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Plasmid profiling of multiple antibiotic-resistant *Pseudomonas aeruginosa* isolated from soil of the industrial area in Chittagong, Bangladesh

Asma Talukder¹, Md. Mijanur Rahman^{2*} , Mohammed Mehadi Hassan Chowdhury²,
Tanha Amina Mobashshera³ and Nazneen Naher Islam³

Abstract

Background: Multiple antibiotic-resistant (MAR) *Pseudomonas aeruginosa* (*P. aeruginosa*) plays a significant role in triggering nosocomial infection in clinical settings. While *P. aeruginosa* isolated from the environment is often regarded as non-pathogenic, the progressive development of antibiotic resistance necessitates exploring the MAR patterns and transposable genetic elements like plasmid in the isolates.

Results: Using *ecfX* gene-based PCR, 32 *P. aeruginosa* isolates among 48 soil samples collected from the industrial region have been confirmed. The antibiotic susceptibility pattern of those isolates revealed that 5 (15.63%) of them were resistant to a range of antibiotics, and they were categorized as MAR isolates. Nevertheless, all MAR isolates were found resistant to piperacillin and gentamicin, but none of them to ceftazidime, aztreonam, and ciprofloxacin. Moreover, the isolates were also showed resistance to amikacin (60%), tobramycin (80%), netilmicin (80%), imipenem (60%), doripenem (40%), meropenem (60%), and cefixime (40%). Furthermore, 60% of MAR isolates possessed double plasmids of 1000–2000 bp sizes which indicates the distribution of antibiotic resistance genes in MAR *P. aeruginosa* might be correlated with the presence of those plasmids. The MAR index's high threshold values (> 0.20) implied that the isolates were from high-risk environmental sites where the presence of numerous antibiotic residues happened.

Conclusions: These findings highlighted the presence of multiple antibiotic resistance in *P. aeruginosa* of the industrial soil and a considerable prospect of transferring antibiotic resistance genes in the microbial community by plasmids. We recommend taking immediate stringent measures to prohibit the unnecessary and overuse of antibiotics in agricultural, industrial, or other purposes.

Keywords: *Pseudomonas aeruginosa*, Multiple antibiotic-resistance, Plasmids, Soil

* Correspondence: mijanur@nstu.edu.bd; mijan.cu91@gmail.com

²Department of Microbiology, Noakhali Science and Technology University, Noakhali 3814, Bangladesh

Full list of author information is available at the end of the article



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1 Background

Pseudomonas aeruginosa is omnipresent in soil, plants, hospital wastewater, bathrooms, tubs, and bathtub faucet [1]. It has been considered a principal causative agent of hospital-acquired infections worldwide and accounts for around 10% of its annual incidence. Typical antibiotic regimens against *P. aeruginosa* are progressively getting unsuccessful owing to the emerging drug resistance that eventually poses a perpetual threat to public health [2]. Moreover, it is also a leading cause of ventilator-associated pneumonia, cystic fibrosis, meningitis, abscess, infections of the cornea, soft tissue and urinary tract, catheter-related infections, and conjunctival erythema [3]. It often induces crippling recurrent lung diseases in cystic fibrosis patients, immunocompromised, and patients undergoing chemotherapy [4]. Furthermore, the Centers for Disease Control and Prevention (CDC) stated that almost 51,000 healthcare-associated *P. aeruginosa* infections arise annually in the USA, in which around 6000 (13%) are triggered by multidrug-resistant (MDR) *P. aeruginosa* responsible for 400 deaths per annum [5]. National Healthcare Safety Network documented that *P. aeruginosa* is the sixth top etiologic agent causing hospital-acquired infection, and it holds the second position in causing ventilator-associated pneumonia within hospitals of the USA [6].

Soil plays a significant role as a source of nutrients in the environment, works as a shelter for plants and other species, and even serves as a big bioreactor, where pollutant decomposition and nutrient alteration occurs. Moreover, it is also a location where an encounter between local microorganisms and antibiotics happened washed with natural fertilizers and wastewaters into the soil [7]. In the past few decades, soil bacteria have been incrementally antibiotic tolerant even though more stringent antibiotic usage laws exist in medicine and agriculture [4]. The regions most influenced by human usage, where the interaction between clinical pathogenic isolates and environmental isolates happened, and the presence of several antimicrobial selective pressure together, is ideal for exchanging and disseminating resistance genes. It has been reported that the prevalence and distribution of antibiotic-resistant genes are comparatively higher in the antibiotics-producing bacterial community. Consequently, bacteria work as a pool of resistant genes integrated into the broad microbial population [8, 9].

