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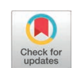
Molecular Characterization and Virulence Profiling of Clinical *Pantoea* spp.: Correlation Between Biofilm Formation and Adhesion Genes



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Abstract

Introduction: *Pantoea* species are emerging opportunistic pathogens that are increasingly implicated in nosocomial infections, particularly among immunocompromised individuals. Understanding their virulence mechanisms, specifically biofilm formation and associated genetic determinants, is essential for addressing these resilient infections.

Objectives: This study aimed to isolate clinical *Pantoea* spp., evaluate their biofilm-forming capacity, and detect the prevalence of virulence-associated genes (*zapA*, *fimH*, and *mrkD*).

Methods: *Pantoea* spp. were isolated from 220 clinical samples and identified using biochemical tests and the VITEK 2 system. Biofilm formation was assessed phenotypically using Congo Red Agar (CRA) and quantified using a Microtiter Plate (MTP) assay. The *zapA*, *fimH*, and *mrkD* genes were detected using Polymerase Chain Reaction (PCR).

Results: Thirty-two *Pantoea* spp. isolates were identified in this study. The MTP assay revealed that 100% of the isolates were strong biofilm producers. The CRA method demonstrated that 56.2% of the isolates produced a strong slime layer. Gelatinase activity was observed in 62% of the isolates. PCR detected *zapA*, *fimH*, and *mrkD* in 37.5%, 56.2%, and 25% of the isolates, respectively.

Conclusion: Clinical *Pantoea* isolates exhibit a robust capacity for biofilm formation, which correlates with the presence of key virulence genes in the biofilm. The synergistic action of adhesion and protease genes likely facilitates persistent colonization and immune evasion, highlighting their pathogenic potential in healthcare settings and underscoring the need for targeted therapeutic strategies against biofilm-mediated infection.

Keywords: Enterobacteriaceae infections, Virulence factors, Fimbriae, Bacterial, Metalloproteases, Cross infection

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Introduction

The genus *Pantoea* comprises pigmented, gram-negative rods that fall within the order Enterobacterales. The genus includes straight, non-capsulated, gram-negative rods, motile via peritrichous flagella, and are facultative anaerobes and non-spore forming(1). *Pantoea* species are infrequent opportunistic pathogens, frequently associated with nosocomial outbreaks, primarily in neonates and immunocompromised patients(2). Plant and opportunistic pathogens cause wounds, bloodstream infections, and urinary tract infections in immunocompromised humans. In the past 20 years, infections caused by these bacteria have caused substantial problems, especially in underdeveloped countries, where they are implicated in high rates of morbidity and mortality and significantly longer hospital stays(3,4). The species most often implicated in human disease is *P. agglomerans*, which can cause a range of infections, including outbreaks in hospitals. There are few reported cases of infection caused by the *Pantoea* genus, although *Pantoea dispersa* has been reported to cause bacteremia(5,6).

Pantoea spp. produce a range of exoproteins that likely aid virulence; gelatinase 31 and serine protease are examples of proteins that have been defined as extracellular factors; they are believed to serve primarily to provide nutrients to the bacteria when degrading host tissue, but they may have some role in the formation of biofilms (7,8). Several potential mechanisms of bacterial biofilm formation have been proposed. These include smear defenses by evading the host immune system and shielding bacteria from hostile environments, foraged habitation in nutritious environments, teaming up to form the quintessential cooperative strategy, and existing as a consortium(9). The primary objective of this study was to determine the genetic basis of biofilm resilience in local *Pantoea* isolates, specifically investigating how the presence of fimbriae and protease genes correlates with their adherence capacity.

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Materials and Methods

Clinical Samples and Patients

A total of 220 clinical specimens were collected from patients with various infections, including urinary tract infections (UTIs), bed ulcers, colitis, otitis media, wounds, burns, and bacteremia. The sampling period will last from March 2025 to June 27, 2025. The sample distribution by type of infection was diabetic foot ulcers (n = 50, 22.7%), UTIs (n = 60, 27.3%), burns (n = 50, 22.7%), gingivitis (n = 35, with 15.9%) and otitis media(n = 25; with 11.4). Clinical information, including age, sex, and type of the infection from each sample.

