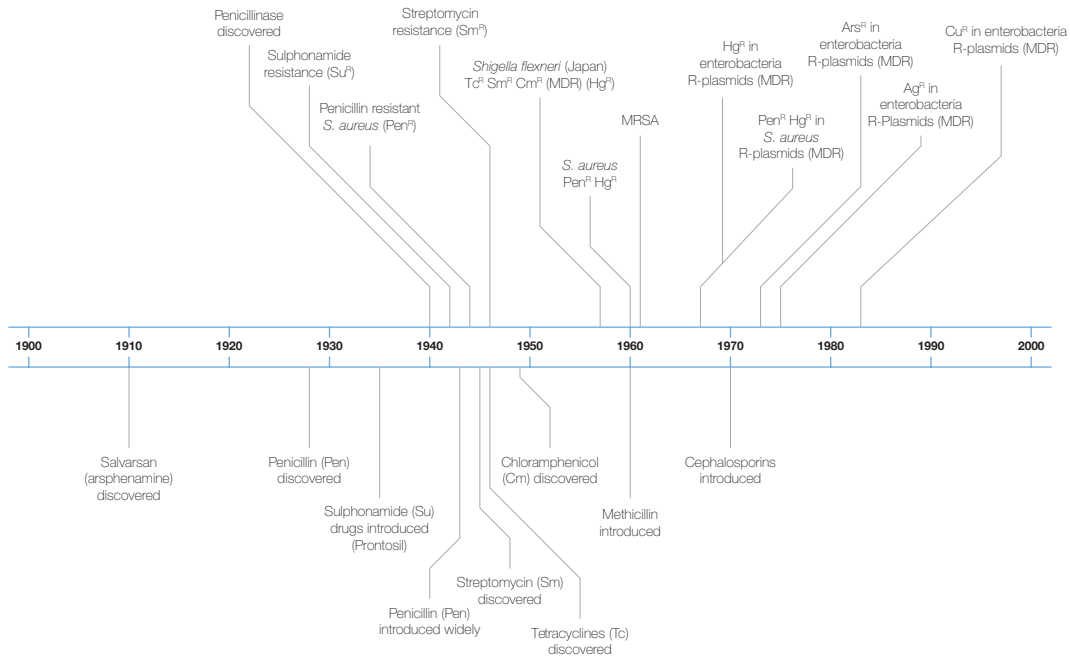

Figure 1 ~ Antibiotic introduction and resistance discovery

Many of these metal based antimicrobials were toxic to the patient, and/or poorly effective. The twentieth century saw some of the most important historical milestones in antimicrobial chemotherapy, but also discoveries of bacterial antibiotic and antimicrobial metal resistance (Figure 1).

Antibiotic resistance developed very quickly after the introduction of antibiotics into clinical usage-penicillinase was reported in 1940[18], before the large scale introduction of penicillin. Penicillin resistant *Staphylococcus aureus* were identified[19] and found in hospitalized patients very quickly after the introduction of penicillin into hospitals[20], followed by community acquired penicillin resistant *S. aureus* by the mid-1950s[21]. Antibiotic resistance in other pathogens also developed rapidly. Sulphonamides were found to be the most effective treatment for *Shigella dysenteriae* and other *Shigella* sp. during outbreaks of dysentery in Japan after World War II, but by 1952, 80% of *Shigella* outbreak isolates were resistant to sulphonamides. In 1950, tetracycline, chloramphenicol and streptomycin were introduced to Japan. Within three years, *Shigella dysenteriae* isolates in Japan were resistant to streptomycin or tetracycline, and by 1956 a *Shigella flexneri* 4a strain resistant to sulphonamides, tetracycline, streptomycin and chloramphenicol was found[22,23,24]. In 1957 multiple drug resistant (MDR) *Shigella* and *Escherichia coli* were being regularly isolated in Japan. At the time the rapid emergence of multiple resistance in different *Shigella* serotypes from different outbreaks was at odds with the idea that antibiotic resistance developed only via simple random mutagenesis of genes[23]. Work pioneered in Japan in the late 1950s to early 1960s led to a realization that antibiotic resistance could move between different bacteria[22,23,24]. The elements transferring resistance genes, which we now know of as plasmids, were called Resistance Transfer Factors, or R-factors. Many of the original clinical isolate plasmids were given R designations such as R100 (which was isolated in the mid-late 1950s and carried multiple antibiotic resistances as well as mercury resistance). Many of these R-plasmids were the focus of intensive studies from the 1960s to 1990s and it became clear that not only did the plasmids contain antibiotic resistance genes, but also conferred resistance to antimicrobial metals and disinfectants[25,26,27,28,29,30]. Multi-drug resistance plasmids are found in bacteria isolated not just in the clinic, but from livestock, wildlife, domestic animals, manures, wastewater treatment plants, and many other terrestrial and aquatic environments.

