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Human health impacts of antibiotic use in agriculture: A push for improved causal inference

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Resistant bacterial infections in humans continue to pose a significant challenge globally. Antibiotic use in agriculture contributes to this problem, but failing to appreciate the relative importance of diverse potential causes represents a significant barrier to effective intervention. Standard epidemiologic methods alone are often insufficient to accurately describe the relationships between agricultural antibiotic use and resistance. The integration of diverse methodologies from multiple disciplines will be essential, including causal network modeling and population dynamics approaches. Because intuition can be a poor guide in directing investigative efforts of these non-linear and interconnected systems, integration of modeling efforts with empirical epidemiology and microbiology in an iterative process may result in more valuable information than either in isolation.

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Introduction

Bacterial infections in humans that are resistant to antibiotics continue to pose a significant challenge globally [1]. The role of antibiotic use in agriculture as a causal factor in this ongoing problem has received considerable discussion over many years. There is no denying that many resistant bacteria are present in agricultural environments and can affect humans through food consumption or through more complex environmental routes of exposure. While agricultural antibiotic use (AAU) can influence resistance in specific bacterial populations, the real challenge is determining which agricultural practices are having the greatest contribution to the emergence, amplification, persistence and dissemination of antimicrobial resistance (AMR). Stated another way, how do we identify those practices that are truly contributing significantly to the antibiotic resistance problem and how do we accurately predict the net benefit to human health that modification or elimination of these practices would have? Incorrectly believing that these links are causal [2], rather than simply correlation, and failing to appreciate the relative importance of the diversity of potential causes, together represent a significant barrier to effective intervention in the agricultural arena.

Without sound science to establish and quantify these causal links, we often rely on assumptions about causality to infer which interventions will be effective. As an example, a recent publication quantified the number of deaths from bloodstream infections caused by third-generation cephalosporin-resistant *Escherichia coli* (G3CREC) that were due to the use of antibiotics, mainly the third-generation cephalosporins, in poultry production [3]. The data on which these calculations were based came predominantly from two sources. One study estimated the excess mortality and prolongation of hospital stay associated with G3CREC bloodstream infections in humans in Europe. A second study in the Netherlands [4] found that '56% of the resistance genes in G3CREC in humans were identical to genes derived from *E. coli* isolated from retail chicken samples' (p. 1339). Collignon *et al.* [3] then calculated the number of excess deaths from G3CREC-associated bloodstream infections caused by antibiotic use in poultry as 56% of the total estimated excess deaths due to G3CREC-associated bloodstream infections.

This simplistic calculation makes several strong and unacknowledged assumptions. First, the authors assumed that all human isolates that have identity to poultry strains (56% for the Netherlands study) were derived from poultry. Second, the authors implicitly assumed that AAU within the poultry production system was the sole cause of all third-generation cephalosporin resistance in these *E. coli*. Finally, the authors assume that the relationship between isolates in the Netherlands can be extrapolated to all of Europe. The need for making such assumptions when attempting to estimate risk is understandable given the challenges of collecting strong, quantitative evidence. However, failure to acknowledge and

validate these assumptions can lead to inaccurate inferences and misguided interventions [5].

