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This week's issue was organized by Guest Editor Yongning Wu.

Preplanned Studies

Dissemination of Antibiotic Resistance Genes Among Patients with Diarrhea — Freetown, Sierra Leone, 2018

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Summary

What is already known about this topic?

Antibiotic resistance (AR) is a serious public health threat worldwide. However, the AR and antibiotic resistance genes (ARGs) data from West Africa, especially from Sierra Leone, are limited.

What is added by this report?

The study revealed ARGs' common dissemination, and multiplex antibiotic resistance genes in one sample. Genes *bla*$_{NDM}$ and *bla*$_{OXA-48-like}$ were first discovered in Sierra Leone.

What are the implications for public health practice?

Basic information is provided for AR research and surveillance and highlights that effective AR surveillance among diarrhea patients is necessary for Sierra Leone and West Africa.

Antibiotic resistance (AR), a major public health problem in developed, developing, and undeveloped countries, has increased with rapid globalization. Due to logistical issues and a rapidly migrating population in Africa, AR has become a complicated issue for the general public. Although medical hygiene and public health system in Africa have improved, and the use of antibiotics has also increased in many African countries (*1*–*3*), AR has become a prevalent issue. Sierra Leone is categorized as one of the most undeveloped countries in the world with bacterial diarrhea as a common and major disease throughout the country. Antibiotics are an effective treatment option for this disease. Since the medical and public health system in Sierra Leone is still in its infancy, the available data related to AR are limited. Here, 17 antibiotic resistance genes (ARGs)/ antibiotic resistance gene (ARG) groups in the stool samples of 56 diarrhea patients were detected in Freetown, Sierra Leone, 2018. Nine ARGs/ARG groups were detected as positive, and most of the samples carried at least 2 ARGs/ARG groups. Two stool samples carrying 7 ARGs/ARG groups highlighted the complexity of ARGs in Freetown, Sierra Leone. Genes *bla*$_{NDM}$ and *bla*$_{OXA-48-like}$ are the first reported ARGs from Sierra Leone. The diversity and dissemination data of ARGs in Freetown of Sierra Leone are expected to complement the antibiotic resistance data of West Africa and highlight the need for continued monitoring of antibiotic resistance.

A total of 56 acute diarrheal stool samples were obtained from six sentinel hospitals of Freetown, Sierra Leone between May 2018 and December 2018 (Table 1). Most samples were collected in July and October (n=10, each), while only two samples were collected in May and December.

A total of 17 ARGs/ARG groups, including 249 genes types/subtypes, were detected using the probe method of real-time PCR. The real-time PCR primers and minor groove binder (MGB)-conjugated fluorescent probes used were reported elsewhere (*4*), except the *tet*(A) gene. The *tet*(A) gene was detected using the forward primer (5'-CAT TCT GCA TTC ACT CGC CCA GGC AAT GAT-3'), reverse primer (5'-GAA GCA AGC AGG ACC ATG ATC GGG AAC GC-3'), and the 6-carboxyfluorescein (FAM)-labeled *tet*A-specific probe (5'-GAT TGC CGA CGG CAC AGG CTA CAT CCT GCT TG-3').

Seventeen ARGs/ARG groups were detected among the 56 diarrhea stool samples, and the ARG positive detection rates ranged from 0 to 92.9% (Table 2). *int*I1, IS*CR*1, *bla*$_{CTX-M}$ E groups, and *tet*A gene positive rates were over 50%. Eight ARGs/ARG groups were not detected, including *bla*$_{CTX-M}$ A, *qnr*S, *aac*(6')-Ib-cr, *cfr*, *fex*A, *mcr*-1, *arm*A, and *aac*(6')-Ie-*aph*(2')-Ia. At least one ARG/ ARG group was detected from all of the stool samples, and most samples showed ARG/ ARG group coexistence. Forty-two (75%) of the stool samples carried more than two ARGs/ARG groups, and two samples carried seven ARGs/ARG groups. Seventeen different ARG coexistent types were detected. Carbapenem resistance encoding genes *bla*$_{NDM}$ and *bla*$_{OXA-48-like}$ were also detected. *bla*$_{NDM}$ and *bla*$_{SHV}$ A genes coexisted in some stool samples.

TABLE 1. Information of diarrheal stool surveillance samples collected from six sentinel hospitals in Sierra Lenoe, 2018.

Month of collection	Sample number	Sentinel hospital
May	2	MHOS (2)
June	9	SZ (1), LH (8)
July	10	SZ (1), LH (3), EH (6)
August	9	SZ (6), LH (3)
September	5	SZ (3), EH (2)
October	10	LH (3), WH (5), RH (2)
November	9	SZ (8), LH (1)
December	2	SZ (1), LH (1)
Subtotal	56	MHOS (2), SZ (20), LH (19), EH (8), WH (5), RH (2)

Abbreviation: SZ=Sierra Leone-China Friendship Hospital; LH=Lumley Hospital; EH=Emergency Hospital; WH=Waterloo Hospital; RH=Rokupa Government Hospital; MHOS=Ministry of Health of Sierra Leone.

TABLE 2. Positivity information of ARGs/ARG groups detected by real-time PCR in 56 acute diarrheal stool samples from Freetown in Sierra Leone, 2018.

ARG	Number of positive samples (%)	ARG	Number of positive samples (%)
bla_{NDM}	6 (10.7)	*cfr*	0 (0)
bla_{CTX-M} A	0 (0)	*fex*A	0 (0)
bla_{CTX-M} E	32 (57.1)	*mcr*-1	0 (0)
bla_{SHV} A	9 (16.1)	*arm*A	0 (0)
$bla_{OXA-48-like}$	1 (1.8)	*aac*(6')-Ie-*aph*(2')-Ia	0 (0)
bla_{PER}	1 (1.8)	*tet*A	44 (78.6)
*qnr*A	1 (1.8)	*int*I1	52 (92.9)
*qnr*S	0 (0)	IS*CR*1	30 (53.6)
aac(6')-Ib-cr	0 (0)		

Abbreviation: ARG=antibiotic resistance gene.

IS*CR*1 and *int*I1 genes always coexisted with other ARGs/ARG groups. bla_{NDM} gene group coexisted with more than four other ARGs/ARG groups and was detected in five stool samples. Gene group $bla_{OXA-48-like}$ was the first group to be observed in Sierra Leone. Further details of ARGs/ARG groups' coexistence are summarized in Table 3.

DISCUSSION

From this short-term surveillance, serious AR has already been noticed, with multidrug resistance and first reported carbapenemase-encoding genes bla_{NDM} and $bla_{OXA-48-like}$ from Sierra Leone.

Isolating bacteria from stool samples is a complex process that includes the probability of bacterial culture having some variable and uncontrollable parameters. In addition, due to the long purchasing period of ordering and receiving special reagents in Sierra Leone, many bacteria cannot be isolated and studied for AR. Considering the existing non-affluent laboratory infrastructure and equipment of Sierra Leone's lab, ARGs/ARG groups were directly detected from the stool samples by real-time PCR.

Previous studies about the rapid detection of ARGs by real-time PCR among samples are limited. These studies mainly reported ARG detection among isolates. For instance, a report from Tunisia reported that class 1 integrons, bla_{TEM}, $bla_{CTX-M-1}$, bla_{OXA-1}, and bla_{CMY-16} were positive in *Klebsiella pneumoniae* isolates, and the bla_{OXA-48} gene was associated with many other ARGs (*5*). A study from Egypt demonstrated a high prevalence of resistance to β-lactam antibiotics through ESBLs and AmpC β-lactamases production among the *Acinetobacter baumannii* isolates (*6*). Rapid detection of ARGs by real-time PCR may reveal the existence of ARGs/ARG groups in diarrhea patients more rapidly and objectively. Our study provided a convenient and accurate method for AR routine surveillance in Sierra Leone, Africa.

TABLE 3. Coexistence of ARGs/ARG groups in 56 acute diarrheal stool samples from Freetown in Sierra Leone, 2018.

Number of ARGs	Coexisting ARGs/ARG groups	Number of samples	Total number of samples
1	*int*I1	6	6
2	$bla_{\text{CTX-M}}$ E-*int*I1	3	8
	*int*I1-IS*CR*1	2	
	*tet*A-*int*I1	3	
3	$bla_{\text{CTX-M}}$ E-bla_{SHV} A-*tet*A	2	18
	$bla_{\text{CTX-M}}$ E-*int*I1-IS*CR*1	1	
	$bla_{\text{CTX-M}}$ E-*tet*A-*int*I1	4	
	bla_{NDM}-*int*I1-IS*CR*1	1	
	*tet*A-*int*I1-IS*CR*1	10	
4	bla_{CTX} E-*tet*A-*int*I1-IS*CR*1	6	14
	$bla_{\text{CTX-M}}$ E-bla_{SHV} A-*tet*A-*int*I1	2	
	$bla_{\text{CTX-M}}$ E-*tet*A-*int*I1-IS*CR*1	5	
	bla_{SHV} A-$bla_{\text{OXA-48 like}}$-*tet*A-*int*I1	1	
5	$bla_{\text{CTX-M}}$ E-bla_{SHV} A-*tet*A-*int*I1-IS*CR*1	3	6
	bla_{NDM}-bla_{CTX} E-*tet*A-*int*I1-IS*CR*1	1	
	bla_{NDM}-$bla_{\text{CTX-M}}$ E-*tet*A-*int*I1-IS*CR*1	2	
7	bla_{NDM}-bla_{CTX} E-bla_{PER}-*qnr*A-*tet*A-*int*I1-IS*CR*1	1	2
	bla_{NDM}-$bla_{\text{CTX-M}}$ E-bla_{SHV}A-*qnr*S-*tet*A-*int*I1-IS*CR*1	1	
	Subtotal		56

Abbreviation: ARG=antibiotic resistance gene.

