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ISOLATION OF MULTIDRUG-RESISTANT *ACINETOBACTER CALCOACETICUS* STRAINS FROM RHIZOSPHERE OF TOMATO

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Abstract

Acinetobacter species have significant challenges as pathogens because of their resistance to various antibiotics. Most studies have focused on clinically significant multidrug-resistant *A. baumannii* strains. This is the first report on multidrug-resistant *A. calcoaceticus* strains from soil in Bangladesh. A total of four tetracycline-resistant *A. calcoaceticus* strains were isolated from the rhizosphere soil of a tomato field. To profile their multidrug resistance patterns, the strains were examined by the Kirby-Bauer disk diffusion method using ten antibiotics from seven different classes. Multidrug-resistant strains were identified by 16S rRNA gene analysis and these strains were found to be highly resistant to ampicillin, erythromycin, and colistin. Among the four strains, the tetracycline-resistant BOTRUEML127 demonstrated the most significant resistance profile against eight antibiotics belong to five different classes. Multidrug-resistant *A. calcoaceticus* indicates a risk of severe infection if the soil isolates are disseminated into the hospital environment.

Introduction

Acinetobacter species are ubiquitous and can be found in various environmental sources such as soil, water, wastewater, landfills, as well as in human, animal, and agricultural products (Doughari *et al*. 2011). These environmental isolates often carry antibiotic resistance genes; therefore, they may serve as an important environmental reservoir for resistance elements that develop into clinically relevant bacteria (Al Atrouni *et al*. 2016). Due to their ability to survive in a diverse environment, *Acinetobacter* species are one of the most common pathogens in hospitals (Espinal *et al*. 2012). Professionals are challenged to formulate strategic treatment plans for patients infected with intrinsically antimicrobial-resistant *Acinetobacter* strains (Vázquez-López *et al*. 2020). *Acinetobacter* strains have shown alarming trends in resistance to several major classes of antimicrobials, including beta-lactams, aminoglycosides, and fluoroquinolones (Weinstein *et al*. 2005). Recent research has reported that *Acinetobacter* spp. can be introduced into the hospital environment via the food chain (Campos *et al*. 2019). Several studies have also highlighted the presence of multidrug-resistant (MDR) *Acinetobacter* spp. in agricultural products (Carvalheira *et al*. 2017). Unfortunately, in recent years, the prevalence of MDR *Acinetobacter* isolates has increased significantly (Weinstein *et al*. 2005), and according to the WHO, *Acinetobacter* are classified among the most serious MDR microorganisms (Boucher *et al*. 2009). Therefore, *Acinetobacter* spp. exposure in the environment should not be ignored as a possible infection agent. The antibiotic resistance and species distribution of *Acinetobacter* spp., especially *A. baumannii* from hospitals have been well studied (Zeana *et al*. 2003). In Bangladesh, most studies reported the prevalence of multidrug-resistant *Acinetobacter* spp. including *A. baumannii* isolated from the clinical environment (Khatun *et al*. 2016). However, data on environmental *Acinetobacter* spp. are scarce in Bangladesh.

Acinetobacter calcoaceticus is one of the members of the *Acinetobacter baumannii-calcoaceticus* (ABC) complex, which is phenotypically similar to *Acinetobacter* species but

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genetically distinct (Glover *et al*. 2022). This complex harbored six species, among which most studies on antimicrobial resistance (AMR) of *Acinetobacter* species have focused on *A. baumannii* in clinical infections (Vázquez-López *et al*. 2020). Strains of *A. calcoaceticus* are also isolated, but less frequently (Glover *et al*. 2022). The occurrence of clinically important MDR *A. calcoaceticus* in agricultural soil is very rare. Therefore, this study aimed to isolate tetracycline-resistant *Acinetobacter calcoaceticus* from agricultural soil and highlight its multidrug resistance profiles to ten antibiotics belonging to seven different classes, particularly fourth-generation antibiotics, which have potential implications for public health.