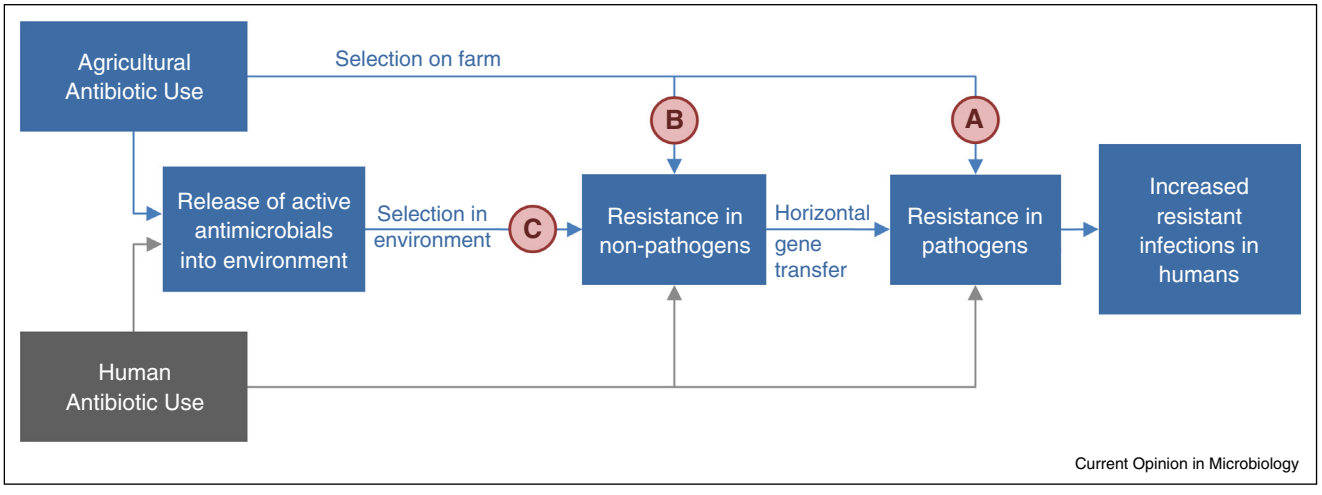
While it is generally acknowledged that much of the resistance in human pathogens is associated with human uses of antibiotics [1], the purpose of this paper is to explore scientific approaches for evaluating and quantifying the causal link between the use of antibiotics in animal agriculture and human health. Specifically, this paper will focus on the theme of attribution, because in effect we are performing attribution analyses on different levels. For example, we are attempting to attribute the observed resistance to specific selection forces. We are also attempting to attribute the human illness to specific sources. The latter has received considerable attention and is a growing area of investigation [6–10], but the former is still in its infancy with respect to the ecology of antimicrobial resistance.

Linking AAU to human health

To motivate this discussion, we first consider the ways in which the exposure (AAU) can cause resistance to increase above background levels and then lead to a negative outcome (human health harm) (Figure 1). We will consider the predominant risk to human health posed by AAU to be treatment failure due to the bacterium being resistant, which then results in increased morbidity, increased duration of illness, or mortality. To link AAU to increased human health impacts, the following three scenarios should, in a general form, provide a sufficient structure for this discussion. In Scenario A, AAU leads to an increase in resistant pathogens which are then transmitted to humans via the food chain or the environment. The selection pressure exerted by the AAU in this scenario occurs on the farm. In Scenario B, AAU selects for resistance in non-pathogens, perhaps commensals or environmental microbes, which then transfer resistance genes to pathogens leading to more resistant infections in humans. Again selection in this case occurs on the farm. Finally, Scenario C involves the release of active antimicrobial compounds into the environment where selection occurs predominantly in non-pathogens, such as soil microbes, and resistance is transferred horizontally to pathogens as in B. Scenarios B and C might be referred to as forces increasing the size of the resistance gene pool.

To estimate the risk to human health in each of these scenarios (or combinations of them), we must establish quantifiable, causal links and determine what the current state of evidence tells us about the relative importance and interconnectedness of these links. For example, knowing that a human illness was caused by a resistant bacterium that originated from an agricultural facility (the proverbial smoking gun) does not necessarily inform the causal relationship between AAU and human health. The resistance in this bacterium was unlikely to have been created *de novo* by the AAU on the source farm. Many of the resistance genotypes, particularly when associated with multidrug resistance plasmids, are conserved and have a global distribution [11,12,13•,14]. The cumulative effect of the AAU over large geographic areas over extended durations of time is likely aiding in the spread of this resistance, but appropriate data and improved analytical approaches are needed to estimate this relationship accurately [13•,15]. Finding resistance genes in the environment, even if it is known that they are emanating