Antimicrobial metals and metal ion resistance

Metals are widely distributed in the Earth's crust and biosphere. Bacteria have been widely exposed to biologically available metal ions, probably since the Great Oxidation Event (GOE) of approximately 2.3–2.4 billion years ago. The increased availability of atmospheric oxygen is believed to have triggered a dramatic increase in the number of metal compounds in one or more oxidized forms in or near the Earth's surface. Some metals such as iron, zinc, and copper were exploited by organisms in enzymes which required redox cycling functions. These metals are essential to life processes, but toxic at higher intracellular concentrations. For these essential metals, cells require homeostasis systems to control intracellular levels. There is increasing evidence that copper and zinc are also intimately associated with pathogen-killing mechanisms in eukaryotes, where copper/oxidative stress bursts are used to destroy engulfed bacteria[31].

Other metals, such as mercury, silver and gold, have no known beneficial effects on cells, and can be extremely toxic to bacteria (and sometimes higher organisms) at even very low concentrations. For these toxic metals, resistance systems have evolved in bacteria.

Geological, and latterly anthropogenic, mobilization of bioavailable metals has released high concentrations of metals that have selected for resistant microorganisms, and have done so over millions of years[32]. Metal pollution events from mining and industrial processes tend to be long-lived because metals are immutable, unlike organic compounds which will eventually be broken down into simple molecules. Although the metal may be converted by microbial or geochemical action into a form that is no-longer bio-available, the metal is still present and could be remobilized, unlike organic compounds. Consequently metals released into the environment centuries ago, could still be exerting a selective pressure.

The recorded use of antimicrobial metal compounds, predates the widespread use of antibiotics, by several thousand years[17], but use of these metals in industrial processes and consequent environmental loss are likely to far outweigh medical use. Historically, antimicrobial metals such as mercury, copper, silver, zinc, and arsenic/antimony have been used in medicine as treatments for human infections, and some still are. Organic arsenic and antimony compounds are used to treat protozoal diseases, as well as in agriculture as pesticides, fungicides, growth promoters, and rodenticides[17,33]. Copper and zinc are widely used in agriculture, either in plant protection (copper salts), animal growth promotion (copper salts), or diarrhoeal disease treatment (copper, and zinc salts), and zinc salts are used to treat diarrhoea in humans. Silver compounds are used in burns treatments and antimicrobial coatings. Mercury was used from the middle ages to treat syphilis infections, mercury compounds were widely used as disinfectants, and mercury is still used in amalgam dental fillings, and organomercurial preservatives in, for example, eyedrops. The use of other antimicrobial metals and biocides in personal care products is also widespread.

The first reports of bacterial metal resistance came much later than reports of antibiotic resistance, because research was focussed on antibiotic resistance (Fig 1). Mercury resistant *S. aureus* were isolated from infected wounds in the early 1960s[34] with reports of mercury resistance following, for example in enterobacteria[25]. Subsequent work found that antibiotic resistance plasmids isolated from pathogens were frequently found to carry metal resistance genes too: for example mercury resistance in *S. aureus*[26,27]; arsenic resistance in *E. coli* [28]; silver resistance in *Salmonella* Typhimurium[29].

