

The Emergence of Global Antimicrobial Resistance: A Formidable Biodefense Consideration?

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Abstract

The emergence of antimicrobial resistance (AMR) represents a global health crisis with profound implications for public health, national security, and biodefense readiness. This study explores the confluence of AMR, water disinfection techniques, and biofilm-mediated resistance in opportunistic bacterial isolates from US wastewater treatment plants. Using phenotypic assays, antimicrobial susceptibility testing, chlorine minimum inhibitory concentration (MIC) studies, and whole-genome sequencing, the authors evaluated and characterized 92 genetically distinct Gram-negative isolates for multidrug resistance (MDR), biofilm formation, and disinfectant tolerance.

Findings reveal that 58% of moderate or strong biofilm-forming isolates exhibited MDR profiles, and over 90% tolerated chlorine concentrations commonly used in water treatment. Chlorine-resistant isolates frequently harbored virulence genes (indicating pathogenicity), suggesting selective pressure linked to disinfection protocols. Biofilm capacity significantly increased the odds of chlorine resistance, demonstrating the resilience of these waterborne bacterial isolates in treated water systems.

Given AMR's potential to threaten total force readiness and even enable adversarial use of biotechnology to target critical infrastructure, these findings highlight a direct threat to force health protection and overall mission readiness. The 2023 Department of Defense *Biodefense Posture Review* identifies AMR as a naturally occurring biological threat with the potential to overwhelm response capacities. This research reinforces that view and highlights the need for integrated medical countermeasures, updated disinfection strategies, and enhanced countering weapons of mass destruction (CWMD) preparedness to mitigate AMR as an emergent weaponizable threat in the 21st century.

Introduction and Relevance

Antimicrobial agents have been used to treat bacterial infections in humans, animals, and plants for the past 90 years. They have also been exploited for diverse purposes including animal husbandry and animal production as preventive measures in many underdeveloped and developing countries for decades. Antimicrobial therapy represents one of the most prescribed class of medications to manage both chronic and acute bacterial diseases in clinical settings.¹

However, the emergence of AMR has escalated into a crisis for modern medicine, representing a critical threat to global health. This rising problem is characterized by a significant increase in the number of antimicrobial resistant strains, "Super Bugs," many of which confer significant levels of multidrug resistance (MDR). In 2019, 569,000 deaths were attributed to AMR-associated infections.² The prevalence of antimicrobial resistant bacteria has gained a high level of attention worldwide and threatens global public health as a silent pandemic, necessitating urgent intervention.

AMR

AMR is an inevitable evolutionary phenomenon resulting from bacteria adapting to the therapeutic antibiotics and developing defenses against the mechanistic effects of antibiotic molecules. Bacterial microbes develop resistance against antibacterial drugs, rendering them ineffective. This adaptation occurs by either chromosomal gene mutations or the acquisition of foreign deoxyribonucleic acid (DNA) through horizontal gene transfer that codes for resistance determinants. AMR proliferates as bacteria acquire resistance genes, often through conjugation, and through internal evolving mechanisms of resistance to withstand and persist despite antibiotic treatments. This phenomenon is also documented with disinfecting agents.³ Bacteria evolve to evade the effects of antimicrobial agents, utilizing various mechanisms such as enzymatic breakdown, chemical modifications rendering agents functionless, inhibition of uptake, activation of efflux pumps, and modifications of target sites. Furthermore, with the aid of biofilm, bacteria are protected from disinfection agents, and biofilms can support communities of clinically relevant bacteria, posing public health threats. The biofilm mode of growth enhances bacterial resistance to antimicrobial agents, presenting a challenge in treating infections.

In the Department of Defense (DOD) 2023 *Biodefense Posture Review* (BPR), the emergence of antimicrobial resistance is described as “a naturally occurring biological threat that can overwhelm response capacities...” The BPR includes novel infectious diseases, the resurgence of once-localized diseases, and zoonotic diseases as growing challenges to outbreak control.⁴ As a result, there is a considerable danger that emerging AMR poses to the health and readiness of the Total Force, as well as the broader US population. While Force Health Protection (FHP) measures are usually effective in countering endemic diseases, some emerging infectious disease threats, such as MDR bacteria, risk rendering medical countermeasures ineffective. Naturally occurring diseases, that

hinder mission effectiveness, pose potentially serious harm to the Total Force and associated missions. Detection and surveillance capabilities addressing emerging disease threats are required for a resilient, ready force, which enables the Total Force to operate through biologically threatened environments.