Some highly prevalent ARGs/ARG groups were previously reported from Africa, such as *tet*(A), *int*1, and $bla_{\text{CTX-M}}$ E. A study that included patients with clinical features of healthcare-associated infections in an urban tertiary hospital in Sierra Leone showed a resistance rate of 1.3% for carbapenem-resistant Enterobacteriaceae but did not reveal related ARGs (*7*). Carbapenemase-encoding genes bla_{NDM} and $bla_{\text{OXA-48-like}}$ were the first reported in Sierra Leone, West Africa. Some investigators have isolated carbapenem-resistant Enterobacteriaceae isolates carrying bla_{NDM} and $bla_{\text{OXA-23}}$ genes from various east-African countries including Kenya, Uganda, Tanzania, Ethiopia, and Rwanda (*2*). The ARGs in our collected stool samples included tetracycline-, fluoroquinolones-, carbapenem-resistance genes, ESBLs and integrase 1 encoding genes. The findings of this study are consistent with those of previously published studies and demonstrate that carbapenemase genes are disseminated in West Africa. Many common ARGs/ARG groups may also spread in Sierra Leone, which could reduce the effectiveness of anti-infective treatment, accelerating the spread of AR. The presence of the carbapenem-resistance gene along with other ARGs in stool samples is critical and may result in the dissemination of ARGs.

Coexistence of multiple ARGs/ARG groups was discovered in the tested samples. 89.3% (50/56) and 75.0% (42/56) of the samples carried more than two and more than three groups of ARGs, respectively. Two stool samples carried seven types of ARGs, simultaneously. Six stool samples carried a single *int*I1 gene that could contribute to ARGs transmission and integration (*5*). The fact that no other ARGs/ARG groups were detected simultaneously might be because of the limited ARG groups screened in this study. Detection of more ARGs should be the focus of future studies. This study found the diversity and complexity of ARGs in stool samples obtained from Freetown, Sierra Leone. Previous studies demonstrated that multidrug resistance had spread in some African countries. A study in Ghana found that >50% and 7.3% of the *Escherichia coli* isolates obtained from pregnant women were positive for bla_{TEM} and *aph*(*3*)-Ia, respectively (*8*). A study from Egypt revealed that 75.4% of the tested gram-negative isolates harbored at least one extended-spectrum β-lactamase-encoding gene — $bla_{\text{CTX-M}}$, and bla_{SHV} (*9*). In Tunisia, an extensively drug-resistant clinical isolate of *Proteus mirabilis* carried plasmid-mediated resistance to

carbapenems (*bla*$_{NDM-1}$), cephalosporins (*bla*$_{CMY-4}$), aminoglycosides (*aph*3-VIa and *aph*3-Ia), and fluoroquinolones (*qnr*A6) (*10*). The dissemination of ARGs in Africa is alarming and should be given more attention in routine surveillance.

Our investigation revealed that the common types of ARGs detected in diarrheal stool samples collected in this study included *tet*(A), *int*1, IS*CR*1, and *bla*$_{CTX-M}$ E. These ARGs might be related to the use or misuse of the above antibiotics. Although no carbapenems antibiotics were available for sale in any hospital or pharmacy during our investigation, carbapenemase-encoding genes *bla*$_{NDM}$ and *bla*$_{OXA-48-like}$ were detected in the diarrheal stool samples. The findings of this study provide the direction of research for future studies. By expanding on the origins of sample collection, types of ARGs screened, and antibiotics investigated in Sierra Leone, further detailed insights can be obtained about the dynamics of AR in Sierra Leone.

This small-scale study revealed AR present situation among a panel of diarrheal stool samples collected from Sierra Leone, but was also subject to some limitations. First, only 56 of stool samples from several public sentinel hospitals in Freetown were involved in this study, because of restrictions for medical technology, sample transportation, and socioeconomic and behavioral factors. Second, limited ARGs were detected here because of long purchase period of special reagents in Africa. AR informantion of *int*1, IS*CR*1, *tet*(A), *bla*$_{CTX-M}$ E common dissemination, *bla*$_{NDM}$ gene existence, and multiplex ARGs in one sample, provides basic information for AR research and surveillance and highlights that continued effective AR surveillance is necessary for Sierra Leone and West Africa.

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Commentary

National Antibiotic Resistance Strategy for Human Health in France

Céline Pulcini[1]; Anne Berger-Carbonne[2]; Bruno Coignard[2]; Jérôme Salomon[3]

France is a country with 67 million inhabitants and is part of the European Union. Antibiotic consumption in humans is higher in France as compared to the European average, especially in outpatient settings (*1*–*2*). The burden of antibiotic resistance is also high, as compared to the European average, with an estimated 5,500 deaths per year associated with multidrug-resistant bacterial infections (*3*). Encouraging results have, however, been observed over the last several years: antibiotic consumption in the community, in nursing homes and in hospitals showed a decreasing trend, as well as certain resistant bacteria, such as 3rd-generation cephalosporin- or fluoroquinolone-resistant *Escherichia coli* (*1*–*2*,*4*–*5*). The COVID-19 pandemic has had a dramatic impact on antibiotic consumption and its long-term impact on antibiotic resistance is being carefully monitored (*1*).

Antimicrobial resistance (AMR), in particular antibiotic resistance, is a long-standing priority for the French government. Action plans in human health coordinated by the Ministry of Health have been in place for more than 20 years, while plans in animal health have been coordinated by the Ministry of Agriculture since 2012 (*6*). The first Interministerial Committee for Health was dedicated to AMR, with a specific focus on antibiotic resistance. In November 2016, the French government adopted the Antibiotic Resistance Interministerial National Action Plan, using a One Health approach (*7*). This action plan is organized around five objectives: 1) raising awareness among the general public and healthcare professionals; 2) education; 3) research and innovation; 4) monitoring and surveillance; and 5) interministerial and international action governance. Regular One Health brochures are published, presenting a brief overview of the implemented initiatives and their impact (*1*,*8*).

The most recent sectorial operational plan in human health was released by the Ministry of Health in February 2022: the 2022–2025 national strategy for preventing infections and antibiotic resistance in human health (*9*). The synergy between antibiotic stewardship and infection prevention and control activities is underlined, encompassing the prevention of viral and bacterial community-acquired and healthcare-associated infections. A series of performance indicators are included, with defined targets, to monitor progress at national and regional levels.

We present below a selection of some actions conducted in human health, that might be of interest to an international readership. For more information on the other actions, annual reports and summary brochures are regularly published and available online (*8*,*10*).

RAISING AWARENESS AMONG THE GENERAL PUBLIC AND HEALTHCARE PROFESSIONALS

Santé publique France (Public Health France) has developed a nationwide communication campaign that started this year targeting healthcare professionals and the general public. These actions will continue during the coming years. The aim of this campaign is to raise awareness among the public and healthcare professionals about the importance of antibiotic stewardship and to empower them to take action. Several preliminary studies have been carried out to better define targets and key messages to change general public and healthcare professionals' behavior. This campaign will be based in particular on the Antibioclic platform (*11*), a therapeutic decision-support tool in antibiotic therapy for healthcare professionals, and Antibio'Malin (*12*), a thematic online space containing practical information for everyone.

Launched in November 2019 on the Santé.fr website of the Ministry of Health, the Antibio'Malin platform offers the general public short and simple thematic information describing all the antibiotics prescribed by healthcare professionals in the community, as well as the most frequent infections

(*12*). It aims to inform everyone on the subject, giving them the means to act individually to prevent antibiotic resistance. Healthcare professionals can also use this resource in their communication with patients.

EDUCATION

Understanding antimicrobial resistance and its drivers is of utmost importance for a sustainable effect of prevention campaigns and measures. Contributing to the teaching of children and teenagers, E-Bug is an online educational resource, initially developed as part of a European project (*13*). It details micro-organisms, common infections, hygiene, and the use of antibiotics and vaccines. E-Bug offers free and fun tools, regularly updated, to facilitate the teaching of infections and antimicrobial resistance to children and teenagers at school, using a One Health approach. This platform is supported by several French ministries and agencies and is available to teachers, with additional resources also targeting parents.

In order to strengthen the training of healthcare students and contribute to raising awareness of the public, the theme of "preventing infections and antibiotic resistance," from a One Health perspective, is now a national priority of the Health Service for Health Students. The introduction of a health service for all health students (medicine, pharmacy, odontology, maieutics, nursing, and physiotherapy) is part of the national health strategy, whose first axis is to implement a policy of prevention and health promotion in their interdisciplinary curriculum.

Funded under the Priority Research Program on Antibiotic Resistance, the PROMISE project is a One Health professional meta-network on antibiotic resistance, bringing together 21 national networks and over 40 academic partners (*14*). One of the main objectives consists in the creation of initial training modules involving veterinarians, medical students, and pharmacists. These training modules have a One Health approach and aim to build bridges between the different scientific communities and reinforce prevention practices by all health professionals.

RESEARCH AND INNOVATION

In 2020, the Priority Research Program on antibiotic resistance was launched, with a funding of 40 million euros, coordinated by the Inserm national research agency (*15*). This program uses a One Health perspective and focuses on three main cross-cutting actions: 1) developing and creating platforms, networks, and observatories dedicated to antibiotic resistance; 2) strengthening research teams through calls for expressions of interest or interdisciplinary calls for projects and human resources; and 3) coordinating a research network on antibiotic resistance for countries with limited resources. Intended for the academic scientific community and industry stakeholders, the related National Antibiotic Resistance Interface website (part of the Priority Research program on antibiotic resistance) is intended to be a common, intersectoral, and interactive entry point, identifying public and private actors, platforms and networks, coordinating and animating activities, and listing research and innovation projects focused on antibiotic resistance (*15*).