P. aeruginosa can survive in divergent environments though typically found in low numbers [10, 11]. Industrial areas, including tanneries, garments, ship breaking, pulp and paper, refineries, food, fertilizer, textile, pharmaceuticals, steel, chemical, and other agro-based industries, are the potent source of environmental pollution as these sites expose their waste byproducts into their

surroundings in a known or unknown way [12]. These industrial zones are enriched with different nutrients, including inorganic and organic residues, toxic organic components, and antibiotic-resistant pathogens [13], which pose severe threats to the environment [14]. *P. aeruginosa* has been commonly found in hydrocarbon-polluted areas and could be superior among hydrocarbon reducing bacteria. As a temporary habitat of bacteria, the functions of non-clinical environments remain in dispute [11, 15]. Although *P. aeruginosa* is recommended for using in bioremediation or plant protection [11], there is a high chance for wide distribution of pathogenic bacteria if it carries virulent genes [16], which will eventually bring adverse effects on public health. Therefore, it is highly demanded to screen the prevalence of antibiotic-resistant *P. aeruginosa* in the soil in the industrial regions. *P. aeruginosa* is also described by inherent multidrug resistance and the capacity to establish high-level (acquired) antibiotic resistance via several processes, including the acquisition of transposable genetic materials, plasmids, and integrons, conclusively crucial for the spread of multidrug resistance in Gram-negative bacteria, particularly in *Pseudomonas* [17]. Plasmid profiling comes up with a fast and reliable means of identifying bacterial isolates of the same strain and assistance to examine bacterial plasmid content [18]. These plasmids could be the potential carriers of antibiotic-resistant genes among bacteria in the environment. Plasmids can be passed in bacteria within the same or different genera by conjugation and transduction though resistant genes are disseminated by replication [19, 20]. Moreover, the plasmid transition requirement such as pilli synthesis is typically encoded in the plasmids by genes. Plasmids may also carry genes that code for other functions, such as bacteriocin synthesis, which triggers antibiotic resistance effectively. Hence, *P. aeruginosa* is a leading critical organism to control due to its intrinsic virulence factors and plasmid-mediated acquired antibiotic resistance [21, 22].

To understand the contribution of *P. aeruginosa* in disseminating antibiotic resistance in environmental bacteria, we have conducted a cross-sectional study in the industrial area of the Chittagong district in Bangladesh. In this study, we screened and characterized multiple antibiotic-resistant (MAR) *P. aeruginosa* based on plasmid profiling and calculated the MAR index value to determine the high-risk contamination source.

2 Methods

2.1 Sample collection and isolation of *Pseudomonas aeruginosa*

A total of 48 soil samples were collected from eight different locations in the industrial area of Chittagong, Bangladesh, following the simple random sampling

method from March 2015 to April 2015. The samples (each sample's weight was 500 grams) were taken from 50 mm underneath the land surface in sterile pots and immediately sent to the laboratory. Serial dilutions of the samples were performed up to 10^{-10} in normal saline. Then, 200 μ L of each dilution was spread to cetri-mide agar media (Oxoid, UK) separately and incubated at 37 °C for 72 h [9]. All isolates have been purified and freshly sub-cultured on Luria-Bertani (LB) agar media at 37 °C for 24 h [23]. Morphological (size, shape, gram reaction) and biochemical (H_2S generation, reduction of nitrates, and fermentation of various carbohydrates) characteristics were determined [24].