Increased AMR prevalence, particularly from multi-drug-resistant “super bugs,” exacerbates public health challenges by limiting therapeutic options, leading to heightened morbidity, mortality, and financial burden. AMR hinders treatment efficacy of microbial infections and threatens the scope of treatment for many common illnesses, resulting in treatment failure, permanent disability, or even death. The emergence of AMR bacteria makes major medical procedures like chemotherapy and organ transplants significantly riskier, as patients become more vulnerable to infections that are increasingly difficult to treat without new antimicrobials. Perhaps more concerning are the evolving advancements in biotechnology that could exacerbate and exploit the AMR challenge. The 2023 BPR notes that developments in biotechnology, including but not limited to gene editing and whole genome sequencing, are driving an increase in the scope and diversity of biothreats. Similarly, the 2023 Department of Defense *Strategy for Countering Weapons of Mass Destruction* points out that the global availability of dual-use technologies, particularly in biotechnology, may erode traditional barriers to proliferation and



ABOVE: (Left to right) Sgt. Monika MacDonald and Private 1st Class Miguel Arroyo, members of the 753rd Quartermaster Company water purification team, drain the cyclone separator as they shut down operations at the Reverse Osmosis Water Purification Unit Rodeo at Fort Story, Virginia, June 15, 2017.

reduce opportunities to deny or disrupt the development of offensive weapons of mass destruction (WMD) programs.⁵ This point implies the potential of adversarial engineering or enhancing of pathogens with increased virulence and antimicrobial resistance using advanced biotechnological techniques.

As advancements in biotechnology expand the potential for pathogen manipulation, the high prevalence of MDR among waterborne pathogens presents a particularly concerning vector of exploitation, posing a threat to both public health and critical infrastructure, especially through the contamination and disruption of water purification and distribution systems. While the vulnerability of water systems to deliberate contamination has long been recognized—from ancient military campaigns to 20th-century conflict—the modern threat posed by AMR pathogens introduces a new dimension of complexity. The possibility of adversarial exploitation of waterborne pathogens (conferring AMR) to disable infrastructure or target military populations raises critical concerns for biodefense planning and strategy. To address this, experimental research has become essential for characterizing resistance profiles in real-world environments. This study, which examined 92 genetically distinct bacterial isolates from US wastewater treatment facilities, revealed a significant overlap between water disinfection resistance (chlorine and ultraviolet (UV) irradiation), biofilm formation, and multidrug resistance. By incorporating phenotypic assays, chlorine minimum inhibitory concentration (MIC) studies, and whole-genome sequencing of *Klebsiella* spp., the findings demonstrate how persistent, biofilm-forming MDR strains could potentially evade standard water disinfection protocols. These results not only highlight the weaknesses in current water treatment defenses but also provide empirical evidence to inform CWMD and FHP strategies as well as biosecurity risk assessments.

Methods and Materials

SAMPLE COLLECTION AND BACTERIAL ISOLATION

Between April and December 2023, surface ($n = 82$) and effluent water samples ($n = 96$) were collected from four wastewater treatment plants (WWTPs) in North Carolina—two employing UV irradiation disinfection and two using chlorination disinfection techniques. Surface swabs were enriched in buffered peptone water and incubated at 37°C for 18–24 hours. Effluent (post-disinfection) water samples were processed using

the World Health Organization Tricycle Surveillance Protocol, plated on MacConkey agar, and incubated for 24 hours at 37°C.⁶ Up to three colonies per plate were isolated and further sub-cultured on blood agar plates. Genera of cultured isolates were identified using Matrix-Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry (MALDI-TOF MS).⁷



ABOVE: Melendez's research produced cultured bacterial colonies isolated on MacConkey agar. Pictured bacterial growth was collected from UV-irradiation WWTP effluent discharge point.⁸

BIOFILM FORMATION ASSAY AND ANTIMICROBIAL SUSCEPTIBILITY TESTING (AST)

