

Choosing Your Battles: Which Resistance Genes Warrant Global Action?

An-Ni Zhang^{1, 4, 5}, Li-Guan Li¹, Xiaole Yin¹, Chengzhen L Dai⁴, Mathieu Groussin⁴⁻⁷, Mathilde Poyet⁴⁻⁷, Edward Topp⁸, Michael R Gillings⁹, William P Hanage¹⁰, James M Tiedje¹¹, Eric J Alm⁴⁻⁷, Tong Zhang^{1-3*}

¹Environmental Microbiome Engineering and Biotechnology Laboratory, The University of Hong Kong, Hong Kong, SAR, China;

²School of Public Health, The University of Hong Kong, SAR, China;

³International Center for Antibiotics and Resistance in Environments, School of Environmental Science and Engineering, Southern University of Science and Technology, China;

⁴Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, USA;

⁵The Global Microbiome Conservancy, Cambridge, USA;

⁶Center for Microbiome Informatics and Therapeutics, Massachusetts Institute of Technology, Cambridge, USA;

⁷The Broad Institute of MIT and Harvard, Cambridge, USA;

⁸London Research and Development Centre (LRDC), Agriculture and Agri-Food Canada, London, Ontario, N5V 4T3, Canada;

⁹Department of Biological Sciences, Macquarie University, Sydney, New South Wales, Australia;

¹⁰Center for Communicable Disease Dynamics, Department of Epidemiology, Harvard TH Chan School of Public Health, Boston, United States;

¹¹Department of Plant, Soil and Microbial Sciences and of Microbiology and
Molecular Genetics, Michigan State University, East Lansing, MI, USA.

*Corresponding author:

Address: Environmental Biotechnology Laboratory, The University of Hong Kong,
Hong Kong

Phone: +852-2857 8551; Fax: +852-2859 8987

Email: zhangt@hku.hk

Abstract

The increasing accumulation of antibiotic resistance genes (ARGs) in pathogens poses a severe threat to the treatment of bacterial infections. However, not all ARGs do not pose the same threats to human health. Here, we present a framework to rank the risk of ARGs based on three factors: "anthropogenic enrichment", "mobility", and "host pathogenicity". The framework is informed by all available bacterial genomes (55,000), plasmids (16,000), integrons (3,000), and 850 metagenomes covering diverse global eco-habitats. The framework prioritizes 3% of all known ARGs in Rank I (the most at risk of dissemination amongst pathogens) and 0.3% of ARGs in Rank II (high potential emergence of new resistance in pathogens). We further validated the framework using a list of 38 ARG families previously identified as high risk by the World Health Organization and published literature, and found that 36 of them were properly identified as top risk (Rank I) in our approach. Furthermore, we identified 43 unreported Rank I ARG families that should be prioritized for public health interventions. Within the same gene family, homologous genes pose different risks, host range, and ecological distributions, indicating the need for high resolution surveillance into their sequence variants. Finally, to help strategize the policy interventions, we studied the impact of industrialization on high risk ARGs in 1,120 human gut microbiome metagenomes of 36 diverse global populations. Our findings suggest that current policies on controlling the clinical antimicrobial consumptions could effectively control Rank I, while greater antibiotic stewardship in veterinary settings could help control Rank II. Overall, our framework offered a straightforward evaluation of the risk posed by

ARGs, and prioritized a shortlist of current and emerging threats for global action to fight ARGs.

Keywords:

Global challenge; epidemiology; bioinformatics; mobile DNA; antibiotic stewardship; environment.

Main

Antibiotic resistance genes (ARGs) are widespread pollutants¹⁻⁵ (Figure S1) threatening human health. The World Health Organization (WHO) calls for global action to fight them⁶, but action cannot realistically be taken against thousands of known (and unknown) ARGs⁷⁻⁹. The risk of ARGs to human health varies considerably according to a number of factors including their genetic context. For example, intrinsic colistin resistance ARGs were found decades ago^{10,11} but their low potential for horizontal gene transfer (HGT) limited their spread. In contrast, the *mcr-1* colistin resistance gene has rapidly spread into eight pathogenic species (“host pathogenicity”)¹² across 31 countries and into 1-20% animal and human gut microbiome samples¹³⁻¹⁶ (“anthropogenic enrichment”), largely driven by its capacity for HGT (“mobility”)¹⁷. These characteristics are typical of high risk ARGs, but current databases and analyses^{7-9,18} do not distinguish ARGs based on them. Mobilizing policymakers to implement interventions on ARGs will require substantial scientific capital, and prioritizing high risk ARGs will allow that capital to be effectively invested.