Figure 1



Conceptual model of the ways in which agricultural antibiotic use (AAU) can cause increased resistant infections in humans. In Scenario A, AAU leads to an increase in resistant pathogens which are then transmitted to humans via the food chain or the environment. In Scenario B, AAU selects for resistance in non-pathogens which then transfer resistance genes to pathogens leading to more resistant infections in humans. In Scenario C, active antimicrobial compounds are released into the environment where selection occurs predominantly in non-pathogens, and resistance is transferred horizontally to pathogens as in B. Human antibiotic use is shown for reference but not discussed.

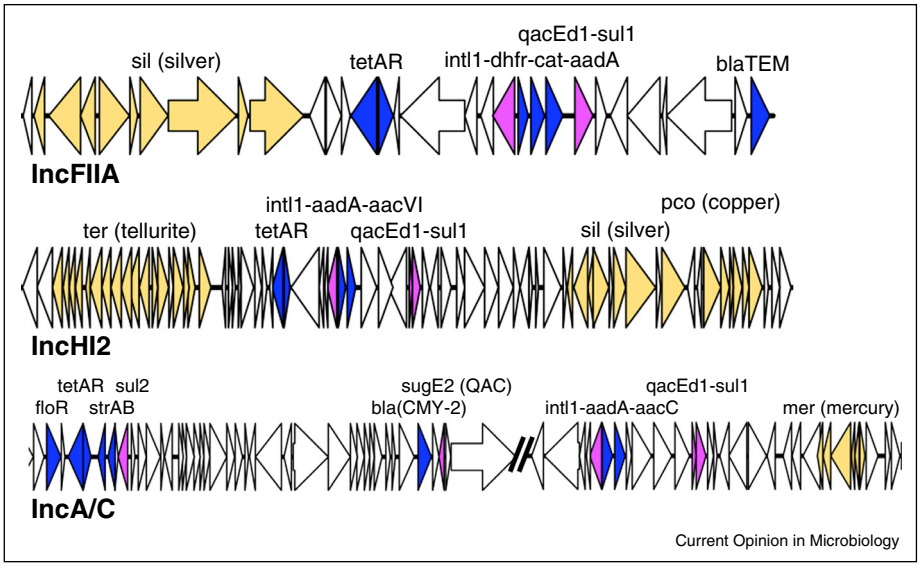
directly from an agricultural facility, does not necessarily indicate that their presence is due to AAU. With an extensive resistome in the environment, including agricultural environments, we need approaches that can estimate the role that AAU is playing in expanding this resistance gene reservoir. Finally, detectable levels of antibiotic metabolites can be found in the environment, for instance in ground and surface water, and some of these may still be capable of exerting a biological effect on bacterial populations. To mitigate this risk, we need methods for attributing these metabolites to specific sources and for determining their ability to impact microbial populations in the environment.

Diverse forces of selection and causal networks

Many factors that can potentially select for antibiotic resistance have been described. These factors include antibiotics, metals and other compounds such as disinfectants [16–18]. Because of the diversity of interrelated potential causes for observed resistance, the relationship between each component cause and observed resistance is likely to be confounded [16]. To understand the reasons for the presence of antibiotic resistance and to predict future changes in resistance [19], it would be ideal to quantify all of the factors that select for resistance genes and resistant organisms. Theoretically, the selection and persistence of an antibiotic resistance gene that has been introduced into the environment may have more to do with the presence of additional genes that confer resistance to chemicals and metals or that provide an ecological fitness advantage to the cell than to the presence of a primary antibiotic selection pressure [20]. This situation is made more complex when resistance is mediated by genes associated with mobile genetic elements such as integrons and plasmids.