The *ecfX* gene was detected as a stable genetic marker encoding an ECF (extracytoplasmic function) sigma factor and confined to *P. aeruginosa* from environmental samples; a species-specific PCR-based *ecfX* gene assay has been performed to validate the detection of *P. aeruginosa* from soil samples [25]. Selected *P. aeruginosa* isolates were then confirmed by *ecfX* gene-based PCR. Bacterial chromosomal DNA was extracted following the conventional heat-thaw method. The PCR has been done employing a thermal cycler (GeneAmpR PCRSystem 9700, Applied Biosystems) following the protocol delineated by Talukder et al. [26]. The forward and reverse primers had the following sequences- Primer 1 (ECF5), 5'-AAGCGTTCGTCCTGCACAA-3', and Primer 2 (ECF2), 5'-TCATCCTTCGCCTCCCTG-3', respectively [25, 26].

2.2 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing has been performed according to the Kirby-Bauer disc diffusion method on Mueller–Hinton agar (Oxoid, UK) following the Clinical Laboratory Standards Institute guidelines [9, 27]. The standard antibiotic discs of piperacillin (PIP) (100 μ g), gentamicin (GEN) (10 μ g), amikacin (AMK) (30 μ g), tobramycin (TOB) (10 μ g), netilmicin (NET) (30 μ g), imipenem (IPM) (10 μ g), doripenem (DOR) (10 μ g), meropenem (MEM) (10 μ g), ceftazidime (CAZ) (30 μ g), cefixime (CFM) (5 μ g), aztreonam (ATM) (30 μ g), and ciprofloxacin (CIP) (5 μ g) were used. *P. aeruginosa* were designated as susceptible (S), intermediate (I), or resistant (R) according to CLSI guidelines [27, 28].

2.3 Multiple antibiotic resistance (MAR) indexing

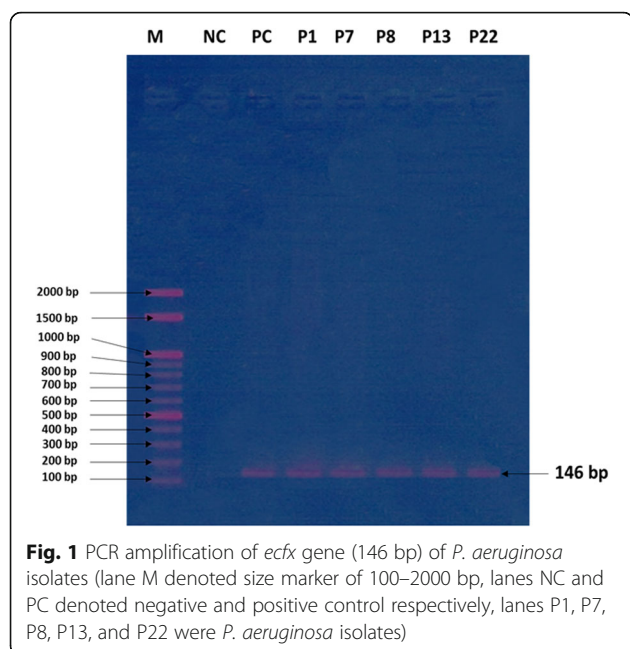
P. aeruginosa were regarded as multiple antibiotic-resistant (MAR) if any of them showed resistance to two or more antimicrobial drugs [9]. MAR index values were calculated following the procedure illustrated by Osundiyi et al. [29]. MAR index for a single isolate was calculated as the following formula: "Number of antibiotics to which isolate is resistant (a)/Total number of antibiotics against which isolate was tested (b)" [29].

2.4 Plasmid profiling of multiple antibiotic-resistant *P. aeruginosa*

Plasmid DNA extraction was carried out employing the standard alkaline lysis protocol delineated by Sambrook et al. [30]. Three complex solutions such as solution-I (50 mM glucose; 10 mM EDTA, pH 8.0; 2 μ M Tris-HCl, pH 8.0), solution-II (0.2 N NaOH; 1% SDS), and solution-III (5 M potassium acetate; 5 M glacial acetic acid) have been prepared for this purpose. Two milliliters of freshly cultured *P. aeruginosa* was centrifuged for 10 min at 10,000 rpm; then, the pellet was suspended in solution-I. The solution-II has been applied to that mixture for lysing the bacterial cells. Then, solution-III was added to the mix and recentrifuged to precipitate genomic DNA. The supernatant was collected and treated with phenol-chloroform solution, and the upper layer of the solution was separated into another Eppendorf tube, then RNase (20 μ L/mL) added to it. The mix was then centrifuged at 4 °C for 10 min at 10,000 rpm. The pellet was taken and washed with ethanol twice. Then, the pellet has been mixed in 50 μ L TE buffer (pH 8.0). Finally, the plasmid DNA has been visualized performing 1.5% agarose gel electrophoresis using ethidium bromide as a staining agent under UV light [12].