Therefore, there exists a need for a framework to categorize the risk of ARGs to human health. Previous attempts^{19,20} at defining such a framework based on critical factors have largely remained theoretical (such as mobility and sub-inhibitory antibiotic concentrations), and thus, difficult to implement. In this study, we developed an empirical approach that combines three factors (“anthropogenic enrichment”; “mobility”, and “host pathogenicity”) to prioritize our efforts and

identify emerging threats.

Results

ARGs enriched by anthropogenic activities, and correlated with antibiotics contamination should pose a higher risk than ARGs that are not enriched. Through investigating ARGs in 854 global metagenomes, we found that ARG composition clustered samples into three habitats along the primary axis of anthropogenicity (Figure 1a) from undisturbed natural habitats, to wastewater treatment plants (WWTPs), to feces. Through a literature review of 30 studies, we observed a 100-fold difference ($p < 0.01$ by kw-test) in the total concentration of antibiotics along the anthropogenicity axis (Figure 1b), posing a potential selection for the transmission and evolution of ARGs^{21–25} that increases bacterial fitness in human-related environments. These observations guided the framework that the anthropogenic impact (i.e., via antibiotics) primarily shaped the ARG composition, and should be assessed first to evaluate ARG risk. Yet the majority of ARGs^{26 27} were not impacted by anthropogenicity with no significant difference across habitats ($p > 0.05$ by kw-test, Figures 1b and S2a). This indicates that the genes we classify as ARGs probably have other primary functions in the environment²⁸. Thus, we prioritize ARGs that are: enriched in anthropogenic environments (Figures 1b and S2b); are more likely to deal with clinically relevant antibiotics; and could be controlled by public health interventions that limit antibiotic usage.

Our risk framework reflected the evolution and emergence of ARGs into human pathogens driven by antibiotics selection (Figure 2a). After assessing the anthropogenic enrichment, we prioritized mobile ARGs that are usually specialized resistance loci responding to antibiotics^{21,29} and are more capable of transferring between lineages³⁰⁻³⁴. We further prioritized ARGs carried by human pathogens, since this indicates a potential ineffective treatment of diseases by antibiotics. Based on these properties, we designed a risk framework that sequentially assessed three criteria (Figure 2b): Namely: being 100-fold more abundant in human dominated ecosystems than other ecosystems; being carried via mobile genetic elements (MGEs); and being resident in pathogens (see supplementary information and Figure S3 for analyses). We ranked all 4,050 ARGs in the Structured ARG Database⁸ (Table S2) and obtained relevant data by searching the ARGs in all available bacterial genomes and plasmids from NCBI, MGEs databases^{35,36}, and 854 global metagenomes by the ARGs Online Searching Platform³⁷. We found that 75% ARGs were not significantly responsive to anthropogenic impact (Rank IV) and were generally non-mobile (83%) and 5-10 fold more abundant in nature (Figure S4). Of all the anthropogenically enriched ARGs, 80% were non-mobile as Rank III ARGs. Rank III largely represented the intrinsic resistance evolved in pathogens as 80% of Rank III ARGs were hosted only by pathogens (50% by ESKAPE pathogens). We found that most mobile ARGs (132 of 145) were already carried by human pathogens as top risk Rank I ARGs. We consider Rank I as an immediate and current threat because they have several dangerous characteristics, such as notable host pathogenicity (93% in ESKAPE

pathogens); wide host range (98% across several genera); and highly prevalent in impacted environments (14%) (Table S2). We also found 13 mobile ARGs that have not yet been carried by any pathogen (Rank II), which could pose a future threat through a potential contact and transfer into a pathogen.

The ranking framework accurately identified 95% of all well-known high risk ARG families as Rank I ARGs and expanded the list by 43 unreported Rank I ARG families. We compared our results against a list of 38 ARG families that were reported to have high clinical concern (to cause treatment failure of healthcare associated infections and/or have been wide-spread phylogenetically and geographically) by the WHO and by literature review (highlighted in purple)^{17,19,20,22,38–47} (Figure 3). For example, we included extended-spectrum beta-lactamases (ESBL) ARGs (i.e., *TEM*, *SHV*, and *CTX-M*), that recently caused the death of fecal microbiota transplantation (FMT) recipients. Our framework properly identified 36 as Rank I and two as Rank IV. Both Rank IV ARGs (*sul1*, *vanA*) met the requirements of “mobility” and “pathogenicity”, but they were 5-20 fold more abundant in nature. More importantly, we automatically identified 43 Rank I ARG families that have not been reported as high risk in previous studies, but which exhibited the same features as frequently reported ARGs. Some of them have shown a strong clinical relevance (such as *IMP-4*, *OXA*^{47–49}) and should also be prioritized for interventions. Rank I ARGs had a wide host range, in that 80% were shared across species and 60% across genera (same sequence variant), while 50% of Rank II-IV ARGs had a narrow host range within single species. We found

