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Where can sequencing and metagenomics aid risk assessment?

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Antibiotic resistance in the food supply chain:

Where can sequencing and metagenomics aid risk assessment?

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Abstract

Antibiotic resistance is a rapidly growing threat to human health. The environment – including animals and plants – functions both as a transmission route for antibiotic resistant pathogens and a source of resistance genes. The food supply chain connects environmental habitats for bacteria with humans through a route that sometimes – due to use of antibiotics in both agri- and aquaculture – includes substantial selection for resistance. According to international food standards, selection and dissemination of foodborne resistance should be considered in the risk analysis of food production. High-throughput sequencing and metagenomics could contribute to understanding these transmission and selection processes in the food supply chain.

Introduction

Antibiotic resistance is a rapidly emerging health crisis, not only threatening our ability to treat diseases now trivially cured, but also complicating medical procedures such as surgery, neonatal care and cancer therapy [1]. While clinical use and misuse of antibiotics are undoubtedly major drivers of the resistance development in pathogens, there is increasing evidence that other settings need to be taken into account to mitigate the rise of resistant bacteria. Resistance genes have been present in the environment since long before the antibiotic era [2,3], and there is evidence that it has likely served as a source for many of the resistance genes we are today facing in pathogens [4,5]. In addition, environmental settings – including animals and plants – function as a transmission route for pathogenic and non-pathogenic resistant bacteria [6]. Here, food production facilities and the food supply chain can play a crucial role, connecting environmental, animal and human habitats for bacteria. International food standards outline that selection and dissemination of foodborne resistance should be considered in the risk analysis of food production, and that monitoring of resistant bacteria is critical for determining effective risk management strategies [7]. High throughput sequencing and metagenomics have been used to provide guidance for risk assessment in various scenarios, both with regards to antibiotic resistance and food safety issues [8,9*,10**,11*,12]. However, the relative risks of finding individual resistance genes in various environments is highly context-dependent, and association to human pathogens is key to determine risks to human health [13*,14*], This review will discuss which information high-throughput sequencing and metagenomics can contribute towards the understanding of dissemination and selection of antibiotic resistance via the food supply chain, along with the limitations of these methods.

Food supply and transmission of antibiotic resistant bacteria

In principle, the risk assessment procedure is the same for the transmission of antibiotic resistant bacteria in food products as for transmission of non-resistant pathogens, and the uses of high-throughput sequencing for these purposes have been excellently discussed and reviewed previously [9*,12,15,16*]. The most important difference specific to antibiotic resistance is that resistance genes may be disseminated by non-pathogenic bacteria, which may then subsequently transfer those resistance genes to human pathogens after the food is consumed. However, such transmission can largely be avoided by the same methods that are used to remove pathogens from food. In addition, infections caused by food-borne resistant bacteria tend to cause more severe disease consequences than infections with susceptible bacteria [17]. Importantly, resistant bacteria, including pathogens, may cause contamination during the processing of food products, opening up an indirect transmission route between humans, and it may be hard to determine whether the presence of resistant bacteria in food is the result of human contamination or exposure to environmental or animal bacteria.

The food supply chain and antibiotic resistance development

A critical aspect in the recruitment of antibiotic resistance genes from animals and the environment to humans is the presence of an antibiotic selection pressure favoring genes contributing to resistance phenotypes. Such selection will enrich resistant bacteria relative to sensitive ones, aiding transmission of the resistance genes they carry. In this respect, the food supply chain represents a special case, as it may present bacteria with transiently high concentrations of antibiotics, particularly in meat production and aquaculture (Figure 1). In addition, antibiotics are still used as growth promoters in many parts of the world, presenting

84 bacteria associated with the animals with, relatively constant, low concentrations of antibiotics,
85 which also may select for antibiotic resistance [18]. The use of antibiotics in food production
86 widens the role of the food supply chain from only being a dispersal route for resistant bacteria
87 and resistance genes, to potentially selecting for resistance determinants and stimulating the
88 sharing of resistance genes through horizontal gene transfer [19,20].

