Lack of evidence for direct linkage of plant agriculture use of oxytetracycline to antibiotic resistance in human pathogens

Oxytetracycline: source and mechanisms of resistance.

Oxytetracycline (Terramycin) is a naturally produced tetracycline antibiotic of *Streptomyces rimosus*, with a spectrum of activity similar to chlortetracycline and tetracycline and remarkable thermostability (16). In plant agriculture, oxytetracycline is used in the USA primarily on pear and apple for fire blight management. Oxytetracycline is also important for the management of a serious disease of stone fruits (e.g. peach and nectarine) in the USA called bacterial spot, caused by *Xanthomonas arboricola* pv. *pruni* (6,14).

Oxytetracycline is bacteriostatic, inhibiting the multiplication of bacterial cells by binding reversibly to the bacterial ribosome blocking the synthesis of proteins (15). As oxytetracycline applied to plant surfaces degrades, the growth of bacteria resumes when oxytetracycline releases naturally from the ribosome (13, 15, 24). Bacteria have three major strategies for developing tolerance to oxytetracycline: efflux pumps, alteration of the ribosome to block binding of oxytetracycline and production of enzymes that inactivate oxytetracycline. Oxytetracycline is not a mutagen, but rather selects for bacterial cells that have developed resistance through acquisition of resistance genes or by accumulation of mutations that occur naturally during replication of its DNA. Over 30 tetracycline resistance genes have been described (5).

Plant agriculture-grade antibiotic formulations did not carry resistance genes or 16S rRNA

After alarming reports that low-grade avoparcin formulations added to animal feed for growth promotion were highly contaminated with *Amycolatopsis orientalis*, the producer organism, and its resistance genes for the glycopeptide antibiotic (12, 32), the cleanliness of antibiotic formulations used in plant agriculture was questioned. An array of commercial streptomycin formulations were tested to determine if plant agriculture-grade formulations were contaminated with the producer strain *Streptomycetes griseus* subsp. *griseus* or its resistance genes for streptomycin (20). Quantitative polymerase chain reaction methods were developed to detect streptomycin resistance genes and 16S rRNA for general bacterial DNA in commercial formulations. Rezzonico *et al*. (20) did not detect bacterial DNA or streptomycin resistance genes in commercial formulations of streptomycin for plant agriculture in samples from the USA, New Zealand and Europe. They concluded that plant agricultural formulations of antibiotics are highly unlikely to introduce resistance genes into the environment.

Regulations reduce direct human exposure to antibiotics used in plant agriculture

The United States Environmental Protection Agency is responsible for setting regulations to minimise exposure to any chemicals applied to crops, including antibiotics (14). In comparison with most pesticides used on plants, antibiotics are relatively non-toxic and were assigned the lowest toxicity rating of pesticides by the agency. Workers are required to wear protective clothing and equipment during handling and application of the antibiotics to crops to mitigate direct exposure to antibiotics. Furthermore, no one is permitted to enter an orchard treated with antibiotics for 12 h after application. The Environmental Protection Agency also regulates the preharvest interval, or the minimum time period permitted between last spray and crop harvest. In the USA, the preharvest interval for application of oxytetracycline and streptomycin varies from between 21 and 60 days, depending upon the compound and the crop (14). The regulations

established in the USA are similar to those established by governmental agencies in other countries. Thus, proper handling of antibiotic materials and required worker protection equipment and procedures used by handlers and applicators in the field, as established by Federal and state regulations, minimize human exposure to antibiotics in the orchard environment, where theoretical risk of human exposure and selection for antibiotic-resistant human-associated bacteria would be most significant.

A direct linkage between antibiotic sprays on plants and antibiotic resistance in clinical bacteria has not been demonstrated

There are numerous reports that the use of antibiotics in animal production is associated with increase of antibiotic-resistant bacteria in animals, waste-water, and manure (for some examples see references 11, 31,33). A direct linkage was reported between infection and colonization of humans by antibiotic resistant bacteria from farm animals (11). No direct linkage has been demonstrated between antibiotic resistant bacteria in humans and antibiotic sprays on plants.

Models generated by the United States Environmental Protection Agency indicate that the potential for direct exposure of humans and their microflora to antibiotics deployed for crop protection are several thousand-fold less than for the medical use of antibiotics (28-30). One potential threat is that antibiotic applications on plants may select for antibiotic-resistant bacterial pathogens, resulting in adverse effects on human health (28-30). Flower tissues of pear and apple are the target of most antibiotics used in plant agriculture. When flowers open, few bacteria are detected (9, 19, 23). Bacteria from environmental sources immigrate and may colonise flowers over time given favourable environmental conditions. As flowers develop and form fruit tissues, detectable populations of bacteria decrease and are restricted to the stem end and the calyx end of the fruit (27). The intact waxy surface of the fruit does not support bacterial growth. The genera of bacteria on flowers that may be treated with antibiotics are common plant-associated bacteria – human pathogens have not been detected in surveys (19, 23,34). Given this, direct enrichment of antibiotic-resistant human pathogens with antibiotic sprays on plants is unlikely.