homologs of one ARG family tended to pose different risks, different host range, and different ecological distributions. Usually only a few Rank I homologs covered a broad host range and others Rank I homologs shared a narrow subset (i.e., 14-16 genera compared to 1-3 genera for *tetM*). However, Rank II ARGs usually did not share the same host range with Rank I homologs (i.e., probiotic bacteria *Lactobacillus* for *tetM*). We also found Rank II ARGs without Rank I homologs and they were hosted by abundant gut commensals and/or close relatives to pathogens that could be a reservoir of new resistance for gut pathogens⁵⁰, especially *aadA* and *vatE* with a host range across two orders. Besides, Rank I-II homologs did not share the same host strain (founder effect) and have distinct sequence variants in different habitats (Figure S5), while multiple Rank III-IV homologs within one genome were quite common. Thus, research and surveillance into ARGs should be conducted at the resolution of their sequence variants, not by just documenting their ARG families.

We observed that Rank I was strongly correlated with the potential exposure of clinical antibiotics, while Rank II was associated with industrialized lifestyles. We applied the ranking framework to 1,120 human gut microbiome metagenomes (36 global populations **ref in preparation** and 85 healthy FMT donors⁵¹) to understand how industrialization impacts the risk of antibiotic resistance. Industrialization dominated over all other factors that shaped the ARG composition across populations (Figure S6). Thus, we classified populations into five levels from non-industrialized rural lifestyles to industrialized urban lifestyles (details in Figure 4).

We roughly estimated the potential exposure to clinical antibiotics considering the access to antibiotics and policy interventions on antimicrobial consumptions⁵²⁻⁵⁴.

Rank I was highly responsive to industrialization and revealed the same pattern with antibiotics exposure. In total of 57 of 65 Rank I ARGs presented this pattern individually (including *mcr-1* and all multidrug resistance), suggesting that this response was robust regardless of different host ranges and resistance mechanisms (Figure S8). Total Rank I was similarly abundant and diverse in non-industrialized rural populations (Level 1 with limited access with antibiotics) and highly industrialized populations (Levels 4-5 with policy interventions on clinical antimicrobial consumptions), and was significantly lower than middle industrialized populations (Levels 3-4 with access but limited interventions on clinical antimicrobial consumptions)⁵⁵. Moreover, Rank I showed a 5-10 fold lower abundance ($p < 0.01$ by kw-test) in FMT donors who had no antibiotic consumptions from 6 months before sampling (Figure 4a). Meanwhile Rank I could maintain its low abundance with low variance in four FMT donors over 150-550 days of surveillance (Figures 4c and S8). These observations suggest that Rank I could be mainly associated with the potential exposure of clinical antibiotics. In addition, we detected 6 Rank II ARGs commonly shared across populations. Three Rank II ARGs with Rank I homologs (*catA*, *ermB*, *tetM*) were loosely correlated with clinical antibiotics. Since they were not carried by pathogens, they could be under a less selection than their Rank I homologs that were carried by pathogens. Another three novel Rank II ARGs (*aadA*, *vatE*, *mdtM*) were found highly abundant in the highest industrialized population (Level 5). Overall, Rank II was more abundant in industrialized populations (Levels 4-5) than high-risk population

(pastoralist population that uses antibiotics in animal farms) and non-industrialized rural populations (Level 1), and most Rank II was less likely to get lost over time in FMT donors without antibiotic selection (Figure 4c). We also observed the transfer of lifestyles from farmers and hunter gatherers to industrialized lifestyles increased the Rank II by 3-10 fold (Figure 4b). Thus, Rank II seemed not to be correlated with clinical antibiotics but industrialized lifestyles. Instead, total ARGs displayed no significant difference across populations.

Discussion

We designed a risk-ranking framework to prioritize high risk ARGs through “anthropogenic enrichment”, “mobility”, and “host pathogenicity”. Our framework successfully identified 36 of 38 high risk ARG that have been reported (including *mcr-1*) and expanded the list by 43 unreported emerging Rank I ARGs. Management strategies should be evaluated and implemented on the basis of their ability to control these high risk ARGs. Moreover, the surveillance of high risk ARGs should target specific sequence variants since homologs (homologous ARGs) in the same ARG family pose different risks and different phylogenetic and ecological preference. The representativeness of available public datasets is the primary limitation of this study. With new sequencing data and clinical data, this ranking framework is expected to supervise the dynamic status of ARG risk and to identify future threats. Finally, to help strategize public health interventions, we studied the impact of industrialization and antibiotics exposure on high risk ARGs in global human gut microbiome. We found that current policy controlling the