Specimens was taken with sterile swabs from each infection site and then put into a transport medium to proceed in laboratory. To reduce confounding effects of antimicrobial therapy, samples were collected only from patients who had not received either prescribed (e.g., antibiotics) or unprescribed (including anti-infective herbal medicines) antibiotic treatment in the preceding week before sample collection. At the laboratory, clinical samples were streaked onto MacConkey agar and blood agar plates and incubated aerobically at 37 °C for 24–48 h for bacterial growth and biochemical identification thereafter.

Isolation and Identification of Pantoea spp.

Cultivation on MacConkey Agar

MacConkey agar (HiMedia, India) was prepared as per the manufacturers' instructions by resuspending 51.5 g of dehydrated medium in 1000 mL of distilled water and then filtered through autoclaving. This is selective and differential medium, containing crystal violet and bile salts which inhibit the growth of gram-positive organisms isolating therefore only some Gram negative bacteria including Pantoea spp (10).

Hemolytic Activity on Blood Agar

Evaluation of hemolytic activity as a primary virulence factor was performed by inoculating 20 uL bacterial isolates directly on Blood Agar base supplemented with 5% sterile human blood. Incubated inoculated plates were transferred for 24 h at aerobic temperature (37 °C) and analyzed for hemolytic patterns (α, β or γ hemolysis).

Automated Biochemical Identification

Presumptive *Pantoea spp*. A definitive identification of the isolates was performed using the VITEK 2 automated system (bioMérieux,), in accordance with the manufacturer's specifications. This system offers fast and very accurate biochemical profiling, along with strong species identification at the level required for clinical diagnostics(11).

Phenotypic Detection of Biofilm Formation

Congo Red Agar Method

Slime production, a qualitative measure of biofilm formation, was assessed by the Congo Red Agar (CRA) method, in accordance with established methods(12). Media were prepared by dissolving 52 g of Brain Heart Infusion (BHI) agar in 1000 mL distilled water, where it was autoclaved at 121 °C (15 psi) for 15 min. Concentrated aqueous solutions of Congo red stain (0.8 g/L) and the sucrose solution (50 g/L) were prepared and sterilized by filtration to avoid heat-induced degradation. After cooling to around 55 °C, sterilized dye and sucrose were aseptically dissolved in BHI agar and poured into sterile Petri dishes. This involved the inoculation of isolates onto CRA plates, which were incubated aerobically at 37 °C for 24 h.

The biofilm-producing strains were determined as black colonies that dried out in a crystalline culture form. Strains that are either weak slime producers or non-slime producing colonies also form pink or red colonies with smooth surfaces. Colonies that darkened at the center without showing the hallmark dry crystalline appearance were classified as indeterminate.

Microtiter Plate Assay

The biofilm-forming capacity quantitatively evaluated using the MTP assay, which is widely regarded as a reliable standard for in vitro biofilm quantification(13).

Molecular Characterization and Virulence Profiling

Genomic DNA Extraction

Total bacterial genomic DNA was extracted from the Pantoea spp. isolates with the help of a rapid boiling method, which was found to be an effective and inexpensive forage for PCR-quality templates(14). In short, 100 µL of sterile distilled water was used to suspend several well-isolated colonies in a sterilized microcentrifuge tube. Suspension was incubated in a boiling water bath at 100 °C for 15 min for cell lysis. The tubes were then immediately cooled at -20 °C for 60 min. To obtain the DNA that remained soluble in water, cellular debris was pelleted by centrifugation at 12,000 × g for 10 min and directly transferred to a new sterile tube and store it at −20 °C to serve as the template in subsequent PCR assays.

Primer Preparation and Polymerase Chain Reaction

Genotypic detection was performed consisting of focused PCRs using distinct primer sets targeting important virulence genes fimH (type 1 fimbriae adhesin), zapA (metalloprotease) and mrkD (type 3 fimbriae adhesin). Per the manufacturer instructions, lyophilized primers were reconstituted in a TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA) buffer to stock concentrations of 100 pmol/µL. Working solution (10 pmol/µL): Combine 10 µL of stock solution in 90 µL TE Buffer. All primer sequences and expected amplicon sizes are provided in Table 1.