Antibiotic and metal cellular targets and resistance mechanisms

The general cellular targets of antibiotics and toxic metals are summarized in Figure 2 (a)[33,35] and general resistance mechanisms to antibiotics and metals are shown in Figure 2 (b)[17,36]. The general cellular targets of antibiotics and metals, and the mechanisms of

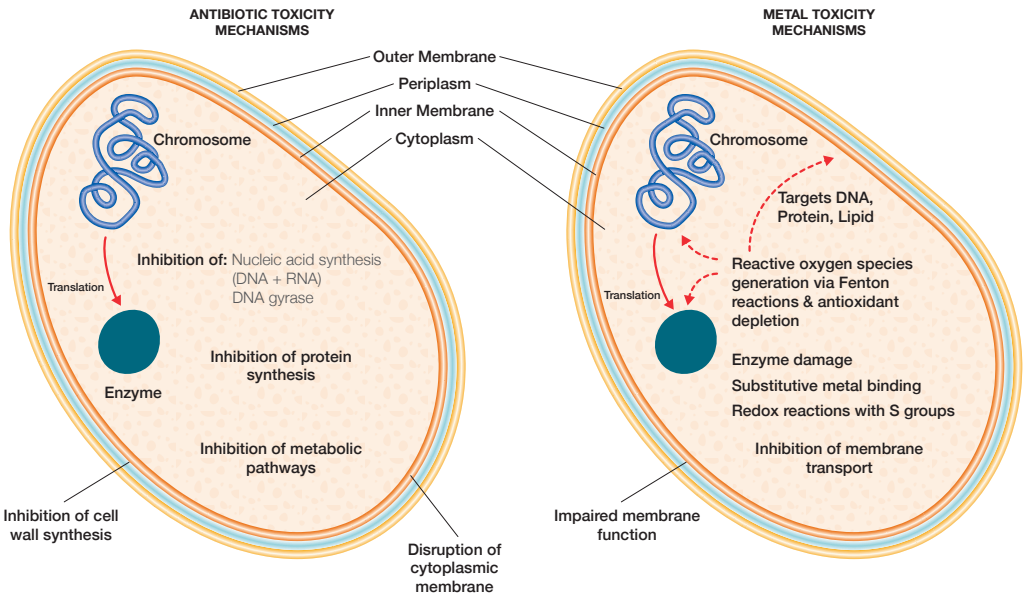

Figure 2a ~ General cellular mechanisms of antibiotic and metal toxicity[33,35]

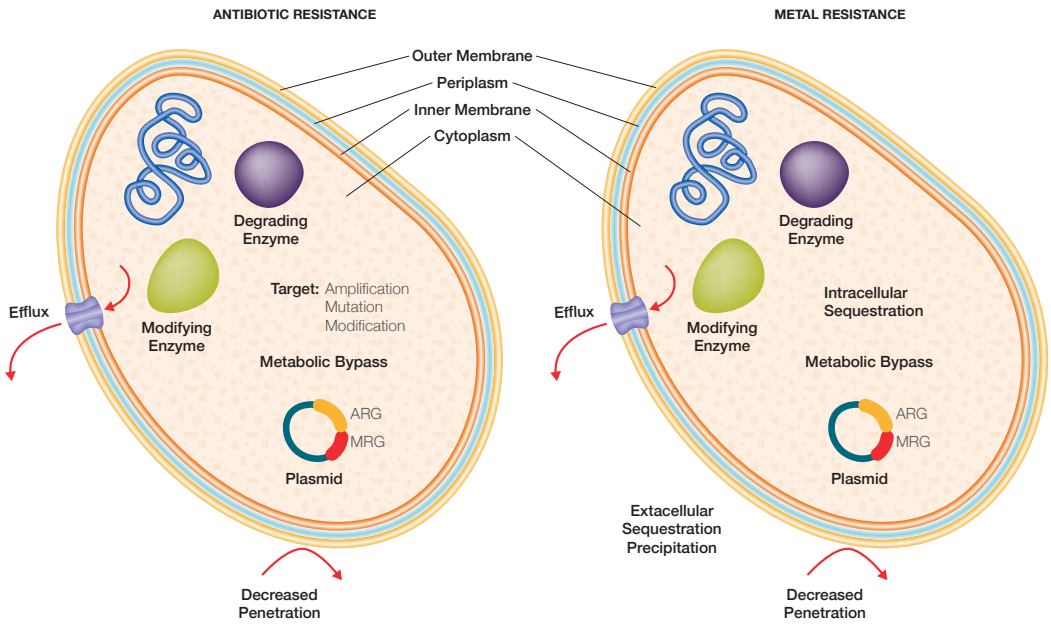

Figure 2b ~ General cellular mechanisms of antibiotic and metal ion resistance[17,36].