3 Results

A total of forty-eight soil samples have been collected from different locations of the industrial area in Chittagong, and 32 *P. aeruginosa* isolates were identified. Antibiotic susceptibility testing of the isolates revealed that 5 (15.63%) of them were multiple antibiotic-resistant (MAR) (Table S1). The MAR *P. aeruginosa* isolates P1, P7, P8, P13, and P22 (Fig. 1) were found resistant to six, eight, seven, and six antibiotics, respectively (Table 1). This study revealed that all MAR *P. aeruginosa* (5, 100%) were resistant to piperacillin which belongs to the penicillin group antibiotics. In the aminoglycosides group, 100% of MAR isolates found resistant to gentamicin, 80% to tobramycin and netilmicin, and 60% to amikacin. Nearly half of the MAR isolates were resistant to carbapenems, where 60% to imipenem and meropenem and 40% to doripenem. Moreover, against third-generation cephalosporins, 40% of MAR isolates were resistant to cefixime and none to ceftazidime. In contrast, all MAR isolates were found sensitive to aztreonam (monobactams group) and ciprofloxacin (fluoroquinolones group) in this study (Fig. 2, Table 1). Nonetheless, though our primary focus on the antibiotic resistance of MAR isolates, it is worth to be mentioned that all the 32 *P. aeruginosa* were sensitive to aztreonam and ciprofloxacin. Furthermore, the MAR index of the isolates revealed that all *P. aeruginosa* were found with greater than



0.20 index values (0.33 to 0.67) which implied that they were from high-risk environments (Table 1). In addition, the plasmid profiling of the MAR *P. aeruginosa* showed that 60% of them possessed double plasmids of 1000–2000 bp sizes, and rest of the isolates did not have any plasmids (Fig. 3).

4 Discussion

A study by Kaszab et al. [31] reported that soil-derived *P. aeruginosa* showed high resistance against two distinct groups of antibiotics at minimum, particularly wide spectrum penicillins, third-generation cephalosporins, carbapenems, and aminoglycosides. These findings supported our observations and indicated a strong and alarming antibiotic resistance profile of environmental isolates of *P. aeruginosa*, which could lead to a massive distribution of antibiotic-resistant isolates in a broad microbial community. Besides this, Deredjian et al. [32] found that 14% of the *P. aeruginosa* were resistant up to five antibiotics (gentamicin, ticarcillin, tobramycin, imipenem, clavulanic acid, and ciprofloxacin), whereas, in contrast, none of the MAR isolates in this study showed

resistance against ciprofloxacin and ceftazidime. Likewise, our findings, a contemporary study [11], stated that the imipenem-resistant *P. aeruginosa* were exclusively isolated from hydrocarbon-contaminated samples. The environmental stress from metals and hydrocarbons could make antibiotic-resistant bacteria more prevalent in polluted soils. There is a crucial little-known association between contaminated soil and antibiotic resistance. It is an eminent need to clarify the danger of horizontal gene transfer among clinical pathogens, soil microorganisms, and commensal bacteria; and a better understanding of environmental triggers for gene transfer [33]. Unlike our findings, a similar study by Pitondo-Silva et al. [9] reported that most *P. aeruginosa* isolated from soil were resistant to aztreonam and ticarcillin significantly and resistant isolates possessed plasmids and class 1 integron.