Opportunistic isolates identified as *Enterobacter* spp., *Klebsiella* spp., *Citrobacter* spp., *Aeromonas* spp., *Pseudomonas aeruginosa*, *Escherichia coli* (*E. coli*), and *Acinetobacter baumannii* ($n = 137$) were assessed for *in vitro* biofilm potential using a crystal violet microtiter plate assay. Cultures adjusted to 0.5 colony forming unit/milliliter (CFU/mL) McFarland standard (1.5×10^8 CFU/mL) were incubated in M9 minimal media supplemented with casamino acids and glucose. Biofilm formation was quantified by crystal violet staining, solubilized in ethanol, and read at Optic Density (OD) value of 570 nanometers. Biofilm potential classification followed established OD cut-off values as described by Leoney et al., 2020.⁹ Isolates identified as moderate ($n = 40$) or

strong ($n = 52$) biofilm producers were subjected to AST using Sensititre Gram-negative plates containing 14 antimicrobials (Thermo Scientific, Fisher). MICs were interpreted according to Clinical and Laboratory Standard Institutes 2023 breakpoints, and MDR was defined as resistance to ≥ 3 antimicrobial classes.¹⁰

CHLORINE SUSCEPTIBILITY AND IMPACT ON BIOFILM FORMATION INVESTIGATION STUDIES

The 92 bacterial isolates previously classified as moderate or strong biofilm formers were evaluated for chlorine susceptibility. A two-fold serial dilution of 12 ppm sodium hypochlorite was prepared in 96-well plates, and approximately 7.5×10^5 CFU/mL of each strain was added per well. Plates were incubated aerobically at 37°C, and the MIC was defined as the lowest chlorine concentration that inhibited visible bacterial growth. Furthermore, each strain was tested for biofilm formation in the presence of sodium hypochlorite using a crystal violet microtiter plate assay. Chlorine dilutions were prepared as described above and added to the wells. After a 24-hour incubation, biofilm biomass was quantified at OD570. OD cut-off values were determined using *E. coli* BAA 2469 as the negative control, and the MIC impacting biofilm was defined as the lowest chlorine concentration where OD570 fell below twice the cut-off threshold.



ABOVE: The 96-well plate with Mueller-Hinton Broth supplement added to columns 2-12 and chlorine solution (sodium hypochlorite solution, 12%) added to columns 1 & 2. Serial dilutions from columns 2-11. *Column 12 left without chlorine disinfectant (growth control). Bacterial suspension added to each well (final concentration 7.5×10^5 CFU/mL). ***ROW E demonstrates strong biofilm potential (in response to chlorine exposure) of pathogenic, multidrug-resistant *P. aeruginosa* strain cultured from chlorine-treated water sample.**¹¹

GENOMIC ANALYSIS OF CHLORINE-TOLERANT ISOLATES

DNA was extracted from selected from MDR *Klebsiella* spp. isolates ($n = 25$) and quantified via Qubit 4.0. Whole genome sequencing was performed on the Illumina MiSeq platform (2 × 250 bp, v3 chemistry).¹² Contigs ≥ 200 bp were assembled using the Bacterial and Viral Bioinformatics Resource Center (BV-BRC), BV-BRC Genome Assembly Services, and analyzed through the Amazon Machine Image Pipeline for antimicrobial resistance and virulence factors.¹³ Phylogenetic analysis was visualized using Interactive Tree of Life (iTOL) software.

Results

Over a 9-month sampling period, 178 environmental samples were collected from four North Carolina WWTPs, with 77% of surface samples and 28% of effluent samples yielding culturable isolates. *Enterobacter* spp. and *Klebsiella* spp. were predominant among surface isolates, while *Aeromonas* spp. represented the preponderance of effluent water samples. Notably, 68% of all surface isolates exhibited moderate or strong biofilm formation potential *in vitro*, with *Klebsiella* spp. showing the highest prevalence of strong biofilm production. AST assays revealed that 58% of moderate and strong biofilm formers were MDR, with chlorine-treated isolates accounting for the majority of MDR phenotypes. Importantly, all isolates remained susceptible to ciprofloxacin, suggesting limited fluoroquinolone resistance and demonstrating its potential anti-biofilm properties. Repetitive-polymerase chain reaction fingerprinting confirmed high genetic diversity across isolates with no clear clustering by treatment type, indicating a heterogeneous bacterial population. These findings