89 The largest uses of antibiotics are in human healthcare and for animal agriculture [21,22].
90 Similarly, heavy use of antibiotics in aquaculture in many parts of the world turns farming of fish
91 and shellfish into arenas for resistance emergence and selection [23]. Indeed, fish farms have
92 been associated with higher abundances of antibiotic resistance genes [24*] and a diversity of
93 mobile genetic elements [25]. Vegetables present another short dissemination route from the
94 environment to humans, and the soils used to grow the crops are often amended with manure,
95 which in turn may derive from animals treated with antibiotics. Furthermore, antibiotics are also
96 used to protect certain fruits from bacterial diseases [26]. The effects of antibiotics on manure
97 and soil amended with manure have been studied using both traditional [27] and metagenomic
98 approaches [28]. Interpretation of such studies is complicated, however, as the effect of selection
99 from antibiotic residues and the effect of adding resistant bacteria from animal feces cannot
100 easily be separated [14]. Similarly, investigation of soils from apple orchards with documented
101 exposure to the antibiotic streptomycin has not generated results in favor of resistance selection
102 [29,30]. It seems, however, that farmland soil can function as a long-term reservoir for antibiotic
103 resistance genes after amendment with manure [27,31], and it is imaginable that the resistant
104 bacteria accumulated in manure-treated soil can be further disseminated with fruits and
105 vegetables and thereby transported globally.

106 Finally, an important aspect of the spread of resistant bacteria through food production is that
107 humans may be exposed directly to bacteria from farmed animals [32-34]. Such sharing of
108 bacteria and resistance genes between humans and animals has been shown to be extensive
109 [11,35,36], and its impact on resistance dissemination may be large.

110 Food preparation

111 In risk assessment of both resistant and non-resistant pathogens in food it is central to recognize
112 the importance of food preparation. Food that is properly cooked, or otherwise prepared in a
113 way that kills bacteria, simply presents an almost impenetrable dispersal barrier to infecting or
114 colonizing humans. This also influences the risk picture, as vegetables and fruits are more likely
115 to be consumed raw or unprepared than meat and fish. Moreover, vegetables are often not
116 substantially prepared before being sold to consumers, who in turn may not wash them
117 sufficiently to avoid intake of (potentially resistant) bacteria. Thus the preparation, preservation
118 and packaging of vegetables and fruits may require additional attention, as they constitute central
119 links between environmental bacteria and the human microbiome, and therefore also potential
120 dispersal barriers to prevent the spread of resistant bacteria.

121 Risk assessment insights from sequencing and metagenomics

122 Many studies have shown the presence of resistance genes in farmed animals, using for example
123 plasmid and whole genome sequencing (WGS) approaches. For example, CTX-M and TEM
124 beta-lactamases have been found on plasmids sequenced from Dutch chickens, and the same
125 gene variants were also found in human samples [37]. WGS has also been used to identify a
126 conjugative megaplasmid encoding ESBL resistance genes in both chickens, pigs, meat and

humans from Italy [38], and to determine clonal relationships, virulence factors and resistance genes among isolates from US feedlot cattle [39]. Plasmid sequencing was also used to identify the cause of observed transferrable colistin resistance – the novel *mcr-1* resistance gene – in isolates from Chinese pigs [40*], on the same plasmid subsequently also identified in isolates from humans [41]. In none of these cases, the transmission route between animals and humans could be decisively determined. It may well be that human exposure to the bacteria carrying these resistance determinants occurred at the farm level rather than through consumed food. In any case, the fact that these studies find virtually identical resistance plasmids in humans and animals, clearly points towards the role of animals and food production as a dissemination route for resistance genes. In support of this, metagenomics confirms that the animal microbiota shares substantial numbers of both resistance genes and taxa with the human gut [11*].

With regards to aquaculture as well as vegetable and fruit production, research employing sequencing approaches is still relatively scarce. A recent sequencing study of an Italian fish farm indicated a complex interplay between phages and bacteria in terms of resistance genes carriage [42]. In another study, WGS of integron-bearing bacteria isolated from fruits and vegetables was used to show that these food products indeed constitute a plausible dispersal route for resistance genes from the environment to the human microbiome [43]. However, it should be noted that those resistant bacteria might as well have originated from contamination of the fruits with human-associated bacteria during handling. Similarly, sequencing of phages from ready-to-eat food in South Korea identified a phage-carried metallo-beta-lactamase [44] and resistance genes have also been detected in Portuguese street food using PCR [45], but it could not be determined if this was the result of human contamination or transmission via the food supply chain.