clinical antimicrobial consumptions could effectively control Rank I (current threat)^{56,57} but not Rank II (future threat). We observed that antibiotics exposure could explain the pattern of Rank I, especially its relatively low diversity and abundance in industrialized populations with strict policy interventions on clinical antimicrobial consumptions⁵⁴. Besides, Rank I was maintained at a significantly low abundance in FMT donors with no antibiotic consumptions. These observations suggested that Rank I could strongly and rapidly respond to clinical antibiotics⁵⁶ and was effectively controlled by current policy in developed countries⁵⁴ (such as therapy guidelines, antibiotic formularies, antibiotic stewardship programmes, and public health interventions⁵⁸). However, we found that Rank II was not directly correlated with the clinical antibiotics and was not observed to be effectively controlled in industrialized countries. The fact that novel Rank II ARGs were usually carried by abundant gut commensals and/or close relatives to gut pathogens, indicated their high potential and consequences to emerge into gut pathogens. We should take actions with new intervention strategies, such as controlling the usage of veterinary antibiotics as growth promoters⁵⁹ (i.e, *vatE*)^{60,61}.

Methods

Details of methods, data and scripts are all available in supplementary information. Briefly, the ARGs Online Searching Platform³⁷ provided the presence and abundance of ARGs in 54,718 all available NCBI bacterial genomes ($\geq 50\%$ completeness and $< 10\%$ contamination) and 854 global metagenomes of Illumina shotgun sequencing. We further searched ARGs in all available NCBI

15,738 plasmids and other mobile genetic element (MGE) databases^{35,36} by usearch v11.0, diamond 0.9.24 and blast 2.5.0+⁶²⁻⁶⁴. The search cutoff was set for genomes and MGEs as e-value 1e-5, 90% aa similarity over 80% aa hit length; and for metagenomes as e-value 1e-7, 80% aa similarity over 75% aa hit length^{7,8,37,65}. The abundance of ARGs was normalized into copy of genes per bacterial cell (ARGs-OAP^{7,8}) using Equation 1. The total bacterial cell number of one metagenomic sample was inferred by counting the average copy number of bacterial essential single copy genes.

$$abu_{ij} \text{ of } ARG_i \text{ in sample}_j = \frac{Reads_{number_{ARG_i}} / Length_{ARG_i}}{Bacterial_cell_number_{sample_i}} \quad (1)$$

We developed a bioinformatics tool (arg_ranker) to assess the risk of ARGs in metagenomes and genomes (https://github.com/caozhichongchong/arg_ranker). The arg_ranker classifies all ARGs in one sample into Rank I-IV and quantifies the risk contributed by each Rank.

We used 460 human gut microbiome metagenomes from 460 donors covering 36 different populations of 9 lifestyles (ref in preparation) and 560 metagenomes from 84 FMT donors⁵¹. The FMT metagenomes consisted of 400 metagenomes of four donors with intensive sampling (231 samples over 536 days, 83 samples over 375 days, 70 samples over 201 days, and 90 samples over 144 days) and 160 metagenomes for 80 donors with sparse sampling (2 samples per individual over 2 to 460 days). We classified the populations into five

industrialization level and we roughly estimated the potential exposure to antibiotics based on the access to antibiotics and public health interventions on clinical antimicrobial consumptions of each population^{52–54}. FMT donors are healthy individuals with no antibiotic consumptions from six months before sampling, which represents the least potential exposure to antibiotics. The high-risk population is a pastoralist population that uses antibiotics in animal farms, which represents the highest potential exposure to antibiotics. Level 1: non-industrialized rural populations with limited access to antibiotics (fisherman, hunter gatherer to farmer, farmer, and hunter gatherer); Level 2: non-industrialized urban populations (farmer to western) with access to antibiotics and little policy interventions⁵⁴; Level 3: industrialized rural population with access to antibiotics and little policy interventions⁵⁴ (hunter gatherer to western); Level 4: industrialized rural populations (western) with access to antibiotics and policy interventions; Level 5: industrialized urban populations (western) with access to antibiotics and policy interventions. The relative abundance difference (abu_{diff}) of an ARG between 2 time-points in one individual was calculated using Equation 2.

$$abu_{diff} \text{ of } ARG_i \text{ between } t0 \text{ to } t1 = \frac{abs(abu_{ARG_{t1}} - abu_{ARG_{t0}})}{mean(aabu_{ARG_{t1}} - abu_{ARG_{t0}})} \quad (2)$$

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Author contributions

A.N.Z. developed the pipeline, analyzed the data, and wrote the manuscript. L.L. contributed suggestions in data analysis and manuscript preparation. C.D. and X.Y. contributed suggestions on data analysis and revised the manuscript. M.G. and M.P. processed and provided gut microbiome metagenomic data, contributed suggestions on data analysis, and revised the manuscript. W.P.H., J.M.T., and E.T. provided valuable encouragement, contributed valuable advice on the pipeline development, and revised this manuscript. E.J.A. and T.Z. guided the pipeline development, data analysis, and revised this manuscript.

Competing interests

The authors declare that they have no competing interests.

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