ENSURING ACCESS TO EXISTING ANTIBIOTICS

A three-year project "Ensure the availability of antibiotics," as part of the Technical Support Instrument co-financed by the European Union, started in November 2020. The aim of the project is to identify and implement pilot measures in France to tackle the root causes of the lack of availability and shortages of off-patent antibiotics used in human and veterinary medicine. The European Commission's Directorate General for Structural Reform Support (DG REFORM) and the WHO provide technical assistance to the French Government with the participation of five ministries and two national agencies (*16*). The first public report presenting the most suitable measures for addressing the root causes of shortages in the human and veterinary sectors will be released soon (*16*).

ANTIBIOTIC STEWARDSHIP AND INFECTION PREVENTION AND CONTROL

In August 2021, the High Council for Public Health (HCSP) was asked by the Ministry of Health to produce scientific recommendations on basic actions everybody could implement in everyday life to prevent common infections. These recommendations are expected in early 2023. They will be used to guide future awareness activities.

Since 2021, access to rapid diagnostic tests for sore

throat is facilitated in community pharmacies. These tests have already been made freely available to medical doctors for many years. They make it possible to determine the viral or bacterial origin of a sore throat in a few minutes, thanks to a throat swab taken by a doctor or a pharmacist. More than 80% of sore throats are viral and do not require antibiotics (*17*). Rapid sore throat tests, therefore, allow antibiotics to be taken only when necessary and preserve their effectiveness.

The national strategy is implemented at the regional level in the three healthcare sectors (hospitals, medico-social facilities and services, and the community) by the Regional Health Agencies (ARS), which are responsible for mobilizing all regional actors involved in the topic. Two types of regional centers provide support to the ARS. To address infection prevention and control (IPC), the centers for the prevention of healthcare-associated infections (CPias) are responsible at the regional level for the prevention of healthcare-associated infections and the control of cross-transmission of infectious agents. They provide expertise and support and run networks of IPC professionals (IPC teams in hospitals and nursing homes). On the other hand, the regional centers for antibiotic stewardship (CRAtb) carry out regional missions of expertise and support, including a strategic mission on antibiotic stewardship, and animation of networks of health professionals in charge of the antibiotic stewardship activities (multidisciplinary teams and referents).

As detailed below, five national surveillance and prevention missions for healthcare-associated infections and antibiotic resistance are coordinated by Santé publique France. The scope of these national missions covers the entire patient healthcare pathway: community, nursing homes, and hospitals. These missions produce not only surveillance data, but also prevention, training, and communication tools for professionals and the general public.

MONITORING AND SURVEILLANCE

The French national public health agency (Santé publique France) is in charge of a national notification system for uncommon healthcare-associated infections and emerging antimicrobial-resistant strains. Every five years, it conducts a national point-prevalence survey on healthcare-associated infections and antimicrobial treatments in hospitals and nursing homes, according to the European Center for Disease Prevention and Control (ECDC) methodology.

In addition, the above-mentioned French network for the prevention of healthcare-associated infections and antibiotic resistance gathers five national missions, coordinated by Santé publique France (*18*). The objectives of these missions are to support the national agency in producing surveillance data on healthcare-associated infections, antibiotic use and resistance, and to produce or make available to the regional IPC and antibiotic stewardship regional centers mentioned before infection prevention and control and media communication tools; the scope will be extended to antibiotic stewardship in 2023. This network contributes to the European surveillance system coordinated by the ECDC.

In order to assess One Health collaborations between surveillance systems in human health, animal health, and the environment, the Surv1Health project has been conducted by the ANSES national agency, in collaboration with Santé publique France (*19*).

INTERNATIONAL ACTIONS

The 2017–2021 European Joint Action on antimicrobial resistance and healthcare-associated infections was a joint action of the European Union (EU) on antimicrobial resistance and healthcare-associated infections, coordinated by France. It brought together 44 partners and 45 stakeholders (e.g., ECDC and OECD) and encouraged synergies between EU Member States in developing and implementing effective health policies to combat the growing threat of antimicrobial resistance and reduce healthcare-associated infections. This joint European action has also facilitated the exchange of best practices and discussion among policymakers to improve the implementation of national action plans. A summary of the main actions taken and recommendations made is available online (*20*).

A One Health ministerial conference on antimicrobial resistance was organized by France in March 2022, within the French Presidency of the Council of the European Union (*21*). The aim was to assess the European Union's progress on this major public health issue and identify areas for improvement and explore the unmet needs at the European level.

In conclusion, we have presented a brief overview of the French strategy to tackle antibiotic resistance in human health that is part of a larger interministerial One Health action plan. Some progress has been made, but room for improvement is still there and the efforts must be maintained and reinforced, in line with the

European and international contexts.

Conflicts of interest: No conflicts of interest.

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Perspectives

Current and Future Landscape of the Antimicrobial Resistance of Nosocomial Infections in China

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ABSTRACT

The rapid increase in antimicrobial resistance driven by the widespread use, abuse, and misuse of antibiotics constitutes one of China's most challenging healthcare problems. In particular, nosocomial infections caused by multidrug-resistant organisms such as methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Acinetobacter baumannii* (CRAB), and carbapenem-resistant Enterobacterales (CRE), which exhibit resistance to most available antibiotics, lead to high mortality and enormous economic and human costs. Here, we summarize the current patterns of the antimicrobial resistance of nosocomial infections in China and address possible interventions to combat antimicrobial resistance.

ANTIMICROBIAL RESISTANCE PATTERNS AMONG SIGNIFICANT CLINICAL PATHOGENS IN CHINA

China's most common hospital-acquired infections are bloodstream infections (BSIs) and hospital-acquired pneumonia (HAP). The proportion of gram-negative isolates was higher than that of gram-positive isolates for both BSIs and HAP, according to data from the Chinese Antimicrobial Resistance Surveillance of Nosocomial Infections (CARES), a national surveillance program monitoring the antibiotic resistance profiles of pathogens causing hospital-acquired infections in China (*1*–*2*). Overall, *Escherichia coli* (29.21%) and *Klebsiella pneumoniae* (12.70%) were the most common BSI-causing pathogens, followed by *Staphylococcus aureus* (9.79%), *Acinetobacter baumannii* (7.03%), and *Pseudomonas aeruginosa* (6.33%) (*1*). For HAP, the most prevalent pathogens were *A. baumannii* (25.6%), *P. aeruginosa* (20.1%), *K. pneumoniae* (15.4%), *S. aureus* (12.6%), and *E. coli* (7.5%) (*2*).

Multidrug resistance of gram-negative bacteria, such as CRAB, carbapenem-resistant *P. aeruginosa* (CRPA), and CRE, presents a particularly critical problem in China. *A. baumannii* has a higher carbapenem resistance rate than *P. aeruginosa* and Enterobacterales, with a proportion of more than 50% among isolates in 2020 (*3*). Furthermore, the rates of resistance to imipenem and meropenem differed by region, with the highest in Henan Province (78.5%), Liaoning Province (69.1%), and Hubei Province (64.1%) (*3*). The carbapenem-resistant proportion of *P. aeruginosa* was relatively stable and the resistance rate was 18.3% in 2020. (*3*).

CRE is an emerging critical public health threat worldwide. Although the overall rates of resistance to carbapenems in Enterobacterales remain at approximately 10%, they continue to rise significantly. Carbapenem resistance rates in *E. coli* and *K. pneumoniae* were 1.6% and 10.9% in 2020, respectively (*3*). A wide variation in the prevalence of carbapenem-resistant *K. pneumoniae* (CRKP) was reported among different regions, with the highest proportion in Henan Province, Shanghai, and Beijing (more than 25%) (*3*). Most CRE strains produce carbapenemase, and *Klebsiella pneumoniae* carbapenemase-2 (KPC-2) is the most common carbapenemase, followed by New Delhi metallo-β-lactamase (NDM).

In China, methicillin-resistant *Staphylococcus aureus* (MRSA) is the main cause of nosocomial infections that increase hospitalization costs and length of stay. The proportion of MRSA in 2005 was 69%, which represented a very high prevalence. The prevalence, however, had steadily declined each year, reaching 29.4% in 2020 (*3*). Between 2005 and 2011, *S. aureus* ST239 was the predominant lineage in hospital infections in China, accounting for 50%–80.8% of MRSA isolates, but has subsequently been declining due to the replacement by ST59. ST59 is more virulent and has been increasing in prevalence since 2013 (*4*). Furthermore, vancomycin-resistant *Enterococcus* (VRE) was observed, with an isolation rate of 1% in 2020 (*3*).

EMERGING RESISTANCE TO LAST-RESORT ANTIMICROBIALS IN GRAM-NEGATIVE BACTERIA IN CHINA

Multidrug resistance has recently increased in prevalence, with novel and complex resistance mechanisms emerging. The increased prevalence of CRKP coproducing KPC-2 and NDM-1 is an example (*5*). Carbapenem-resistant hypervirulent *K. pneumoniae* has been reported worldwide in current statistics. Notably, this novel *K. pneumoniae* clone was more prevalent than previously assumed, especially among KPC-2-producing ST11, exhibiting an isolation rate rising from 2.1% in 2015 to 7.0% in 2017 (*6*).