149 Metagenomic sequencing has so far not been much applied for food safety assessment of
150 resistant bacteria, despite that it could play a central role in hazard identification [47], where it
151 could be used for pathogen detection [48], as well as in hazard characterization, particularly for
152 identification of virulence factors [47] and resistance patterns [8,49,50]. Microbial quantitative risk
153 assessment requires determination of the concentrations of bacteria at the exposure sites and
154 dose estimates after human consumption of food products containing resistant bacteria [6].
155 While metagenomics only can contribute relative abundances, spiking of samples with known
156 doses of bacteria can partially alleviate this problem [48]. If appropriately applied, metagenomics
157 has the potential to fill in quantitative gaps in risk assessment models for example pertaining to
158 microbial presence in raw materials, efficiency of contaminant removal during food processing,
159 and the severity of the hazard (e.g. antibiotic resistance and virulence).

160 Interestingly, metagenomic sequencing could not identify a single resistance gene in
161 slaughterhouses, despite finding several in feces, water, soil and transport trucks associated with
162 the same animals – a difference that could not solely be explained by the much higher proportion
163 of host DNA in the slaughterhouse samples [10**]. This highlights that the food preparation
164 process is important for preventing the presence of resistant bacteria in food, and that proper
165 hygiene and preparation measures vastly lower the risk of human exposure to antibiotic resistant
166 bacteria originating from animals and external environments via food products. It deserves to be
167 noted that if transmission of resistant bacteria is avoided in the food preparation process, this
168 increases the relative importance of direct contact between live animals and humans for the risk
169 of dissemination of resistant bacteria (Figure 1). In fact, transmission of bacteria directly from

170 farmed animals to farmers may be the epidemiologically most relevant exposure route for
171 humans to resistance determinants from animals.

172 ## Future prospects for the use of sequencing in food safety

173 It is clear that we stand at a turning point where large-scale sequencing is becoming a commodity
174 technique to study microbes and microbial communities, which also has significant implications
175 for food safety [15]. Already, the added value of whole genome sequencing over traditional
176 methods, such as multilocus sequence tagging (MLST), for delineating the causes of foodborne
177 disease outbreaks has been recognized [46], and metagenomic sequencing is knocking on the
178 door to enter as a serious contender for being a broad, all-encompassing, method to screen
179 samples for multiple pathogens as well as resistance and virulence genes at once. However, a
180 number of important hurdles exist preventing implementation of metagenomics as a screening
181 tool. First of all, if food products are investigated, it is very hard to only extract bacterial DNA
182 from the samples. Most often, the vast majority of the reads will be derived from the food itself.
183 This was the case also in the slaughterhouse study mentioned earlier, in which more than 97% of
184 the high-quality reads from meat originated from the genomes of the slaughtered animals [10**].
185 Furthermore, even very high sequencing depths may not be sufficient to detect clinically
186 important pathogens and resistance genes even in fecal samples [49]. Recent statistical
187 developments in this area [51,52] may nevertheless make count data from shotgun metagenomics
188 investigation usable in quantitative risk assessment models based on e.g. Bayesian statistics [53].
189 Second, it is generally not possible to identify the specific pathogen from which a sequenced read
190 originated, which in principle would be required for regulatory and confirmatory purposes
191 [7,16*]. Although assembly of metagenomic sequence data could partially alleviate this problem,

several obstacles remain before assembly of e.g. antibiotic resistance regions becomes straightforward [8,50]. Third, shotgun metagenomics only provides relative abundance information, which may be insufficient to discern whether a detected pathogen or resistance gene is present in quantities relevant to the infectious dose. This problem can to some extent be overcome by spiking the samples with material of known concentration [48]. In addition, similar to other molecular methods, metagenomic sequencing makes no difference between DNA from dead and living bacteria, which further complicates risk assessment. Finally, metagenomic sequencing and data analysis are time-consuming and results can take weeks to obtain, which may be too slow for many food safety purposes. Nevertheless, metagenomic approaches can still be useful for highlighting specific routes of resistance dissemination requiring more attention [6]. Metagenomic screening for resistance genes may also guide the prioritization of interventions to reduce the propagation of resistance genes in the food supply chain [10**,54*]. It is, however, unclear if there are enough benefits of shotgun metagenomics to justify the costs of its implementation beyond sporadic reports into systematic screening. That said, with sequencing costs quickly dropping [55], the feasibility of sequencing-based approaches may improve rapidly. In addition, metagenomic sequencing allows retrospective investigation of antibiotic resistance genes as novel resistance factors are discovered [56*]. This enables estimation of the degree of penetration newly discovered resistance genes already have achieved, as well as tracing of their dissemination history, for example through the food supply chain.