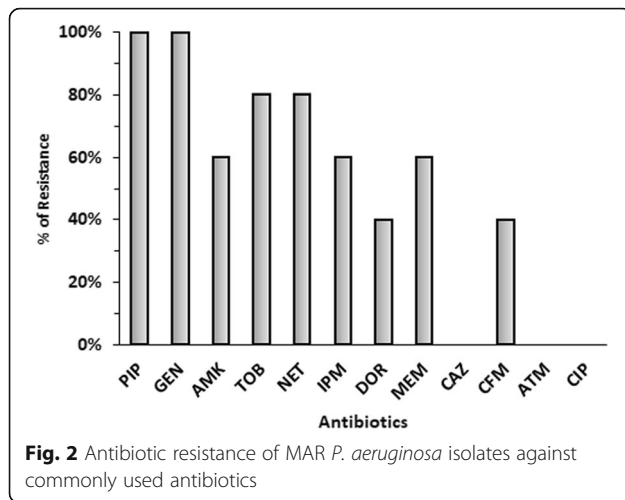
In the USA, it has been estimated that almost 26,000 *P. aeruginosa* isolates were recovered annually. Among them, around 19.3% were resistant or intermediately susceptible to one carbapenem antibiotics at a minimum level. In contrast, roughly 14% of them found as multidrug-resistant exhibited extreme resistance facilitated by several antibiotic resistance mechanisms such as modification of the target sites, beta-lactamase production, porin alterations, and antibiotic efflux [34]. Beta-lactamase enzymes production is considered an essential mechanism of resistance commonly used by *P. aeruginosa* [35], even though it can demonstrate tolerance to non-beta-lactam antibiotics via the modification of the antibiotic target site. However, fluoroquinolones and aminoglycosides are two antibiotic groups affected by the resistance process [34, 36].

The MAR index of bacteria is commonly considered to determine the sampling sites targeting high-risk contamination sources [37, 38]. It is a practical, cost-effective, and justifiable technique of tracking the source of multiple antibiotic-resistant bacteria by “calculating the ratio of the number of resistant antibiotics to which the organism is resistant to, and the total number of antibiotics to which the organism is exposed” [39]. The multiple antibiotic resistance (MAR) indexing values of isolated *P. aeruginosa* were recorded from 0.33 to 0.67. The value of the MAR index above 0.2 admitted the

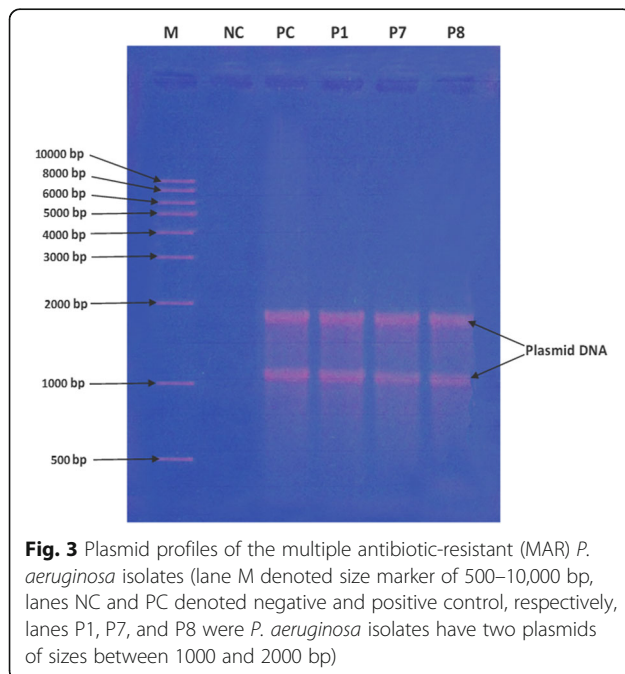
Table 1 Multiple antibiotic resistance (MAR) pattern of *Pseudomonas aeruginosa* isolates with MAR index values

| Sample ID | Isolate ID | Resistance pattern | Number of resistant antibiotics (a) | MAR index (a/b) |
|-----------|------------|--|-------------------------------------|-----------------|
| CIS-3 | P1 | PIP, GEN, AMK, NET, IPM, MEM | 6 | 0.5 |
| | P7 | PIP, GEN, AMK, TOB, NET, IPM, MEM, CFM | 8 | 0.67 |
| CIS-18 | P8 | PIP, GEN, AMK, TOB, NET, DOR, CFM | 7 | 0.58 |
| | P13 | PIP, GEN, TOB, NET, DOR, MEM | 6 | 0.5 |
| CIS-31 | P22 | PIP, GEN, TOB, IPM | 4 | 0.33 |