Materials and Methods

Fresh rhizosphere and bulk soil samples were collected from four commercial tomato fields at Nowhata (24°45′76″N, 88°61′66″E) and Katakhali (24°21′13″N, 89°22′60″E), Rajshahi. A total of eight soil samples of approximately 15 g were collected. The samples were carried to the laboratory on ice. The soil samples were sieved and stored at 4°C in zip-lock bags until further processing.

For each soil sample, a ten-fold serial dilution was made. Then, 100 µL of each diluted sample was inoculated onto nutrient agar supplemented with 32 and 64 µg/ml tetracycline (TC) (Sigma pharmaceuticals). Strains resistant to $\geq$ 32 µg/ml TC were defined as "TC-resistant strain" (Neela *et al.* 2007). The plates were incubated at 37°C for 24 hrs. Finally, morphologically different colonies were selected and purified and kept at -20°C.

The isolated TC-resistant bacteria were tested for their antimicrobial susceptibility to ten antibiotics (HiMedia, India) belonging to seven different classes by the Kirby-Bauer disk diffusion method on Mueller-Hinton medium (HiMedia, India), according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2020). The measured zones of inhibition were compared with the CLSI standard interpretation table and designated as susceptible (S), intermediate (I), and resistant (R). In this study, the term "multidrug-resistant" (MDR) was used for isolates resistant to antibiotics of at least three different antimicrobial classes (Maravić *et al.* 2016).

Among the 48 TC-resistant isolates, 22 isolates were selected for identification based on their antibiogram profiles. To identify the isolates, PCR analysis was performed at a reference laboratory (Central Dogma Lab., Invent Technologies Ltd., Mirpur 2, Dhaka). The 16S rRNA gene sequence was approximately 1,500 base pairs (bp) long. PCR amplification of the 16S rRNA gene was performed using primers 27F (5'-CGGTTACCTTGTTACGACTT-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') as performed by Suresh *et al.* (2022). Sequence data were retrieved, and consensus sequences were created by merging forward and reverse reads using BioEdit Sequence Alignment Editor Version 7.2 (https://bioedit.software.informer.com/7.2/). To identify bacterial species, online similarity searches were performed using the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov/). Phylogenetic analysis was done by using the NCBI database and Phylogeny.fr web-based software (https://www.phylogeny.fr).

Results and Discussion

A total of four multidrug-resistant *A. calcoaceticus* strains were isolated from the rhizosphere soil of a tomato field and identified by 16S rRNA gene sequencing in this study. The raw nucleotide sequences obtained from these four strains were analyzed using BLAST programs and confirmed that the isolates belonged to *A. calcoaceticus.* The phylogenetic tree of the 4 isolated strains was constructed, showing their relationship with each other (Fig. 1). Partial 16S rRNA sequences have been deposited in GenBank, detailed data are provided in the supplementary table. Some reports also mention the isolation of *A. calcoaceticus* from soil in Canada (Ho *et al*. 2020) and from the rhizosphere zone of *Zea mays* and *Brassica oleracea* (Uniyal *et al*. 2016, Ghoreishi and Etemadifar 2017). In Bangladesh, *A. calcoaceticus* was also reported from rhizosphere soil of sugarcane as diazotrophic bacteria (Hossain *et al*. 2020).

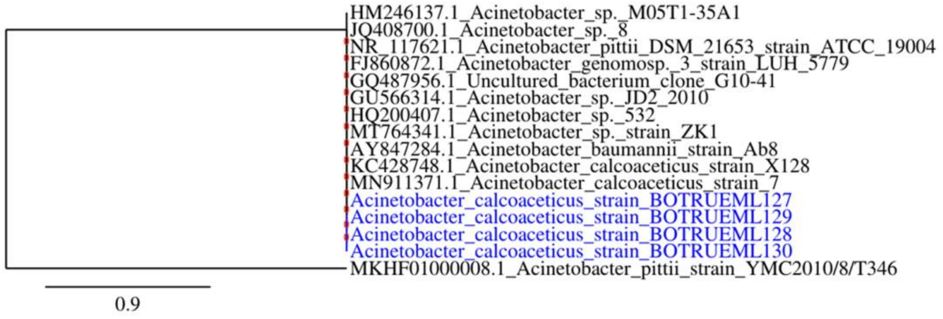

Fig. 1. Phylogenetic tree. Taxa labeled with colored (light blue) indicate the bacterial isolates in the present study. Other sequences were obtained from the NCBI database.