Colistin and tigecycline, as well as the latest drugs ceftazidime-avibactam and cefiderocol, are considered the last-line antimicrobials against carbapenem-resistant gram-negative pathogens. The mechanism of colistin resistance mainly involves the modification of lipid A, and most of these modifications are chromosomally mediated by the inactivation of *mgrB*. The plasmid-mediated colistin resistance gene *mcr-1*, which has a prevalence of 10%–20% in animals and 1%–2% in clinical isolates, was discovered for the first time in China (*7*). Clinical settings have also shown the presence of *mcr* and carbapenemase genes, including bla_{NDM} and bla_{KPC}. Furthermore, clinical isolates have been reported to exhibit tigecycline resistance mediated by the *tmexCD1-toprJ1* or *tet*(X) plasmid. More seriously, the "super plasmids" carrying *tmexCD1-toprJ1* and *mcr-8* can cause resistance to both tigecycline and colistin (*8*). Before ceftazidime-avibactam and cefiderocol were clinically available in China, the resistance had already developed in Enterobacterales. Ceftazidime-avibactam resistance was largely attributed to the production of metallo-β-lactamases, the $bla_{\text{KPC-2}}$ mutation, or high expression, whereas carbapenem-resistant *E. coli* exhibits cefiderocol resistance through the *cirA* inactivation combined with PBP3 mutation (*9*–*10*).

INTERVENTIONS AND STRATEGIES TO COMBAT ANTIMICROBIAL RESISTANCE

Diagnosis and Management of Infections Caused by Multidrug-Resistant Bacteria

The antibiotic resistance crisis is expected to be alleviated with the integration of advanced multidisciplinary technologies, including genome sequencing and metagenomics, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, spectroscopy, microfluidic technology, biosensing technology, and artificial intelligence. Metagenomic next-generation sequencing (mNGS) is used increasingly frequently in clinical laboratories for culture-independent diagnosis, particularly for identifying uncommon, novel, challenging-to-culture, and coinfected pathogens. It also has excellent potential for resistance prediction by analyzing antibiotic resistance genes. In addition to mNGS being considered a last resort to address clinical infection issues by some physicians, several obstacles, such as workflow validation, quality control, method standardization, and data interpretation, still need to be overcome (*11*); otherwise, mNGS results can mislead the diagnosis and management of infections in clinical settings. Utilizing the "Five Rights" — the Right patient, the Right drug, the Right dose, the Right route, and the Right time — is one of the recommendations made to reduce medication errors, harm, antimicrobial resistance, and hospital expenses.

Furthermore, to combat antimicrobial resistance in China, the National Health Commission (NHC), as well as academic committees and associations, have published a series of Regulations, Notices, Standards, Guidelines, and Consensus to strengthen the prevention and control of multidrug-resistant organism (MDRO) infections in hospitals. Namely, handwash protocols, environment management, patient isolation, aseptic techniques, and rational use of antimicrobial agents. Over the past three decades, China has made significant strides in infection control for MDROs with the help of the antimicrobial stewardship program.

Establishment of the Surveillance System for Antimicrobial Resistance in Hospitals

Several multicenter surveillance systems, mainly based on the susceptibility patterns of clinical bacterial and fungal strains, have been established to monitor the resistance profiles and dynamics of clinical strains, especially MDROs of concern in China. A national surveillance network, the China Antimicrobial Resistance Surveillance System (CARSS), was established by the former Ministry of Health (now known as the National Health Commission, NHC) in 2005 for hospital antimicrobial resistance surveillance, and until recently, over 1,400 centers in 31 provincial-

level administrative divisions (PLADs) throughout China have participated. However, most surveillance systems are strain- and laboratory-based and focus on the microbiological features of MDROs in China, and few have examined the patient-based disease burden in China.

In addition to conventional approaches for resistance phenotype surveillance, novel technologies, such as whole-genome sequencing (WGS) and culture-independent diagnostic tests (CIDTs), are providing new ideas for the surveillance of antimicrobial resistance. WGS can quickly screen for basic information, such as bacterial genus, species, and serotypes, and trace and screen resistance genes. Moreover, WGS can be used to find new drug resistance genes after testing for phenotypic susceptibility of some isolates or even genes conferring resistance to drugs that are not under surveillance.

'One Health' Approach to Combating Antimicrobial Resistance in China

Antibiotic abuse and misuse in humans, animals, and environmental reservoirs are the main triggers of the emergence of antimicrobial resistance. The overuse of antimicrobials in agriculture has made China one of the hotspots of antimicrobial resistance in the world (*12*). Colistin was one of the first effective antibiotics against gram-negative bacteria, and it was replaced due to its nephrotoxicity and neurotoxicity in the 1970s. However, the quantity of colistin used in agriculture was high in China. To combat the spread of resistance to colistin, particularly plasmid-mediated resistance by *mcr-1*, China banned the use of colistin as an animal feed additive. It has been demonstrated that complete reductions in colistin use significantly reduced the *mcr-1* prevalence, suggesting the effectiveness of colistin stewardship in reducing colistin resistance in both livestock and humans (*13*).

Furthermore, in recent years, an increasing number of investigators have recognized the mutual transmission of resistant bacteria and resistance genes among humans, animal host populations, and the environment (i.e., soil, water, and air). When anthropogenic factors cause a significant increase in antibiotic content in the environment, a large decrease in the population of susceptible bacteria results, and this unintended artificial selection simultaneously facilitates the survival of bacteria with antibiotic resistance genes and mutations. Considering the tight and interconnected association among human, animal, and environmental elements, it is essential to take the 'One Health' approach to integrate multisectoral and multidisciplinary efforts to address the serious problem of antimicrobial resistance.

Basic Research on Resistance and Transmission Mechanisms

China is challenged by the rapid dissemination of antimicrobial resistance genes (ARGs) and the remarkable development of multidrug-resistant bacteria. It is crucial to examine the mechanisms of resistance transmission to prevent, control, and eradicate the spread of antimicrobial resistance. Mobile genetic elements (MGEs), which include insertion sequences (IS), transposons (Tn), integrons (In) as well as genetic elements such as plasmids and integrated conjugative elements that can move between bacterial cells, cooperate in causing drug resistance. These components are essential for promoting horizontal genetic exchange, which aids in spreading and acquiring resistance genes in these strains (*14*). Furthermore, phages can transfer genetic material without the requirement for direct contact with bacteria, facilitating the development of bacterial resistance and the transfer of resistance genes. The prevalence of numerous ARG-carrying phages in the environment has been demonstrated, indicating that phages can act as environmental carriers for the horizontal transfer of ARGs. Gene transfer agents (GTAs) are phage-like particles containing DNA produced by particular bacteria and archaea (*15*). GTAs are a novel strategy for HGT and seem to be a cross between phage transduction and organic transformation. The distribution of virulence and resistance genes is one way that GTAs may influence bacterial evolution and genome plasticity.

New Antibacterial Therapy Strategies

Peptide treatment, immunotherapy, and phage therapy are emerging therapeutics for antimicrobial resistance. Antimicrobial peptides primarily act to suppress or kill pathogenic microorganisms by processes such as cell wall and membrane destruction, induction of changes in metabolic enzyme activity, and host immunological control (*16*). The current issue, in which the progress of antibiotic development cannot keep pace with the increase in resistance to new antibiotics, may be resolved by the development of immunotherapy (*17*). The most commonly explored bacterial vaccines are whole-bacteria-inactivated or

attenuated vaccines, outer membrane vesicles, recombinant DNA, and capsular polysaccharide vaccines. The first recombinant *S. aureus* vaccine has been approved by China's State Food and Drug Administration for phase III clinical research (*18*). Furthermore, the phage can self-replicate and, when used therapeutically, improve the effectiveness of antibiotics. To increase clinical efficacy, several researchers in China have used phage cocktail therapy, the combination of phage with antibiotics, phage lyase, and antibacterial medications. Additionally, new antibacterial approaches include probiotic therapy, antibacterial nanotechnology, antibacterial photodynamic therapy, and gene editing technologies.

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Perspectives

Minimizing Risks of Antimicrobial Resistance Development in the Environment from a Public One Health Perspective

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ABSTRACT

Antimicrobial resistance (AMR) is a globally recognized crisis with meaningful engagement across humans, animals, and the environment as in the One Health approach. The environment is the potential source, reservoir, and transmission route of AMR, and it plays a key role in AMR development from the One Health perspective. Animal farming, hospitals, and the pharmaceutical industry are identified as the main emission sources in the environment. Minimizing emissions and determining antimicrobial emission limits are priorities in the containment of environmental AMR development. From the perspectives of environmental management and environmental engineering, some important actions to minimize risks of AMR development are summarized, including the recent progress in enhanced hydrolysis pre-treatment technology to control the development of antibiotic resistance genes (ARGs) during biological wastewater treatment. It is desirable to establish a holistic framework to coordinate international actions on the containment of environmental AMR development. To establish a community with a shared future for humanity, China should and could play an important role in international cooperation to cope with AMR challenges.

THE ROLE OF ENVIRONMENT IN AMR DEVELOPMENT FROM A ONE HEALTH PERSPECTIVE

Antimicrobial resistance (AMR) refers to microorganisms (i.e., bacteria, fungi, viruses, and parasites) that can become resistant to antimicrobials through a variety of mechanisms such as mutation or genetic exchange. The world is facing high rates of AMR — particularly antibiotic resistance — which threaten the core of modern medicine and the sustainability of an effective, global public health response to the enduring threat of infectious diseases. The response to the AMR crisis has been spearheaded through the One Health AMR Globe Action Plan (GAP) (*1*), which was developed by the World Health Organization (WHO) in close collaboration with the Food and Agriculture Organization of the United Nations (FAO) and the World Organization for Animal Health (WOAH). The One Health perspective emphasizes close connections among humans, animals, and the environment (*2*). It is frequently demonstrated that clinically important resistance genes or resistant bacteria could be disseminated among humans, animals, and the environment. For example, carbapenemase-producing *Escherichia coli* were reported to transmit among humans and backyard animals (*3*); *tet*(X)-variant genes were reported to disseminate from layer farms to manure-receiving soil and corresponding lettuce (*4*). The generation and spread of resistant bacteria and antibiotic resistance genes (ARGs) in the environment will have direct and immediate health implications for both humans and animals.