Conclusions

Currently, the main use of high-throughput sequencing in food safety is the adoption of whole genome sequencing to precisely delineate which strains are involved in particular foodborne

disease outbreaks, and which resistance factors they carry. The rapidly evolving long-read sequencing technologies look very promising for this purpose, particularly if their comparably high error rates are improved. Additionally, the use of shotgun metagenomics to screen samples for a multitude of genes involved in resistance and virulence is emerging as a suggested tool to monitor the transmission of resistance factors and pathogens through the food supply chain. However, it would at present be both costly and of doubtful benefit to employ metagenomics as a part of routine screening programs. Still, metagenomics can contribute scientific knowledge to the quantitative risk assessment of antibiotic resistance in the food supply chain. Thus, we can expect a partial transition towards DNA-based techniques in food safety in the coming years, and with that increasing understanding of how food production contributes to the development of antibiotic resistance among human pathogens.

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Figures

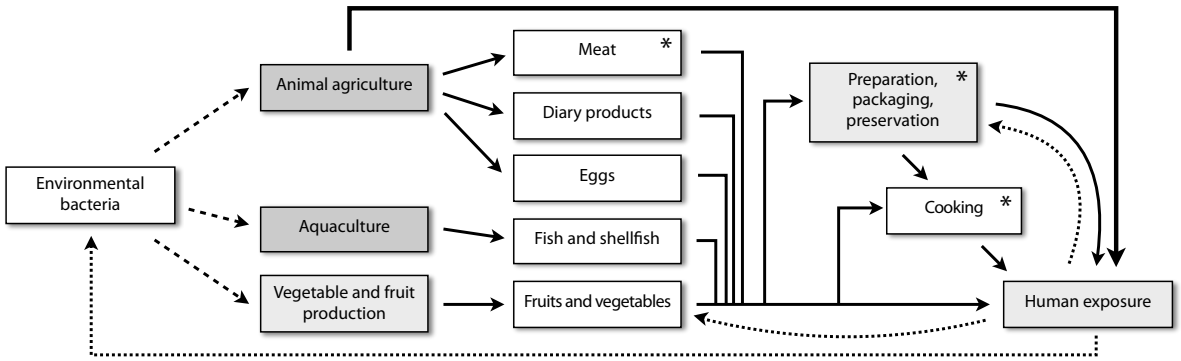

Figure 1. Simplified schematic overview of the flow of resistant bacteria and resistance genes in the food supply chain. Steps where strong selection pressure for antibiotic resistance (i.e. antibiotic usage) is commonly encountered are indicated with a dark grey background, while steps where such selection occurs but is less common is indicted by light grey background. Note that direct exposure to resistant bacteria in animal agriculture is an important dispersal route, perhaps more important than exposure through the entire food supply chain. Although much food passes through a preparation, packaging and preservation process, many food products are also consumed directly, with or without cooking. Also, (resistant) human pathogens can contaminate food both in this process, as well as in the handling of fruits and vegetables (dotted lines). Steps that present important barriers to transmission of resistance have been indicated with an asterisk.

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This study is probably the most thorough to date utilizing metagenomic sequencing to

address antibiotic resistance risks related to food safety. Through comprehensive sampling of the beef production process, including animal feces, soil, water, transport trucks and slaughter, the authors highlight where in the food production chain the abundance and diversity of resistance genes is highest. The study also underscores several obstacles that face metagenomics if it were to be applied in routine screening.

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This study is the most comprehensive screening for resistance genes in deeply sequenced environmental, animal and human metagenomes published to date, investigating in total 864 samples. The study detected a substantial overlap of both resistance genes and bacterial taxa between the animal microbiome and the human gut, highlighting the interplay between these two bacterial habitats.

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This study is a neat example of that metagenomic data can be re-used to screen for resistance genes that may be discovered in the future. Here, the authors find the novel *mcr-1* colistin resistance gene described in [38*] in metagenomes from the human gut, originally sequenced years before the discovery of *mcr-1*. This emphasizes the power of metagenomics for retrospective analysis of resistance.