TABLE 1: PHENOTYPIC AST RESULTS

Treatment/ Sample Type	MDR Count	MDR Percent
Chlorine/Surface	24	45%
Chlorine/Water	10	19%
UV/Surface	19	36%
UV/Water	0	0%

TABLE 1: Phenotypic AST Results: 58% ($n = 53$ of 92) of the moderate and strong biofilm potential strains displayed MDR.¹⁴

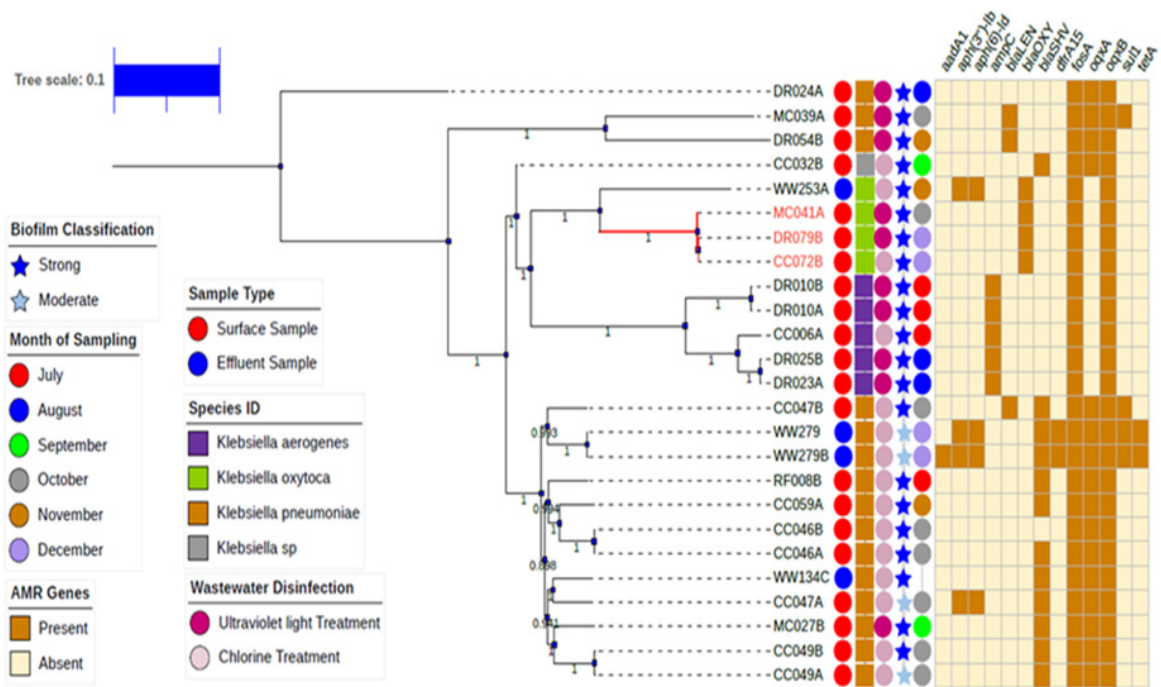


FIGURE 1: Phylogeny tree comprised of *Klebsiella* spp. strains constructed with iTOL (version 6.9.1) demonstrates AMR gene prevalence and sample collection factors.¹⁵

highlight the limitations of both UV and chlorine disinfection in mitigating biofilm formation and antimicrobial resistance, reinforcing the need for integrated control strategies and deeper exploration of molecular resistance mechanisms in water treatment systems.

Minimum inhibitory concentration (MIC) assays revealed that over half of isolates from both chlorine-treated (57%) and UV-irradiated (50%) WWTPs maintained a chlorine tolerance of 12 ppm. No significant differences were observed in MIC values across treatment types, sample sources, or initial biofilm classifications. However, follow-up biofilm assays under chlorine stress showed statistically significant associations: isolates from UV-treated WWTPs and surface samples exhibited higher chlorine MICs and biofilm formation capacity ($p < 0.05$). Strong biofilm producers had notably higher odds (OR = 5.42) of chlorine tolerance. These findings suggest that biofilm-forming capacity may confer increased resistance to disinfection, with UV-treated isolates potentially compensating for lack of residual disinfectant by enhancing biofilm development. Interestingly, several effluent water isolates from chlorine WWTPs—despite showing high MICs—failed to produce biofilm under chlorine exposure, indicating potential chlorine-induced damage beyond recovery thresholds.