As an example of this possibility, consider sequenced plasmids from avian pathogenic *E. coli* (APEC) [12,21–23]. These plasmids, shown in Figure 2, possess genes that confer resistance to tetracycline, streptomycin, gentamicin, sulfisoxazole, copper sulfate, and benzalkonium chloride (a quaternary ammonium compound, QAC), among others. The QAC resistance observed in the APEC plasmids is not unique; QAC resistance genes are found in a variety of bacteria and are often linked to antibiotic resistance genes [24,25]. These compounds are used at different stages of poultry production. Although bacteria may require metals for survival, high levels can be toxic to the cell, and thus many bacteria possess mechanisms of resistance to specific metals. Copper [26] has been shown to select for antibiotic resistant bacteria in natural environments [27] and can also be present at different stages of poultry production. The presence of integrons within these plasmids can further foster the spread of resistance traits, possibly independent of cell replication or plasmid transfer. To understand the reasons for the presence of antibiotic resistance and to predict future changes in resistance, it is necessary to attribute observed resistance genes and resistant organisms to the specific factors that can select for these traits. To develop effective interventions, the

Figure 2



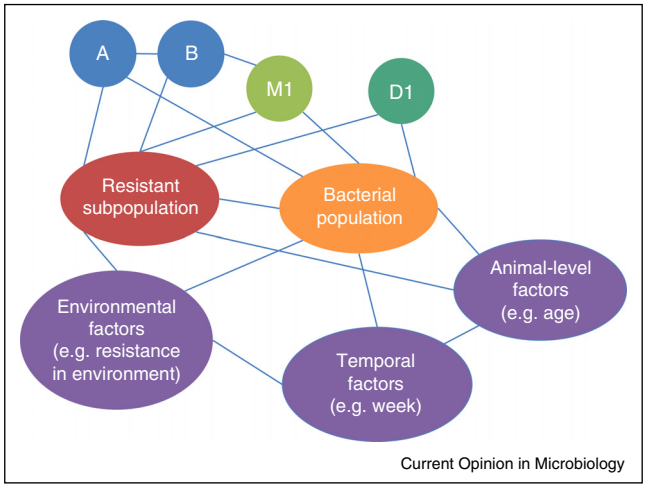
Regions of avian pathogenic *E. coli* (APEC) plasmids that encode for resistance to multiple antimicrobial agents. All plasmids were isolated from APEC from diseased broiler chickens in the US [12,22,23,63]. The IncFIIA plasmid is pAPEC-O2-R (101 kb), the IncHI2 plasmid is pAPEC-O1-R (241 kb), and the IncA/C plasmid is pAPEC199061_160 (160 kb). Selected genetic modules are depicted from each plasmid with arrows illustrating genes with their direction of transcription. Heavy metal resistances are colored tan, antibiotic resistances are colored blue, and class 1 integron components are colored pink.

relative importance of these causal factors needs to be estimated.

Due to the complex interactions of exposures, confounders and resistance outcomes, even carefully applied epidemiologic methods are often insufficient to accurately describe the relationships between AAU and resistance. Relationships between exposures, including AAU, and observed resistance patterns are not constants but will vary considerably with changing cofactor distributions, geography and time [16,28]. An approach that may help overcome the limitations of traditional analytical methods is the use of causal network modeling [29]. In general, these methods examine distributions of variables to estimate the direction and strength of the most plausible causal connections. For example, a directed acyclic graph (DAG) [30], which has been used in many other epidemiological areas, depicts all causal relationships and uses data to estimate the strength of these causal links. Figure 3 shows a hypothetical undirected causal network, which could be developed prior to the collection of data regarding the frequency and extent of specific selection forces, the animal, farm and environment, and the population of microorganisms and their resistance genes. After the data collection and analysis, Figure 3 would be revised to show the direction and importance of each causal relationship.