a, number of antibiotics to which isolates are resistant; b = 12, total number of antibiotics against which isolates were tested



survival ability of the isolates in a highly polluted atmosphere, and this higher contamination could result from an abundance of multiple antibiotic residues in sites [30, 40]. Resistance to multiple antibiotics in environmental bacteria without direct selection pressure could be implicated for several instances, such as the introduction of antibiotics in the environment and spreading resistance gene sequences. Some considerable ways that contribute to disseminating antibiotics and resistant strains of *P. aeruginosa* could be patients, public antibiotic usage, the flow of waste and sewage to the environment, employing bacteria in bioremediation purposes, use of antibiotics in agriculture, and certain actinomycetes in soil. In the coming days, as we see already, the multiple antibiotic-



resistant *P. aeruginosa* would be increased alarmingly in both the hospital settings and in the environment [41].

Plasmid profiling of the *P. aeruginosa* showed that 60% of the MAR isolates in this study had double plasmids of 1000–2000 bp sizes. The findings of Akingbade et al. [18] showed that eight (36.4%) multidrug-resistant *P. aeruginosa* isolated from human wound specimens possessed plasmid, wherein two (25%) of them had double, and six (75%) of them contained a single plasmid. All plasmids harboring isolates showed resistance to amoxicillin, ampicillin, erythromycin, tetracycline, cotrimoxazole, and cloxacillin [18]. Likewise, our findings, Odumosu et al. [42] reported that the *P. aeruginosa* isolated from humans, animals, and plants had versatile plasmid profiles with copies of 1 to 5 and size ranges < 1000 bp to \geq 23,000 bp. Furthermore, a study has shown that *P. aeruginosa* lost their resistance markers after losing their plasmids [43]. Thus, it can be construed that a plasmid possibly facilitated the antibiotic resistance of those bacteria. Mobile genetic materials like plasmids are responsible for disseminating resistance genes among the bacterial community and act as a transporter for other resistance mechanisms [42]. In Gram-negative bacteria, plasmids are harboring antibiotic-resistant genes that facilitate numerous antibiotics resistance [44]. These self-governing DNA molecules can transmit between cells and turn on a portion of the chromosome via high-recurrence recombination [20]. Analysis of plasmids and their functions provide essential insight into antibiotic resistance. Genotyping and other molecular characterization of plasmids could help to determine the reasons for occurring antibiotic-resistant genes. Plasmids are significantly implicated in horizontal gene transfer in intimate bacterial families disseminating antibiotic-resistant genes in the environment. Therefore, plasmid profiling could assist in the routine surveillance of outbreaks and tracking antibiotics resistance [22].

5 Conclusion

The occurrence of multiple antibiotic-resistant *P. aeruginosa* with harboring plasmids in environmental samples such as soil from the industrial area signifies the possibility of widespread dissemination of antibiotic resistance in microbial communities through *P. aeruginosa*. The obtained data in this study is not only alarming but also scary for the public health sector. Effective control measures are highly recommended to reduce contamination in environmental sites. The spread of antibiotic-resistant pathogens must be controlled in constructive and realistic ways. Nonetheless, to figure out the whole scenario of the antibiotic resistance pattern of *P. aeruginosa* and its possible consequences in both environment and health sector, region-specific, large-scale, and more comprehensive studies are needed.

6 Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s43088-021-00131-w>.

Additional file 1: Table S1. Antibiotic susceptibility test results of all 32 *Pseudomonas aeruginosa* isolates.

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Authors' contributions

AT and NNI designed the study. AT and TAM have carried out the collection of soil samples and laboratory research. MMR and AT have done the data analysis and prepared the draft manuscript. NNI, MMHC, and MMR edited and critically reviewed the manuscript. The authors have read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Author details

¹Department of Biotechnology and Genetic Engineering, Noakhali Science and Technology University, Noakhali 3814, Bangladesh. ²Department of Microbiology, Noakhali Science and Technology University, Noakhali 3814, Bangladesh. ³Department of Genetic Engineering and Biotechnology, University of Chittagong, Chattogram 4331, Bangladesh.

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