Whole genome sequencing of 25 *Klebsiella* spp. MDR strains (with moderate or strong biofilm potential) confirmed high genetic diversity and widespread resistance. All strains harbored at least one AMR gene, and 84% amplified AMR gene presence, with β -lactam and fosfomycin resistance being most prevalent. Virulence genes (indicating pathogenicity) such as *fimH*, *iutA*, *iroN*, and *traT* were commonly detected, with chlorine-derived strains showing higher frequencies of *fimH* and *traT*, suggesting possible selection pressures from residual chlorine. The presence of *fimH*, associated with adherence and biofilm initiation, was significantly higher among chlorine-treated isolates (80%) versus UV-treated (30%). These results strongly indicate the potential role of disinfectant type in shaping both phenotypic resistance and virulence traits in *Klebsiella* spp. and necessitate the need for targeted biofilm-disruption strategies and deeper molecular characterization in water disinfection management.

Conclusion

The threat posed by AMR is no longer a distant theoretical risk; it is an emerging biological reality with profound implications for military readiness, national security, and global public health. AMR has demonstrated the potential to erode the effectiveness

of current medical countermeasures, compromise water safety, and effectively overwhelm healthcare and logistical systems during conflict or humanitarian crisis.

The findings from the recent experimental studies further reinforce the urgency of this challenge. Characterization of chlorine-resistant Gram-negative bacteria isolated from US wastewater treatment plants revealed a concerning prevalence of multidrug resistance (58%) among moderate-to-strong biofilm-forming isolates, with 92% demonstrating resistance to chlorine concentrations commonly employed for water disinfection. The persistence of these bacterial populations under chemical disinfection pressures, compounded by enhanced survival through biofilm formation, highlights a dual challenge: the erosion of existing public health defenses and the growing opportunity for adversarial exploitation of resilient pathogens.

These experimental outcomes emphasize that combating the AMR threat demands not only innovation in medical countermeasure development, but also a fundamental re-examination of disinfection protocols, surveillance strategies, and biodefense frameworks. Bridging laboratory evidence with operational preparedness is essential. Only through integrated research, policy adaptation, and proactive security environment-focused initiatives can we preserve operational resilience and effectively mitigate the evolving biological threats of the 21st century as WMD threats expand beyond the nuclear domain. ■

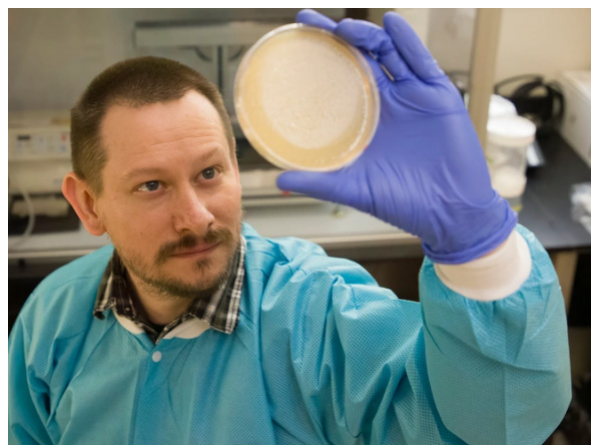


FIGURE 2: Matthew Henry, a research associate with the Naval Medical Research Command's (NMRC) Biological Defense Research Program (BDRD) Directorate, observes signs of successful infection of bacterial agents by isolated bacteriophage products during a separate study. (U.S. Navy photo by Mike Wilson/Released)

Key Points:

AMR is a critical factor in the DOD's biological defense efforts due to its potential to:

- Undermine the health and readiness of the Total Force as a naturally occurring threat.
- Possible exploitation as a characteristic of biological weapons targeting water systems and populations.
- Enhancement or engineering through advancing biotechnology, leading to more complex and resistant biothreats.
- Influencing by current disinfection practices and lack of antibiotic stewardship, exacerbating the problem.
- AMR necessitates the development of novel and adaptive medical countermeasures.
- AMR require ongoing advanced research and development to anticipate and counter future AMR threats.

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