Forward primer (10 pmol/µL) 2.5 µL, reverse primer (10 pmol/µL) 2.5 µL, ready-to-use PCR premix 5 µ L, extracted genomic DNA template in a total reaction volume of 20 µL was used for the PCR amplifications and diluted to 5 µL using nuclease-free water. In each PCR run, a negative control (containing all of the components of the reaction but template DNA, which was replaced with

sterile distilled water) was also included. They were then vortexed briefly and centrifuged before transfer into the thermal cycler. The details of each specific thermocycling conditions are summarized in the Table 2 per target gene.

Sequencing and Data Analysis

In order to confirm the specificity of PCR amplification, Sanger sequencing was performed for representative PCR amplicons of each target gene. The raw sequence data were filtered to remove background noise and low-quality reads. To accurately validate the identity of these amplified genes, processed nucleotide sequences were aligned by use of Basic Local Alignment Search Tool (BLAST) algorithm with sequences in the National Center for Biotechnology Information (NCBI) GenBank where it is confirmed that these nucleotide sequences are homologous(15).

Statistical Analysis

All experimental data were systematically recorded and statistically evaluated. Categorical variables, such as the prevalence of specific genes or phenotypic traits, were expressed as observed counts and percentage frequencies. Statistical analyses, including correlation assessments to evaluate the relationship between biofilm formation capacity and the presence of specific virulence genes, were performed using PSS software.

Results

Of the 220 specimens obtained from patients, 118 (53.6%) were gram-negative bacteria, and 32 (14.5%) were identified as *Pantoea* spp. There were 60 (27.3%) gram-positive specimens and 42 (19.1%) with no growth on MacConkey and blood agar Figure (1).

Colonies of *Pantoea* spp. on MacConkey agar ranged from pink to pale circles in medium size, smooth with some being mucoid, and others being swarming, punctate, and sluggish with lactose fermentation. On blood agar, the colonies were pale pink to white, smooth, convex, rounded, mucoid, serrated, and non-hemolytic. Under the microscope, *Pantoea* spp. showed the presence of gram-negative bacilli. The drying and biochemical testing results for *Pantoea* isolates are shown in Table (3). Similar characteristics were observed, including oxidase-negative and catalase-positive results. The isolates were able to ferment sugar on Kligler iron agar, producing (Alkaline or Acidic) slants and bottoms (acidic, no H2S present).

The ability of *Pantoea* spp. isolates to produce biofilms was evaluated using two methods: the CRA method and the MTP method.

The ability of 32 isolates of *Pantoea* spp. to produce biofilms was successfully detected using MTP. Biofilms were quantified by measuring the absorbance of the stained biofilms at 630 nm using a MTP reader. Whereas, 100% of the *Pantoea* spp. isolates showed high biofilm production (strong positive adherence) (Table 4).

The results showed that 18/32 (56.2%) of the isolates produced a strong slime layer, as demonstrated by colonies that were dark in color and dry with a crystalline appearance Figure (2), and 10/32 (31.2%) of the isolates produced brown colonies resulting from slime producttion (Figure 3).

A specific PCR primer set was used to detect gene markers, as illustrated in Figure 5. The results indicated that eight (25%) of *the Pantoea* spp. isolates carried the *mrkD* gene.

Pantoea spp. isolates, 12 (37%) were *zapA* gene positive as shown in Figure 6.

Pantoea spp. isolates 18 out of 32 (57%) Pantoea spp. isolates were *fimH* gene positive, as shown in Figure (7).

Discussion

Our findings confirm the substantial pathogenic potential of clinical *Pantoea spp*. that reveals their strong capability for biofilm formation and the presence of critical virulence-associated genes. Among these, we identified as *Pantoea spp*. in 32 (14.5%) of 220 clinical specimens from healthcare-associated infections, highlighting emergence of new opportunistic pathogens in the strains responsible for deteriorating health conditions. This broader capacity for biofilm formation seen in this cohort (100% strong biofilm producers by MTP assay) together with the difference in adhesion genes and protease genes provides evidence that these pathogens employ a multifactorial virulence strategy which may facilitate prolonged colonization and resistance to antibiotic therapy and immune clearance mechanisms.

Table 1. Primer sequences and expected product sizes for the detection of virulence genes in Pantoea spp.