The discipline of causal network modeling, often termed Bayesian network modeling, is a growing area of research that has direct relevance to the antibiotic use — resistance relationship. Given the diversity of potential causes for observed resistance, whether in a single strain, a population of cultured bacteria, or in a metagenomic analysis of genes, data regarding these potential causes can be collected in well-designed studies and then used to inform these causal networks. The iterative approach to these networks enables the model to 'learn' the most likely causal structure and refine these estimates with additional data. In AMR research, valid network modeling will rely on the ability to accurately measure relevant variables, including resistance determinants. Current advancements in microbiological detection, including culture-independent methods in combination with quantitative PCR [31–33], will support the use of this approach. Causal networks have been used extensively in other fields and have recently been applied to problems such as risk factors for colonization with vancomycin-resistant *Enterococcus* [34] and the linkage characteristics of observed resistance traits in *E. coli* from swine [35].

Figure 3



Hypothetical Un-directed Acyclic Graph. In this environment, two antimicrobials (A, B), one metal (M1), and one disinfectant (D1) are used. Bacterial populations are regularly sampled and analyzed, and the resistant subpopulation can be determined. Records related to animal-level, environmental and temporal factors are recorded. The iterative Bayesian network model then learns the positions, directions and importance of the connections based on the data, enabling the estimation of cause and effect relationships.

Population dynamics and the challenge of predicting co-selection

Even when we know the linkages that exist among diverse resistance genes, predicting changes in resistance following specific exposures (such as AAU) can be challenging. It is possible for one antibiotic to be a strong co-selection force for a second antibiotic but the reverse situation can be much weaker. A series of informative field trials examining third-generation cephalosporin (G3C) and tetracycline resistance in *E. coli* of beef cattle highlights this challenge. In an initial study, the investigators found a transient increase in resistance to G3C and tetracycline as well other antibiotics following administration of G3C [36]. The investigators therefore logically hypothesized that the use of tetracycline compounds in these cattle would lead to an increase in both tetracycline and G3C resistance. Yet after administration of chlortetracycline, while tetracycline resistance increased as expected, G3C resistance decreased [37]. Further investigation revealed the underlying population genetics that produced this unexpected effect. A third trial, wherein G3C use preceded a chlortetracycline administration, showed an increase in resistance to both antibiotics [38•]. Genetic characterization of the isolates revealed that tetracycline resistance was due primarily to two tetracycline resistance genes, *tet*(A) and *tet*(B); *tet*(A) was linked to the G3C resistance gene $bla_{\text{CMY-2}}$, whereas *tet*(B) was not. This differential linkage in conjunction with the temporal sequence of administered antibiotics meant that use of G3C increased the population of isolates possessing $bla_{\text{CMY-2}}$ and therefore co-selected for *tet*(A). In contrast, the use of chlortetracycline alone favored the *tet*(B) gene and resulted in a decrease in the $bla_{\text{CMY-2}}$/*tet*(A) isolates. However, when chlortetracycline followed the G3C administration, it seemed to augment the already elevated population of G3CREC

possessing *tet*(A). This series of investigations demonstrates that even prior knowledge of genetic linkages between resistance genes may be insufficient to predict how the antibiotic use — resistance relationship will manifest. Although progress has been made in our understanding of the bacterial ecology of agricultural settings [39], more remains to be done to understand the detailed population dynamics occurring at the farm level.

In addition to co-selection potential, there are other evolutionary considerations that may play a role in resistance dynamics. Although the evolution of resistance can occur simultaneously at multiple hierarchical levels (e.g. genes, integrons, transposons, plasmids, cells and bacterial communities), selection can act to produce dissimilar resistance dynamics at these different levels [13•]. Genetic drift, which results in changes to population genetics through random chance, is another overlooked evolutionary process with relevance to the AAU-resistance relationship, particularly when the size of the microbial population has been significantly reduced, such as in the presence of any biocide [13•]. Temporal and spatial heterogeneity in the environment of a given microbial population can strongly influence the dominating evolutionary processes and, thus, the resulting dynamics of resistance [16,40]. In short, as stated by Fernando Baquero, 'the evolution of resistance is therefore the evolution of a complex system' [40].