resistance, have some distinct similarities. Resistance to metals frequently involves specific efflux pumps, but broad spectrum efflux pumps are also carried by bacteria, allowing them to remove toxic lipophilic and amphiphilic compounds from the cytoplasm. These pumps have been implicated in various aspects of pathogenesis, but they play an important role in efflux of a wide range of compounds including biocides, antibiotics, and other antimicrobial compounds. This broad-spectrum efflux ability is a function of their lack of specificity. Specific modifying or degrading enzymes can be used to alter the toxicity of metal or antibiotic compounds. A significant difference between metal and antibiotic compounds is specificity of targets. An antibiotic normally has a specific cellular target (e.g. a cell wall or ribosome component), whilst toxic metal targets are general—their chemistry drives their toxicity, and the resistance mechanisms to them. This does lead to some differences in resistance—metal ions can be sequestered intracellularly, whilst antibiotic targets can be amplified, mutated or modified.

Why did antibiotic resistance emerge so rapidly once widespread use of antibiotics occurred?

The evidence suggests antibiotic resistance was already circulating in bacteria. It has been found in 'pre-antibiotic era' bacterial strains, and isolated human populations with no antibiotic exposure[23]. Recent evidence shows that antibiotic resistance genes are ancient: in some instances >3 million years old[16] and are part of the environmental resistome. This is not especially surprising because many classes of antibiotics are naturally produced by soil-borne bacteria, such as the actinomycetes. Antimicrobial metals are also widespread in the environment, either from geogenic or anthropogenic sources, and bacterial antimicrobial metal resistance genes have been found in subterranean isolates[37], >15,000 year old permafrost bacteria[38], and ancient arctic sediment cores[39]. Studies on pre-antibiotic era plasmids from enterobacteria in the Murray collection (clinical isolates collected between 1917–1954 at the Hammersmith Hospital in London) found very low levels of antibiotic resistance, but significantly higher levels of metal resistances in plasmids belonging to the same incompatability groups as current R-plasmids[40,41]. All of which is consistent with the idea that level of usage of antibiotics is correlated with incidence of resistance.

The rapid spread of multi-drug resistance in Japan in the 1950s was due to self-transmissible plasmids transferring antibiotic resistance genes between enterobacteria. The biology of plasmids, their ability to transfer, and their ability to persist within strains is now well known. Other mobile genetic elements (MGEs) such as transposons, insertion sequences (IS), integrons, bacteriophages, integrative conjugative elements, and genomic islands are involved in the acquisition of foreign DNA, including resistance genes. Some of these MGEs have acquired and mobilised AMR genes allowing them to move between plasmids and chromosomes. IS elements and transposons classically mobilize antibiotic resistance genes either as single genes or sometimes as arrays of genes. Integrons have the ability to acquire, express and re-assort antimicrobial resistance gene cassettes, and are found in the chromosomes of environmental Gram-negative bacteria. Chromosomal integrons became mobile through their association with transposable elements and conjugative plasmids, and rely on these MGEs to move them around. Integrons are now widely considered as the major causative agents in the rise of MDR in enterobacteria[42]. The classic example of this is In2 carried by the mercury resistance

transposon Tn*21*, where a mercury resistance transposon carries the In2 integron which confers resistance to sulphonamides, partial resistance to quaternary ammonium compound resistance, and aminoglycosides. Multiple variants of integron containing mercury resistances, and copper/silver resistances found on MDR plasmids are found in enterobacteria[17].

Pre-existing AMR resistance genes, genetic elements capable of acquiring, expressing and mobilizing these genes, plus a rapid expansion in lethal and sub-lethal selection applied by widespread use of antimicrobials is likely to have led to the current situation. For some time now, there has been concern that co-selection of antibiotic resistance by antimicrobial metals[7] or by other biocides[43] could be occurring.

Co-selection, co-carriage, co-resistance, co-occurrence, cross-resistance

Co-selection – selection of more than one antimicrobial resistance gene when one is selected. An example of this is an integron where there is one promoter driving the coordinated expression of multiple antibiotic resistance cassettes.

Co-carriage–the carriage of more than one antimicrobial resistance gene in the same strain or mobile genetic element (plasmid, transposon).