It has been recognized that the environment is the potential source, reservoir, and transmission route of AMR. Antibiotic resistant bacteria (ARB), antibiotics, and other selective agents will be discharged into the environment. ARGs can be shared between bacteria under selective pressure from antimicrobials along with other selective agents. The sharing allows for spread of AMR across diverse populations of environmental bacteria and pathogens. People and animals can be exposed to AMR through the intake of food, water, and air.

IDENTIFICATION OF MAIN EMISSION SOURCES

Natural AMR is common among environmental bacteria, including in pristine locations relatively untouched by anthropogenic activities (*5*–*7*). However, the use of antimicrobial agents in humans

and animals has been associated with the evolution and amplification of antimicrobial resistant pathogens and their respective ARGs. Anthropogenic activities are increasing the importance of the environment as a pathway for human AMR exposure. Release of antimicrobials or other selective agents into the environment via excreta from humans, terrestrial or aquatic animals, or from antimicrobial manufacturing waste and wastewater promotes resistance by creating an environment that is favorable for the transfer or emergence of new resistance genes (*8*). There are three main emission sources in the environment: animal farming, hospital/community sewage, and the pharmaceutical industry (Figure 1).

Human consumption of antimicrobials can result in antimicrobial resistant pathogens and ARGs being discharged to the environment. Wastewater from hospitals, intensive livestock farms, and aquaculture is likely to contain particularly elevated concentrations of antimicrobials, ARB and ARGs, which might influence AMR spread (*8*). Wastewater discharge from antimicrobial production is a hotspot for AMR development. Antibiotics in water downstream of some antibiotic manufacturing sites have been found at higher concentrations than those found in the blood of patients taking medicines (*9*), facilitating AMR selection in the local environment. The conventional wastewater treatment processes are typically not designed for treating high concentrations of antimicrobials. Thus, the development of AMR during pharmaceutical wastewater treatment is a universal problem.

PRIORITY IN THE CONTAINMENT OF ENVIRONMENTAL AMR DEVELOPMENT

Minimizing Emissions

To contain AMR dissemination in the environment, the priority is to minimize the emission of antimicrobials and ARB/ARGs during the discharge of waste and wastewater from animal farming, hospitals, and pharmaceutical manufacturing (Figure 1). Since antimicrobials could become selective agents for AMR once released into the environment, the most important approach is to reduce the residual level in wastewater as much as possible. However, the reduction of antimicrobials has not yet been set as a target for wastewater treatment. Meanwhile, AMR development could also occur during wastewater treatment under the selective pressure of the antimicrobials. Thus, pre-treatment of production wastewater to remove antimicrobials is the best way to control the development of ARGs during biological wastewater treatment; this has been implemented in some manufacturing sites in China (*8*). It is also important to prevent the release of the ARB and ARGs in wastewater to the environment. Different disinfection technologies could be used for this purpose. On the other hand, rapidly developing membrane technology could provide an attractive option because, unlike disinfectants, it will impose no selective pressure on bacteria.

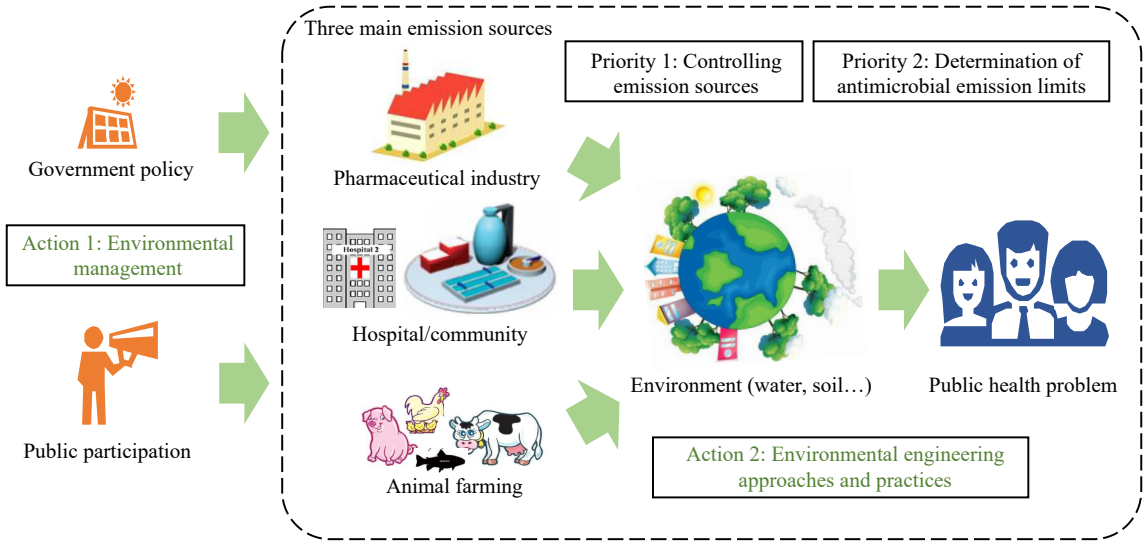

FIGURE 1. Environmental mission sources, priorities, and actions for minimizing risk of developing AMR in the environment.
Abbreviation: AMR=antimicrobial resistance.

Determining Antimicrobial Emission Limits

To minimize the emissions of antimicrobials, it is necessary to establish a sound emission limit (Figure 1). Currently, there are no global effluent water quality guidelines based on health risk assessment. Recently, a voluntary industry group named the AMR Industry Alliance has developed initiatives to establish a common manufacturing framework for managing the discharge of antimicrobial compounds into waterways and apply it across manufacturing and supply chains among their members. One of the important points in this framework is to determine predicted no-effect concentrations (PNECs) to mitigate against AMR spread (*10*). The PNECs for resistance selection were acquired by modeling based on the Minimal Inhibitory Concentrations of 111 antibiotics from the public European Committee for Antimicrobial Susceptibility Testing (EUCAST) database (*11*). The current list stands at 125 antibiotics (*12*). However, transforming such a list into internationally accepted emission targets is still a challenge. While this list only focuses on emission control, it is also important to establish a guideline for the pre-treatment of production wastewater.

PROGRESS IN THE CONTAINMENT OF ENVIRONMENTAL AMR DEVELOPMENT

Environmental Management

Table 1 summarizes some important actions related to AMR containment in the environment. Many countries are taking actions to limit emissions. For example, China has included waste antibiotic fermentation residue in the national hazardous waste list, and the disposal and discharge of antibiotic residues are strictly regulated. Norway and Sweden have added emission control as part of their antimicrobial procurement criteria. WHO and other agencies have summarized recent progress of AMR control in a technical brief on antimicrobial resistance in 2020 (*8*). The list of antibiotic manufacturing

TABLE 1. Summary of some important international and Chinese actions related to AMR containment in the environment since 2002.

Action types	Year	Actions
International actions	2013	Green procurement for pharmaceutical manufacturing (WHO and UNEP meeting)
	2015	Global Action Plan on AMR (WHO)
	2016	Creation of the AMR Industry Alliance
	2017	Developing priorities for WHO activities on anti-microbial resistance and the environment (WHO expert meeting)
	2018	List of antibiotic manufacturing discharge targets following the launch of the Common Antibiotic Manufacturing Framework (AMR Industry Alliance)
	2019	Antibiotic use and wastewater residue (WHO expert meeting)
	2020	Technical brief on water, sanitation, hygiene and wastewater management to prevent infections and reduce the spread of antimicrobial resistance (WHO/FAO/WOAH)
	2022	Antibiotic manufacturing standards: Minimizing risk of developing antibiotic resistance and aquatic ecotoxicity in the environment resulting from the manufacturing of human antibiotics (AMR Industry Alliance)
Actions of China	2002	Antibiotic fermentation residues were listed as prohibited drugs in forage and animal drinking water
	2008	Antibiotic fermentation residues were officially included in Directory of National Hazardous Wastes in 2008 and remained in the revised versions in 2016 and 2021
	2016	National action plan to contain antimicrobial resistance in China (2016–2020)
	2017	Ministry of Agriculture and Rural Affairs of China formally banned colistin as an animal growth promoter
	2019	Ministry of Agriculture and Rural Affairs withdrew all types of growth promoters from animal feed except Chinese medicine on July 10, 2019, and the ban took effect on January 1, 2020
	2020	Pharmaceutical Industry EHS Guide (CPEA)
	2020	Annual Report on the Development of China's pharmaceutical Industry with a chapter on “Synergistic control of antibiotics, resistance genes and conventional pollutants in pharmaceutical wastewater” (CPIA)
	2021	The Biosecurity Law of the People’s Republic of China was issued. Containment of AMR was emphasized
	2021	Standards for determination of erythromycin, cephalosporin and penicillin in antibiotic fermentation residue, raw fertilizer material, crop, and related environments (CPIA)
	2022	The National action plan to contain antimicrobial resistance in China (2022–2025)

Abbreviation: WHO=World Health Organization; UNEP=United Nations Environment Programme; FAO=Food and Agriculture Organization of the United Nations; WOAH=World Organization for Animal Health; CPEA=China pharmaceutical Enterprises Association; EHS=Environment, Health and Safety; CPIA=China Pharmaceutical Industry Association.

discharge targets established by the AMR Industry Alliance was adopted by the Pharmaceutical Supply Chain Initiative (PSCI). A pharmaceutical industry Environment, Health, and Safety (EHS) guideline issued by the China Pharmaceutical Enterprises Association (CPEA) suggested monitoring the active pharmaceutical ingredients (APIs) in waste and wastewater, establishing an effective management system, and formulating emission control targets in accordance with PNECs. Pretreatment of antibiotic production wastewater to remove the residual antibacterial activity was included in the WHO technical brief (*8*) and the Blue Book of China's Pharmaceutical Industry (2020), published by the China Pharmaceutical Industry Association (CPIA), as an approach to ensure the synergistic control of antibiotics, ARGs, and conventional pollutants (*13*).