The application of complex systems methodologies from various fields to the epidemiology of AMR may allow us not only to separate out individual drivers of AMR trends but also to describe general principles of the system that can inform public health action [41]. Evolutionary biology, for example, offers a rich literature examining the implications of complicated population genetics for a variety of evolutionary situations with relevance to AMR [42•]. The integration of insights derived from empirical and mathematical evolutionary experiments with epidemiologic methods provides a path for understanding the behavior of the AMR system, in agriculture and more widely. Recent developments in evolutionary epidemiologic models, such as the ability to model fitness dynamics of microbial populations that are not at steady state, represent a promising area for future incorporation into AMR research [43].

Future directions

Researchers face many challenges when seeking to establish causal relationships between antibiotic use practices and AMR, particularly in open systems such as agricultural environments. Intuition can be a poor guide in directing investigative efforts of these non-linear and interconnected systems, and consequently, integration of modeling efforts with empirical epidemiology and microbiology in an iterative process has been found to result in more valuable information than either in isolation [44••]. This 'model-guided fieldwork' framework, originally proposed in the context of wildlife ecology, dictates that models should be constructed early and inform all stages of the investigative effort from generation of hypotheses through the design of experimental trials and data analysis. Models resulting from this framework are data driven, specific to the system under study and able to provide highly useful insights.

Arriving at valid causal inferences in this context is difficult but is becoming increasingly manageable with novel methods and careful attention to potential biases and limitations of different study designs [45••]. Evolutionary biologists with expertise in bacterial population genetics should have a place on multi-disciplinary teams evaluating this issue, as population genetic theory and methods will help to illuminate the mechanisms by which resistance is emerging and persisting [13•,40,42•,43,46,47].

Much of this discussion has been focused on the direct selection of resistance following the administration of the antibiotic. However, an area of considerable importance for potential selection of resistance that requires continued investigation and intervention is waste management within human and agricultural sectors [48,49,50••]. Much of the administered antibiotic will be excreted in a potentially active form, and yet we have little published data on the excretion, fate and transport of these compounds [51]. Methods of attribution need to be developed to link these environmental residues to their originating source [52]. Although metabolite concentrations in the environment are often low and might not have considerable biological effect on bacteria in water [53], the effect on microbial populations in sediments and soils is real but impacts are uncertain [39,54••,55–57].

In the United States, the growth promotion/feed efficiency administration of antibiotics in animal agriculture is officially being phased out [58,59]. Regardless of one's opinion on this matter, we can all agree that the agricultural sector of the U.S. is about to embark on a substantial natural experiment, given that antibiotics have been used in this manner for over six decades. Agricultural systems will make compensatory changes, but will this intervention produce the desired effect and will there be any unintended consequences that augment potential human health risks? One possible example of this occurred in Denmark where the potential selection of methicillin-resistant *Staphylococcus aureus* strains with linked zinc resistance was observed after the removal of growth promoters and the subsequent increase of zinc oxide use in swine [60,61]. The scientific and agricultural communities should be ready with well-designed, rigorous studies to evaluate resistance dynamics as the growth promoters are phased out, so that we are not forced to rely

on correlations and before–after comparisons to infer potential causal relationships.

Utilizing more holistic, system-based approaches will hopefully result in the selection of interventions that have the largest capacity for reducing risks associated with resistance. Importantly, these interventions will not be implemented in isolation and will impact other management decisions. A successful intervention should be one that will optimize human, animal and environmental health. Success should not be measured by implementation of the policy itself [62] but rather through documented health improvements. This does not mean that we should wait until our knowledge of the problem is complete before we act. Rather we need to make a concerted effort to collect relevant information and interpret the data openly and fairly. Only through open and honest discourse of what options are available, what the intended goals of action are, how individual stakeholders would be impacted through action, and what metrics will be used to evaluate efficacy can we begin to make a concerted and collaborative effort to mitigating the human health impacts of resistance that are caused by the use of antibiotics in animal agriculture.

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