Table 1. Multidrug-resistance profile of TC-resistant *Acinetobacter calcoaceticus*.

			Acinetobacter calcoaceticus							
Class	Antibiotics	Concen-tration	BOTRUEML 127	Category	BOTRUEML 128	Category	BOTRUEML 129	Category	BOTRUEML 130	Category
Tetracyclines	Oxytetracycline	30 µg	14*	I**	11	R	9	R	10	R
Carbapenem	Meropenem	10 µg	6	R	20	S	12	R	20	S
Penicillin	Ampicillin	10 µg	0	R	0	R	0	R	0	R
Penicillin	Amoxicillin	30 µg	0	R	37	S	23	S	28	S
Aminoglycoside	Gentamicin	10 µg	0	R	28	S	25	S	29	S
Aminoglycoside	Kanamycin	30 µg	0	R	16	I	0	R	16	I
Macrolides	Azithromycin	15 µg	0	R	11	R	15	I	15	I
Macrolides	Erythromycin	15 µg	0	R	12	R	0	R	10	R
Polymixins	Colistin	10 µg	0	R	10	R	0	R	8	R
Fluoroquinolone	Ciprofloxacin	5 µg	24	S	26	S	26	S	28	S

*Zone of inhibition (mm), **I: Intermediate, R: Resistant, S: Sensitive.

In this study, the antimicrobial resistance profile of TC-resistant *A. calcoaceticus* strains was presented in Table 1, indicating that all isolates were multidrug-resistant as they were resistant to ≥ 3 different antimicrobial classes (Maravić *et al.* 2016). Here, all TC-resistant *Acinetobacter* strains were resistant to ampicillin, erythromycin, and colistin. One study that directly focused on commercially available clinical *A. calcoaceticus* was resistant to erythromycin and colistin (Glover *et al*. 2022), consistent with the results of the present study. Among the four TC-resistant *A. calcoaceticus* isolates, BOTRUEML127 showed resistance to eight antibiotics belonging to five different classes (Fig. 2). The morphological characteristics of *A. calcoaceticus* BOTRUEML127 strain was presented in Fig. 3. Multidrug-resistant environmental strains of *A. baumannii* from the soil of Mangaluru city, India showed resistance to aminoglycosides, tetracycline, and carbapenems (Suresh *et al.* 2022). *Acinetobacter baumannii* ATCC 19606 and *A. calcoaceticus* share a similar antibiotic resistance profile (Glover *et al*. 2022). In this study, *A. calcoaceticus* BOTRUEML127 showed a similar resistance profile to those reported *A. baumannii* suggesting that isolate BOTRUEML127 could cause significant diseases like *A. baumannii* if it spreads from soil to the hospital settings. The antimicrobial resistance pattern of four *A. calcoaceticus* strains in this study was different, despite being isolated from the same geographic area and phylogenetically closely related, suggesting that antibiotic resistance varies by strain. Strain-dependent and specific antibiotic resistance have also been observed in *Acinetobacter* species (Glover *et al*. 2022). Different resistance patterns of *Acinetobacter* sp. have been isolated from very close geographic areas (Neela *et al*. 2015). This implies that this bacterium has demonstrated exceptional resistance patterns to a wide range of drugs.