On the other hand, some actions from one health perspective have been adopted. Following the discovery of *mcr-1* (*14*), the Ministry of Agriculture and Rural Affairs of China banned colistin as an animal growth promoter. The colistin withdrawal policy and the decreasing use of colistin in agriculture have had a significant effect on reducing colistin resistance in both animals and humans in China (*15*). China has decided to ban antibiotics as growth promoters in animal feed. However, there are no internationally agreed actions on the containment of the emissions of antimicrobials and ARB/ARGs to the environment. It is desirable to establish a holistic framework to coordinate international actions on the containment of environmental AMR development. Recently, China has announced a new national action plan (NAP 2022–2025, Table 1) to combat antimicrobial resistance. The plan embraces the one-health concept, with health, agriculture and environmental protection departments dedicated. It also details measures to contain the dissemination of antimicrobial resistance in the environment, such as enhancing the control of antimicrobial pollution discharge and carrying out trials to monitor antimicrobials in water environments. In addition, cross-resistance of antibiotics used in animals and humans becomes an increasingly serious problem. More actions on reasonable arrangement of prophylactic and therapeutic veterinary antibiotics from the one health perspective are needed.

Environmental Engineering Approaches and Practices

The biological processes — a combination of anaerobic digestion and an activated sludge process — widely used in wastewater treatment are vulnerable to the presence of extremely high concentrations of antibiotics in antibiotic production wastewater. The presence of high concentrations of antibiotics in wastewater has been found to cause treatment failure by disturbing wastewater treatment microbial communities. Particularly, multidrug resistance can be developed during wastewater treatment due to horizontal gene transfer of ARGs among the bacterial community mainly through the enrichment of plasmids harboring multidrug resistance regions (*16–17*). Motivated by the ease of hydrolysis of most antibiotics, a novel pretreatment technology based on enhanced hydrolysis has been developed using homogeneous or heterogeneous acid/base catalysts for targeted elimination of antibiotic potencies from wastewater (*18–19*). The enhanced hydrolysis pre-treatment technology has been successfully applied in full-scale plants in Hebei Province, China (*13*). The antibiotic concentrations could be reduced from around 1,000 mg/L to less than 1 mg/L through the pre-treatment. The abundance of ARGs in the biological treatment units could be reduced by >80%, and the biological wastewater treatment systems could be operated under stable conditions. In addition, hydrothermal treatment based on enhanced hydrolysis was also applied in Chinese full-scale plants for recycling waste erythromycin and cephalosporin fermentation residues (*20*).

Similarly, livestock waste is another major source of ARGs in the environment. Some prevalent high-risk ARGs in animal manure, such as CTX-M-type extended spectrum β-lactamase genes ($bla_{\text{CTX-M}}$), the ABC transporter gene conferring resistance to florfenicol and linezolid (*optrA*), and mobile tigecycline resistance gene [*tet*(X) variants], were found to persist in animal manure, mesophilic anaerobic digestion and composting systems, and the related environment (*21–23*). Hyperthermophilic anaerobic treatment or composting could reduce the abundance of most high-risk ARGs and inactivate the fecal bacteria effectively, indicating that effective management of operating temperatures in anaerobic digestion or composting might be an effective way to prevent the discharge of the high-risk ARGs from animal manure treatment systems.

The role of the environment in the rise, spread, and health risks of AMR have been intensively investigated. However, environmental AMR development is an extremely complicated issue. There are still many questions to be answered: How and to what extent do different sources of antimicrobial residues and ARB contribute to developing AMR in the environment? How can an internationally coordinated action plan be

established to contain environmental AMR development? To better answer these questions, coordinated monitoring, research, and actions are required. In 2016 and 2022, China developed its own National Action Plan with contributions from multisectoral departments for 2016–2020 and 2022–2025, respectively (Table 1). China is advocating establishing a community with a shared future for humanity; therefore, an important role in such critical international cooperation to cope with AMR challenges could and should be played.

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Methods and Applications

The Establishment and Application of a Kraken Classifier for *Salmonella* Plasmid Sequence Prediction

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ABSTRACT

Introduction: *Salmonella* is a key intestinal pathogen of foodborne disease, and the plasmids in *Salmonella* are related to many biological characteristics, including virulence and drug resistance. A large number of plasmid contigs have been sequenced in bacterial draft genomes, however, these are often difficult to distinguish from chromosomal contigs.

Methods: In this study, three different customized Kraken databases were used to build three different Kraken classifiers. Complete genome benchmark datasets and simulated draft genome benchmark datasets were constructed. Five-fold cross-validation was used to evaluate the performance of the three different Kraken classifiers by two benchmark datasets.

Results: The predictive performance of the classifier based on all National Center for Biotechnology Information plasmids and *Salmonella* complete genomes was optimal. This optimal Kraken classifier was performed with *Salmonella* isolated in China. The plasmid carrying rate of *Salmonella* in China is 91.01%, and it was found that the Kraken classifier could find more plasmid contigs and antibiotic resistance genes (ARGs) than results derived from a plasmid replicon-based method (PlasmidFinder). Moreover, it was found that in the strains carrying ARGs, plasmids carried more ARGs [three, 95% confidence interval (*CI*): 1–14] than chromosomes (one, 95% *CI*: 1–7).

Discussion: We found building a high-quality customized database as a Kraken classifier to be ideal for the prediction of *Salmonella* plasmid sequences from bacterial draft genomes. In the future, the Kraken classifier established in this study will play a significant role in ARG monitoring.

Salmonella is an important intestinal pathogen of foodborne disease, causing enteritis and bloodstream infections, among other serious consequences, transmitted by food and water. Plasmid genome sizes in *Salmonella enterica* are generally between 2 kb and 200 kb and are biased based on serotype (*1*). As an important mobile genetic element (MGE), plasmids in *Salmonella* endow strains with many biological characteristics, including toxin production, resistance to heavy metals, antibiotic resistance genes (ARGs), and prophage integration (*2*–*4*). The spread of plasmid-borne ARGs has become a global public health problem, and plasmids, as reservoirs of ARGs, can spread rapidly between different species, including human pathogens (*5*–*6*). Therefore, it is necessary to monitor the ARGs carried by plasmids for the evaluation of ARG transmission.

Salmonella genome analyses based on next-generation sequence techniques have become an important tool for infectious disease surveillance, prevention and control, and food safety management. Currently, it is challenging to distinguish the full genomes of the chromosomes versus the plasmids without using long-read sequencing. It is very important to obtain the complete genomes of these MGEs for understanding plasmid origins and contributions to strain adaptability. To solve this problem, several plasmid sequence prediction methods have been developed, including Kraken (*7*), cBar (*8*), PlasFlow (*9*), RFPlasmid (*10*), mlplasmids (*11*) and PlasmidFinder (*12*). The Kraken classifier is an ultra-fast and highly accurate species classification program for sequences, and the Kraken classifier-based method has the highest accuracy and balanced performance in terms of overall sensitivity and specificity among the compared methods in the prediction of plasmid sequences in *Klebsiella pneumoniae* (*13*).

In our study, three customized Kraken databases were constructed using three different plasmid datasets and a *Salmonella* chromosomal dataset. These formed three different Kraken classifiers. A five-fold cross-validation method was used to evaluate the performance of the three Kraken classifiers using two different benchmark datasets. Finally, the optimal

Kraken classifier was used to predict the plasmid sequence contigs from the genomes of *Salmonella* strains isolated in China, and plasmid-carrying prevalence and plasmid-borne ARGs were estimated.

METHODS

Three Customized Kraken Databases

Three customized Kraken databases were constructed (Figure 1A); each dataset included a plasmid dataset and a chromosome dataset. The plasmid datasets were the National Center for Biotechnology Information (NCBI) plasmid dataset, an *Enterobacteriaceae* bacterial plasmid dataset, and a *Salmonella* plasmid dataset, which contained 46,033, 19,853, and 1,591 plasmid sequences, respectively. The chromosome dataset comprised 2,001 *Salmonella* complete genomes. Therefore, our customized Kraken database A was composed of 2,001 *Salmonella* complete genomes and 1,591 *Salmonella* plasmids. Our customized Kraken database B was composed of 2,001 *Salmonella* complete genomes and 19,853 *Enterobacteriaceae* bacterial plasmids. And our customized Kraken database C was composed of 2,001 *Salmonella* complete genomes and all 46,033 NCBI bacterial plasmids. The download address of the NCBI plasmid dataset is https://ftp.ncbi.nlm.nih.gov/genomes/refseq/plasmid. The *Salmonella* complete genomes were downloaded from NCBI ftp (https://ftp.ncbi.nlm.nih.gov/genomes/genbank/). The *Enterobacteriaceae* bacterial plasmid dataset was constructed by extracting all sequences belonging to the *Enterobacteriaceae* from the NCBI plasmid dataset. Likewise, the *Salmonella* plasmid dataset was extracted in a similar manner. Kraken version 1.0 (*7*) was used to build our Kraken classifier. Operation of the Kraken classifier followed the Kraken manual (http://ccb.jhu.edu/software/kraken/).