Gene	Primer Sequence (5′–3′)	Product Size	Reference
fimH	F: ATGCGTACTGCTGAACTG R: TCAGGCTTTGATCGTACC	688bp	Designed for Pantoea spp.
zapA	F: GCTGACCTGATGATCAAG R: CTTCTTGGGTCAGCTTCA	563bp	Designed for Pantoea spp.
mrkD	F: GGGGGTGCACAATAGCAGCCACGCGATAGT R: GGGGAAGCTTTGTTTATCAGCGATGCGAAC	240bp	Designed for Pantoea spp.

Table 2. Thermocycling conditions for the amplification of *zapA*, *fimH*, and *mrkD* genes.

Target Gene	Initial Denaturation	Denaturation	Annealing	Extension	Final Extension	Cycles
zapA	95 °C for 5 min	95 °C for 45 sec	58 °C for 45 sec	72 °C for 1 min	72 °C for 10 min	35
fimH	94 °C for 4 min	94 °C for 30 sec	55 °C for 40 sec	72 °C for 60 sec	72 °C for 10 min	30
mrkD	95 °C for 4 min	95 °C for 45 sec	55 °C for 45 sec	72 °C for 45 sec	72 °C for 7 min	30

Table 3. shows conventional biochemical assays for Pantoea spp isolates.

P. stewartti	*P. calida*	*P. ananatis*	*P. agglomerans*	Test
-	-	-	-	Urease
/-+	+	/-+	+	Citrate
-	-/+	-	-	Indole
+	+	+	+	Motility
-	-	-	-	Oxidase
+	+	+	+	Catalase
-	-	-	-	Hemolysis
/+-	+	-/+	+	MR
-/+	+	-/+	+	VP
K/A	K/A, A/A	K/A, A/A	K/A	TSI
-/+	-/+	-/+	-/+	Gas
-	-	-	-	H2S
+	+	+	-	Lactose
-	+	-	+	Maltose
+-/	+	-	-	Sorbitol
+	+	+	+	Sucrose
-	+	-	-	Raffinose
-	-	+	-	Inositol
+	+	+	-	Mannitol
-	+	+	-	Cellibiose
+	-	-	-	Melibiose

(+): 100% strain positive, (-): 100% strain negative (+/-): 50-90% strain positive, (-/+): 50-90% strain negative (A/A): Acidic/Acidic TSI agar

Table 4. Biofilm formation in by MTP Method.

Pantoea spp.	Strong Biofilm (OD range>0.240) No(%)
P. agglomerans	19(100)
P. ananatis	5 (100)
P. calida	7 (100)
Total	32(100)

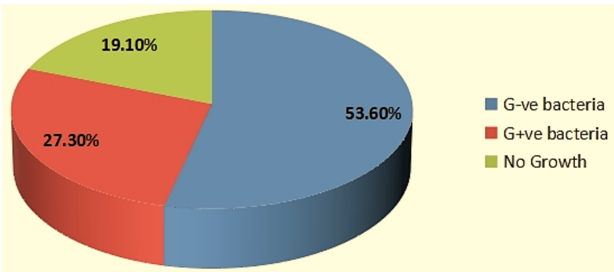

Figure 1. Distribution of pathogenic bacteria revealed a positive culture in 220 specimens

Figure 2. Slime layer production by *Pantoea* spp. on CRA after 24 hours of incubation at 37 °C

Figure 3. Screening of *Pantoea* spp. gelatinase Production

The remarkable 100% prevalence of strong biofilm formation among the clinical isolates aligns with recent genomic analyses identifying biofilms as a primary virulence attribute of *Pantoea* species during host infection(16). Biofilms are complex multicellular communities embedded in an extracellular polymeric matrix that convey increased tolerance to environmental stress, antibiotics, and host immune responses(17). The high rate of slime layer production (56.2%) observed on CRA further corroborates the structural integrity of these biofilms. In clinical settings, the ability to form robust biofilms is a critical factor in the pathogenesis of nosocomial infections, particularly those associated with indwelling medical devices, such as central venous catheters(18). This is particularly relevant given that *Pantoea* agglomerans, the most frequently isolated species in human infections, is often implicated in catheter-related bacteremia and soft tissue infections following penetrating trauma(19).