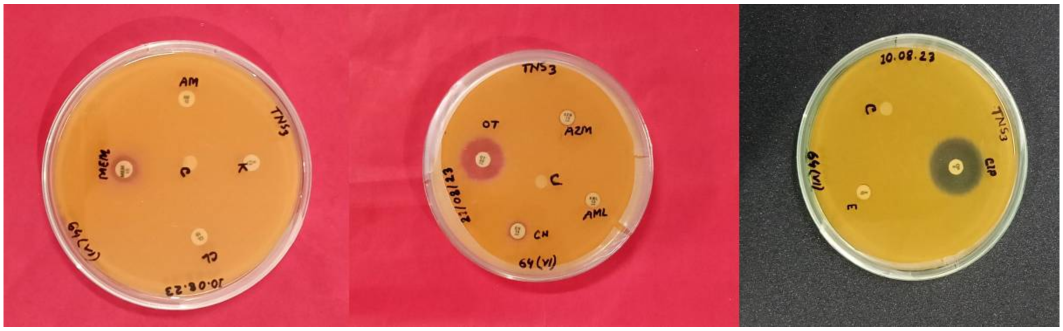

Fig. 2. Multidrug-resistant *Acinetobacter calcoaceticus* BOTRUEML127 strain. MEM: Meropenem, AM: Ampicillin, K: Kanamycin, E: Erythromycin, CL: Colistin, AML: Amoxicillin, CN: Gentamicin, AZM: Azithromycin, OT: Oxytetracycline and CIP: Ciprofloxacin. C: Negative control.

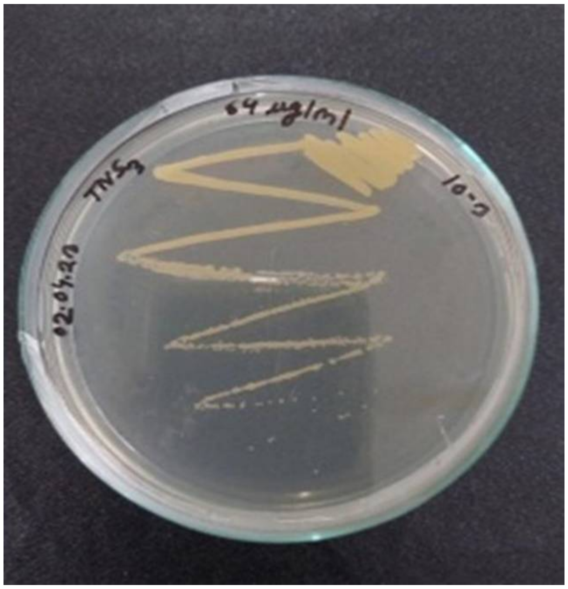

Fig. 3. Representing the morphological characteristics of *A. calcoaceticus* BOTRUEML127 strain.

The emergence of colistin resistance in rhizosphere soil in this study could be due to the application of poultry litter and manure as organic fertilizer. Colistin is commonly used in Bangladesh in poultry farms and animal husbandry to prevent infection and as a growth promoter (Chowdhury *et al*. 2021). Considering the widespread and extensive use of antibiotics as pesticides in South Korea, it is noteworthy that *Acinetobacter* isolates from agricultural areas are mainly resistant to colistin, chloramphenicol, and especially streptomycin (Son *et al*. 2024). In terms of antibiotic resistance, this result implies that environmental strains of *A. calcoaceticus* respond to antibiotics in the same way as clinical strains of *A. calcoaceticus*. Colistin resistance of *A. calcoaceticus* in the agroecosystem can be transmitted to humans via agricultural products, which is a human health concern because, according to Oliveira *et al*. (2019), colistin is currently considered the antibiotic of last resort for the treatment of multidrug-resistant bacterial infections.

In this study, the antibiotic ciprofloxacin significantly reduced the growth of four multidrug-resistant *A. calcoaceticus* strains, consistent with the results of Stenstrom *et al*. (2016). Another study also found that among eight *Acinetobacter* spp. strains, all were susceptible to ciprofloxacin (Sun *et al*. 2014). These data suggest that fluoroquinolone antibiotics may be a potential treatment for patients with *Acinetobacter* infections. To our knowledge, this is the only study specifically examining antibiotic resistance of *A. calcoaceticus* strains from soil in Bangladesh.

This study reported the isolation and identification of MDR environmental *A. calcoaceticus* strains from rhizosphere soil. Their multidrug-resistant nature indicates severe disease if these isolates can be transmitted from soil to hospital. Further studies are needed to identify the antibiotic resistance gene(s) and the pathogenicity of environmental strains with hospital isolates.

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