Two Benchmark Datasets

Two different benchmark datasets were constructed. Benchmark dataset I includes complete genomes, and benchmark dataset II includes simulated draft genomes. Benchmark dataset I consists of 2,001 Salmonella complete chromosomes from NCBI GenBank and all NCBI RefSeq plasmids. Benchmark dataset II was created as follows: One thousand *Salmonella* draft genomes were randomly selected from NCBI GenBank, all draft contig lengths were obtained, deciles were calculated according to the lengths, and ten intervals were formed by the deciles. For each sequence in benchmark dataset I, each time one interval was randomly selected, an integer value was randomly selected within the interval. The process was repeated until the total length exceeded the sequence length. The sequence was then broken into a series of sequential fragments according to the selected integer values. Following this method, each sequence was fragmented.

Kraken Classifiers Evaluation

Five-fold cross validation was used to evaluate the

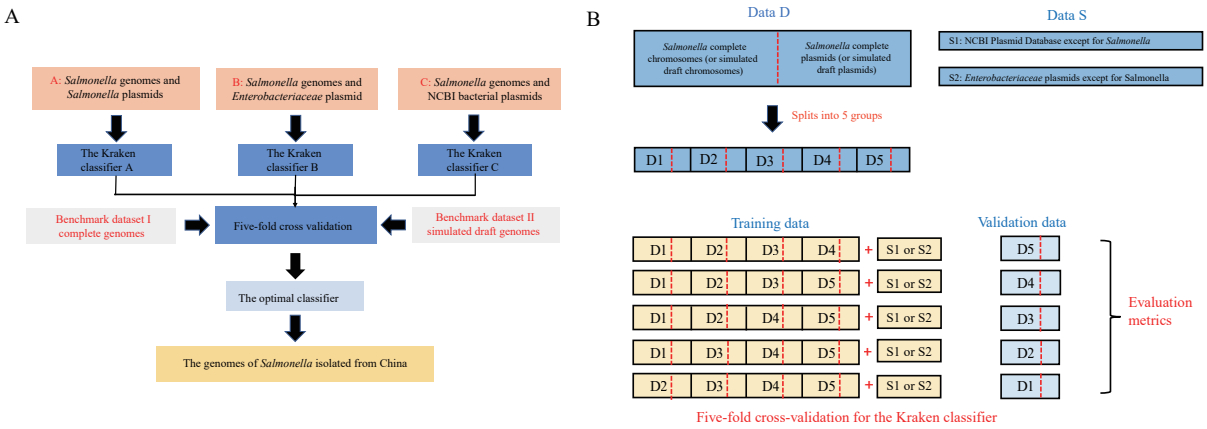

FIGURE 1. The building and evaluation flow charts of our Kraken classifier for plasmid sequence prediction. (A) Scheme for building and evaluating. (B) Details of the Kraken classifier five-fold cross-validation.
Note: The detailed procedure for building and evaluating our kraken classifier is illustrated. *Salmonella* genomes (Data D), both chromosomes and plasmids, were divided into five groups. Four groups, together with the National Center for Biotechnology Information (NCBI) bacterial plasmid database (without Salmonella plasmids) (Data S1) or *Enterobacteriaceae* plasmid database (without *Salmonella* plasmids) (Data S2), were used as training data to build the Kraken classifier, the remaining group was used as validation data.

three Kraken classifiers constructed in this study (Figure 1B). For the NCBI plasmid dataset, the *Salmonella* plasmid sequence and chromosome sequence were divided into five groups, took out four groups and added the NCBI plasmid dataset (without *Salmonella* plasmids) to construct the training database, and validated the Kraken classifiers by using the other group. For the *Enterobacteriaceae* bacterial plasmid dataset, the *Enterobacteriaceae* bacterial plasmid sequences and chromosome sequences were also divided into five groups, took out four groups, and added *Enterobacteriaceae* bacterial plasmids (without *Salmonella* plasmids) to build the training database. For the *Salmonella* dataset, the *Salmonella* plasmid sequence and chromosome sequence were divided into five groups, we took out four groups to build the training database, and validated the classifiers with the other group.

The Metrics of the Kraken Classifier Evaluation

A series of evaluation metrics were used to evaluate the classifier, including accuracy, precision, recall, specificity and false predictive value:

Accuracy=(TP+TN)/(TP+TN+FP+FN)

Precision=TP/(TP+FP)

Recall=TP/(TP+FN)

Specificity=TN/(TN+FP)

False predictive value=TN/(TN+FN)

(TP: The number of sequences that were predicted to be plasmids, and actually were plasmids. FP: The number of sequences that were predicted to be plasmids, but actually were not plasmids. TN: The number of sequences that were predicted to be chromosomes, and actually were chromosomes. FN: The number of sequences that were predicted to be chromosomes, but actually were not chromosomes.)

Plasmid Prediction Based on PlasmidFinder

PlasmidFinder is based on an *Enterobacteriaceae* plasmid replicon sequence database (*12*). The minimum coverage threshold was set to 60% and the minimum identity threshold was set to 80%.

Statistical analysis and plotting

All Statistical Analyses were done using R programming language. The ggridges (https://wilkelab.org/ggridges/), ggplot2 (https://ggplot2.tidyverse.org), and eulerr (https://github.com/jolars/eulerr) packages were used to generate ridgeline plots, violin plots, and Venn diagrams. Kolmogorov-Smirnov test was used to evaluated the distribution differences between two variables. Fisher's exact test was used to test the proportion difference.

RESULTS

Classifier Evaluation Based on the Complete Genome Benchmark Dataset (Benchmark Dataset I)

Evaluation results for the three Kraken classifiers showed that the third Kraken classifier, C, which was composed of complete *Salmonella* genomes and all NCBI bacterial plasmids, had the highest accuracy (98.94%) and the highest recall rate (97.67%), with relatively high precision (99.94%) and specificity (99.95%). The recall rate and precision of the other classifiers were lower (Table 1).

Classifier Evaluation Based on the Simulated Draft Genomes Benchmark Dataset (Benchmark Dataset II)

Here, according to *Salmonella* draft genome contig length distributions in the NCBI database, the complete genomes in the benchmark dataset (benchmark dataset I) were broken into fragments according to empirical contig distributions to construct a simulated draft genome benchmark dataset

TABLE 1. Evaluation results for Kraken classifier-based plasmid sequence prediction.

Dataset	Classifier type	Accurate	Precision	Recall	Specificity	False predictive value
Benchmark dataset I	Kraken classifier A	98.41%	100.00%	96.42%	100.00%	98.57%
	Kraken classifier B	98.89%	100.00%	97.49%	100.00%	98.96%
	Kraken classifier C	98.94%	99.94%	97.67%	99.95%	98.86%
Benchmark dataset II	Kraken classifier A	99.20%	99.80%	91.23%	99.65%	99.87%
	Kraken classifier B	99.25%	99.64%	92.38%	99.65%	99.90%
	Kraken classifier C	99.28%	99.48%	92.68%	99.66%	99.90%

(benchmark dataset II). The distribution of chromosomal fragment lengths and the distribution of plasmid fragment lengths in our simulated *Salmonella* draft genome benchmark dataset showed similar distributions as the distribution of contig lengths in 1,000 randomly selected *Salmonella* draft genomes from GenBank, indicating that our benchmark dataset II is a good simulation of actual data (Figure 2A).

Results showed that the third Kraken classifier, C, which was created from databases based on all bacterial plasmids and complete *Salmonella* genomes in NCBI, had the highest accuracy (99.28%). Other metrics were also relatively higher than the other two Kraken classifiers. Therefore, Kraken classifier C was selected as the optimal Kraken classifier obtained in this study.

Analysis of Plasmid Carrying Prevalence and Plasmid Carrying ARGs for *Salmonella* Isolated From China

A total of 4,036 draft *Salmonella* genomes isolated from China were collected from GenBank. Our optimal Kraken classifier was then used to predict plasmid contigs from them. Among all strains 3,673 (91.01%) were predicted to have plasmid contigs, with