It is well established that bacteria harbouring transmissible antibiotic resistance genes are common in the environment, even in environments that have never been exposed to antibiotics applied by humans (1, 2, 7, 8, 18, 22, 26). Antibiotic-resistant bacteria that are competent phyllosphere colonisers can persist in the environment, evidently independent of antibiotic use, as shown by Yashiro and McManus (34). They demonstrated that long-term applications of streptomycin alone did not alter the bacterial communities on apple leaves. They sampled leaves from four orchards that were treated with spring-time applications of streptomycin over 10 years and from four orchards that were not sprayed with antibiotics. The bacterial genera *Massilia*, *Methylobacterium*, *Pantoea*, *Pseudomonas*, and *Sphingomonas* were detected from all orchards, regardless of spray history. More streptomycin-resistant isolates (65%) were cultured from non-sprayed orchards compared to sprayed orchards (50%). They concluded that factors other than streptomycin influence both the proportion of streptomycin-resistant bacteria and phylogenetic makeup of bacterial communities on apple leaves (34).

Erwinia amylovora, a flower colonist and plant pathogen, could be considered a model for acquisition of antibiotic resistance genes by other enteric bacteria in orchard environments. Cases demonstrating acquisition of antibiotic resistance genes by *E. amylovora* has been limited

to *strA*/*strB*, which encodes for an enzyme that inactivates streptomycin, in Michigan and California (3, 17). Acquired resistance to streptomycin in this prevalent floral bacterium is uncommon. In most locations, streptomycin resistance in *E. amylovora* is due to spontaneous mutation of its chromosome (4, 15). In Michigan, where *E. amylovora* was demonstrated to acquire the streptomycin resistance genes, streptomycin-resistant bacteria were common on flowers and leaves; between 22 to 88% of the native bacterial populations were resistant to streptomycin (22). To date, isolates of *E. amylovora* with resistance to moderate concentrations (>20 ppm) of oxytetracycline have not been detected in orchards in the USA (15). Unlike the situation with streptomycin, this plant pathogen does not develop resistance to oxytetracycline via spontaneous mutation (10). In the laboratory, introduction of tetracycline resistance genes into *E. amylovora* confers resistance to the antibiotic, so resistance to oxytetracycline would involve acquisition of resistance genes (10). The observed lack of oxytetracycline-resistant strains of *E. amylovora* in orchards may be due, in part, to low populations of tetracycline-resistant bacteria on flowers that could be a source of resistance genes (22). In orchards treated with antibiotics, only 5% of the bacteria isolated from flowers or leaves were resistant to oxytetracycline (10 μg/ml) (22). Given that human pathogens are not common colonisers of pome fruit flowers, the probability of direct acquisition of antibiotic resistance genes from resident phyllosphere bacteria in the tree canopy is reduced.

While spraying trees with antibiotics, a portion of the material settles on the orchard floor and potentially could select for pools of antibiotic resistance genes in the soil, but this supposition has not been supported by recent studies (7, 18, 31). Rodriguez-Sanchez *et al.* (21) repeatedly applied gentamicin and oxytetracycline to coriander plots, and the abundance of antibiotic-resistant bacteria, resistance genes or plasmids was not influenced by antibiotic treatment. This finding is not surprising because tetracycline is adsorbed on soil particles and rapidly rendered inactive (25). From these studies, we speculate that antibiotic residues settling on soil surfaces from spraying trees would have minor effects, if any, on the prevalence of antibiotic resistance genes in soils.

Overall, there is no direct evidence that applications of antibiotics to orchards during bloom contribute to antibiotic resistance in human pathogens. Adherence to federal and state regulations minimizes the direct exposure of workers to antibiotics. Human pathogenic bacteria have not been reported as common colonists of flowers, the tissue treated directly with antibiotics. The antibiotics are active on plant tissues for a limited time period, long before harvest (6, 24). Antibiotic formulations for plant agriculture are not contaminated with resistance genes. Finally, naturally-occurring tetracycline-resistant bacteria are minor components of the communities found on apple flowers and leaves and their presence is independent of antibiotic applications. Overall, springtime applications of oxytetracycline on plants are unlikely to impact the development of antibiotic resistant human pathogens.

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Drafted by Virginia Stockwell and David Granatstein on behalf of the Organic Tree Fruit Industry Work Group, January 2013.