Co-resistance–resistance to more than one antimicrobial in the same bacterial strain.

Co-occurrence–where more than one resistance gene is found in the same bacterial strain or mobile genetic element.

Cross-resistance–where resistance to more than one antimicrobial from the same class is conferred by one gene product (e.g. aminoglycoside resistance). This can also mean resistance to different antimicrobials conferred by one gene product. An example of this would be a multi-drug efflux protein, or there are overlapping drug targets, which if modified confers cross-resistance to more than one antibiotic.

Co-selection/co-occurrence of AMR? What is driving it?

Lethal levels of antibiotics select for carriage of antibiotic resistance genes. Studies have shown in the laboratory that sub-lethal levels of antibiotics and antimicrobial metals (as low as 140-fold below MIC levels) will maintain selection of a large multi-drug resistance plasmid[44]. So, as well as the use of antibiotics, the use of other antimicrobials can select for carriage of multi-drug resistance plasmids. Non-antibiotic antimicrobials are widely used in agriculture, medicine, and consumer items. The use of antimicrobial metals (particularly copper and zinc, but organic arsenicals are allowed in certain countries) in agricultural animal feeds is associated with increased levels of antibiotic resistance in the microbial populations from farm animals or in animal faeces[8,10,45,46,47,48]. The co-carriage of metal resistance (copper and silver resistance) and antibiotic resistance in plasmids from enterobacteria of food-producing animals is increasingly noted[49,50,51,52,53].

Another contributor to multi-drug resistance associated with metal ion resistance are integrons which have been mobilized by mercury resistance transposons, such as Tn*21* (first found on plasmid R100, which was isolated from MDR *Shigella*) in the early 1950s in Japan. Variants of the class I integron In2, containing the *qac*ΔE1 gene (efflux pump protein) as well as various combinations of antibiotic resistance gene cassettes, are found worldwide. The close association of class I integrons with antibiotic resistance and mercury resistance in enterobacteria is now being used as a measurement of anthropogenic pollution[54,55]. Association of mercury resistance with antibiotic resistance has been found in human populations exposed to mercury, and also in fish[56,57]. These findings suggest that co-resistance (co-occurrence, of metal and antibiotic resistance genes on the same genetic elements), rather than cross-resistance, may be the major mechanism of co-selection between metal and antibiotic resistances[9].

Case study

Zhu Y-G *et al.* (2013) Diverse and abundant antibiotic resistance genes in Chinese swine farms. Proc. Natl Acad Sci USA 110: 3435-3440

This paper measured the concentrations of antibiotics and metals in swine manure, compost and manure amended soil from three Chinese pig farms, and studied the number and abundance of Antibiotic Resistance Genes (ARGs) and Metal Resistance Genes (MRGs) in the manure. The samples from farms where a range of antibiotics and copper, zinc and arsenic were fed to the pigs showed several thousand-fold enrichment of ARGs compared to controls, and enrichment correlated with concentrations of antibiotics and metals in the diet. 149 unique ARGs were detected among all the samples including ARGs to antibiotics not used on the farms.

Conclusion

Already strategies to reduce antibiotic use and spread in the environment are recommended[5]. These interventions include reducing demand for, and unnecessary use of, antibiotics; and reduction of antibiotic dissemination into the environment from manufacture[5]. The continuing widespread presence of antimicrobial metal resistance genes, often co-carried on transposons or plasmids with antibiotic resistance genes, suggests that we must take resistance gene co-carriage/co-occurrence and co-selection into account when we think about strategies

to combat antimicrobial and antibiotic resistance. We need to consider what the effect of use of these metals as antimicrobials will have on the bacterial flora of animals and humans and what effects the environmental release of non-antibiotic antimicrobials (particularly metals) will have on multi-drug resistance carriage. The evidence from published work shows a correlation of higher levels of metals in manures and soils with higher levels of antibiotic resistance genes. We need to better understand the nature of co-selection and how to interfere with this process. If low levels of metals, biocides and antibiotics are driving selection for multi-drug resistance, then a re-examination of our use of antimicrobials in non-critical applications, particularly at sub-lethal levels needs to be conducted. Increasing the use of non-antibiotic antimicrobials in order to compensate for reduced antibiotic usage may drive co-selection.

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