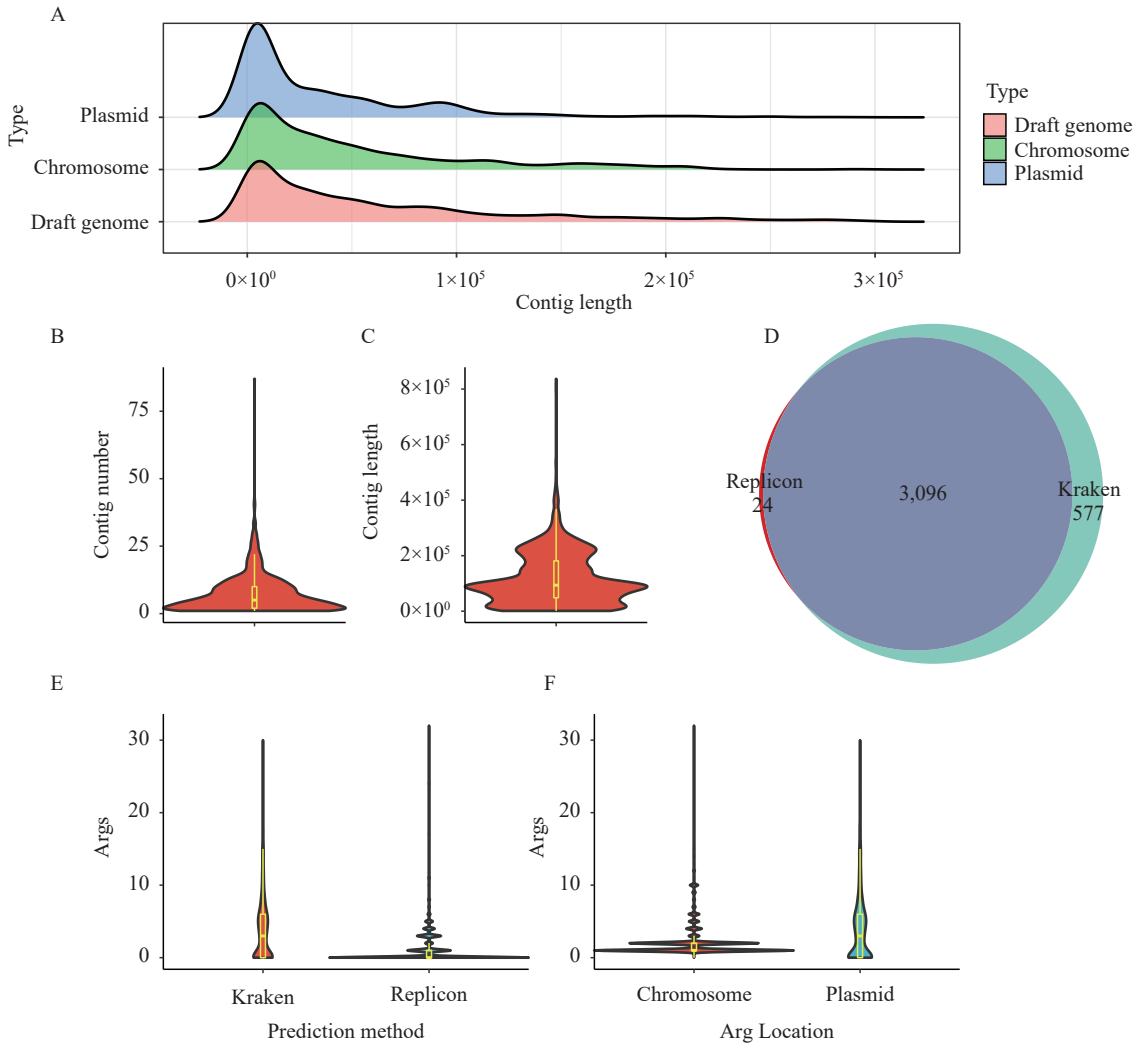

FIGURE 2. The application and evaluation of Kraken classifier for Salmonella strains isolated in China. (A) Distribution of contig lengths of 1,000 randomly selected *Salmonella* draft genomes compared to the chromosomal and plasmid length distributions in our simulated draft genome benchmark dataset (benchmark dataset II). (B) Number of plasmid contigs per strain for *Salmonella* with plasmids isolated from China. (C) Total length distribution of plasmid contigs per strain for *Salmonella* with plasmids isolated from China. (D) Venn diagram displaying the overlap of strains containing plasmids predicted by a replicon-based method (PlasmidFinder) and the Kraken classifier. (E) Comparison of the number of ARGs carried by plasmids predicted by the Kraken classifier and the replicon-based method, respectively. (F) Comparison of the number of ARGs located in plasmids and those located in the chromosome.

a median contig number of five [95% confidence interval (*CI*): 1–21] for plasmids (Figure 2B), and a median total plasmid length of 93,740 bp per strain (95% *CI*: 4,657–26,7721 bp) (Figure 2C).

To compare the Kraken classifier established in this study with a conventionally used replicon-based method, PlasmidFinder was also used to predict plasmid contigs. Among the 4,036 *Salmonella* strain draft genomes, 3,145 strains (72.72%) were predicted to contain plasmid contigs. Compared with PlasmidFinder, our Kraken classifier discovered that another 556 strains harbor plasmids, while the replicon-based method found 24 strains that our Kraken classifier did not (Figure 2D). Among these 24 strains, four strains had very long (>4 Mb) contigs, which may be due to the integration of plasmids into chromosomes. Additionally, contigs carrying replicons in the other 20 strains are quite short (<5 kb) and harbor extensive mobile genetic elements, making it difficult to distinguish whether these contigs belong to chromosomes or plasmids, or are the result of assembling error.

Simultaneously, the predictive ability to discover ARGs between the replicon-based method and the Kraken classifier was compared and it was found that the replicon-based method evaluated the median number of plasmid-borne ARGs to be zero (95% *CI*: 0–5). The Kraken classifier assessed the median number of ARGs carried by plasmids to be three (95% *CI*: 0–14), which is significantly different (*P* value <0.001, Kolmogorov-Smirnov test) (Figure 2E), suggesting the Kraken classifier established in this study can predict more ARGs carried on plasmids than other methods.

Using our Kraken classifier predictor, the median number of chromosome-carrying ARGs of each strain was one (95% *CI*: 1–7), and the median number of plasmid-borne ARGs was three (95% *CI*: 1–14). This is a significant difference in ARG distribution between chromosomes and plasmids in these *Salmonella* strains (*P* value <0.001, Kolmogorov-Smirnov test) (Figure 2F).

Quinolone and third-generation cephalosporins are commonly used antibiotics in clinics. ARGs can be carried on chromosome and plasmids in *Salmonella*. Here, our Kraken classifier was used to predict chromosomal and plasmid locations of these ARGs in 4,036 *Salmonella* strains. It was found that 1.88% of the strains have the acquired quinolone-related resistance genes on chromosomes, while 11.90% of the

TABLE 2. Comparison of quinolone and third-generation cephalosporin-related ARGs prediction results.

Antibiotic type	ARG	Number of ARGs	Number of ARGs isolated on chromosome	Number of ARGs isolated on plasmids	Number of ARGs isolated on both chromosome and plasmids	Undefined	*P* value
Quinolone resistance	*qnrA*	4	0	4	0	0	0.02
	qnrB	182	0	182	0	0	<0.001
	qnrD	3	0	3	0	0	0.06
	qnrS	1,054	19	778	2	259	<0.001
	qnrVC	4	0	4	0	0	0.02
	qepA	29	0	29	0	0	<0.001
	aac(6')-Ib-cr	942	76	299	3	570	<0.001
	oqxA	797	13	221	0	563	<0.001
	oqxB	798	13	225	0	560	<0.001
	qnrS	1,054	19	778	2	259	<0.001
Third-generation cephalosporins resistance	*blaTEM*	1,607	94	838	7	682	<0.001
	blaCTX-M	863	192	408	17	280	<0.001
	blaOXA	854	80	205	2	571	<0.001
	blaCMY	27	1	23	1	4	<0.001
	blaDHA	24	0	24	0	0	<0.001
	blaNDM	10	2	8	0	0	0.02
	blaSHV	5	0	5	0	0	0.01

Abbreviation: ARGs=antibiotic resistance genes.

strains carry acquired quinolone-related resistance genes on plasmids. Besides 7.71% of the strains carry third-generation cephalosporin-related resistance genes on chromosomes, while 62.61% of the strains carry the gene on plasmids (Table 2). The number of strains carrying quinolone-related resistance genes or the acquired third-generation cephalosporin-related resistance genes on plasmids is significantly higher than that carrying the corresponding resistance genes on chromosomes (*P* value <0.001, Fisher's exact test).

DISCUSSION

The Kraken classifier can be a useful tool in metagenomic species identification because of its ultra-fast speed and high accuracy (*7*). Kraken-based plasmid prediction methods demonstrated the highest accuracy and F1 score (an evaluation metric for the performance of a machine learning model) compared to other methods (Centrifuge, RFPlasmid, mlplasmids, PlaScope, and Platon), with balanced sensitivity and specificity (*13*). Our results also show that our Kraken classifier has high accuracy, precision, and sensitivity when applied to the prediction of plasmid sequences carried by *Salmonella*. Furthermore, our optimal Kraken classifier, built with all NCBI bacterial plasmids and *Salmonella* complete genomes, proved to be an ideal method for *Salmonella* plasmid sequence prediction.

In our study, three kinds of customized Kraken databases were used to construct three different Kraken classifiers. It is challenging to choose the ideal database. It was found that the Kraken classifier based on NCBI bacterial plasmids and *Salmonella* complete genome data had the highest prediction accuracy and could be used as an optimal customized Kraken database for *Salmonella* plasmid prediction.

The optimal Kraken classifier was used to predict the plasmid sequences from *Salmonella* strains isolated from China and it was found that 91.01% of these strains carried plasmids. PlasmidFinder is a traditional and easy-to-use tool for plasmid sequence and type detection that relies on the fact that most plasmids have identifiable replicon sequences (*14*). However, finding plasmid contigs containing undiscovered replicons with replicon-based methods is quite difficult. In this study, plasmid contigs from *Salmonella* sequences isolated from China were predicted and it was found that our Kraken classifier could find more strains that carried plasmids than plasmid-based replicon methods, suggesting the Kraken classifier we established in this study would be a useful tool for determining plasmid contigs in bacterial draft sequences. Those plasmid contigs not detected by our Kraken classifier may be due to the integration of plasmids into the chromosomes or the presence of a large number of MGE-related genes.

Plasmids are an important reservoir of ARGs and a vector of resistance transmission (*15*). Our newly developed Kraken classifier can find more plasmid contigs than the existing methods, and, therefore, can find more ARGs carried on plasmids. In *Salmonella* strains isolated from China, the number of plasmid-borne ARGs was higher than that of chromosomes-carrying ARGs. Currently, obtaining an entire complete genome sequence remains somewhat challenging. It was expected that the Kraken classifier developed in this study will become crucial for monitoring ARGs in the future.

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