Genotypic profiling revealed that *fimH* (56.2%) was the most prevalent virulence gene, followed by *zapA* (37.5%) and *mrkD* (25%). The predominance of *fimH* over *mrkD* indicates that type 1 fimbriae-mediated adherence may be the primary mechanism facilitating biofilm initiation in clinical *Pantoea* spp. The *fimH* gene encodes a D-mannose-specific adhesin located at the tip of type 1 fimbriae, which mediates binding to host cell receptors and abiotic surfaces, playing a crucial role in the initial stages of colonization and biofilm formation in Enterobacteriaceae (20). Conversely, the *mrkD* gene, which encodes the adhesin of type 3 fimbriae, was present in a quarter of the isolates. Type 3 fimbriae are heavily implicated in facilitating biofilm formation on both biotic and abiotic surfaces, including medical implants, which is a hallmark of device-associated infections(21). The presence of both adhesin types in the clinical cohort suggests a versatile attachment strategy that allows *Pantoea* spp. to colonize diverse niches within the human host.

Furthermore, the detection of the metalloprotease gene *zapA* in 37.5% of the isolates, coupled with the phenotypic

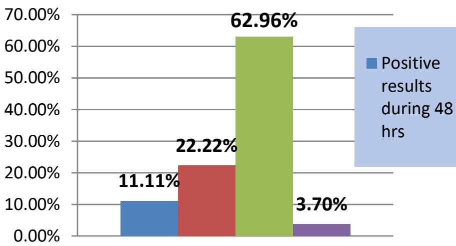

Figure 4. Gelatinase production in *Pantoea* species

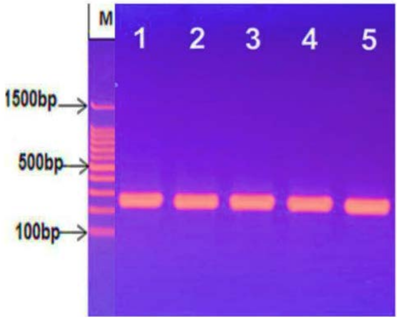

Figure 5. The PCR product of *mrkD* gene in 1% agarose gel electrophoresis, voltage (80 V), time 90 min, and 7μL of PCR product loaded for each well. Lane M: DNA Ladder (10000bp) Lanes 1-5: PCR product (positive case band 240bp)

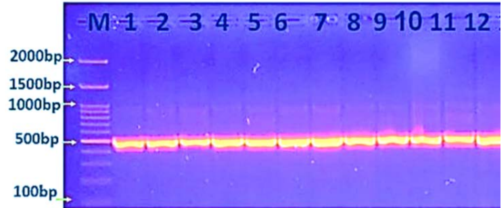

Figure 6. The PCR product of intelligence of *zapA* gene in 1% agarose gel electrophoresis, 80V for 90 minutes sliding 7ul of PCR product in gel as a sample for loading PCR reaction. Lane M DNA Ladder (10,000 bp) Lanes 1-12 :PCR product (positive case 565band bp)

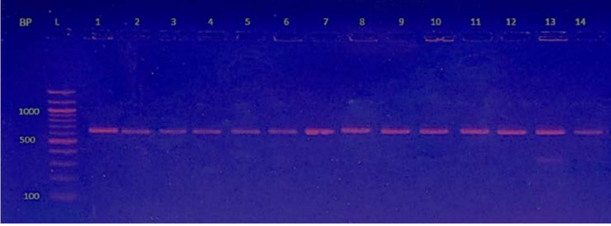

Figure 7. PCR product of *fimH* gene resolved on a 1% agarose gel electrophoresis, voltage (80 V), time 90 min. and loaded 7 μL of PCR product in each well. Lane M: DNA Ladder (10000bp) Lanes,1-14 PCR product (positive case band 688 bp)

observation of gelatinase activity in 62% of the strains, indicates the significant role of secreted proteases in *Pantoea* virulence. Secreted bacterial proteases are key virulence factors that degrade host connective tissue, facilitating tissue invasion and nutrient acquisition(22). Moreover, proteases such as *ZapA* have been shown to degrade a broad spectrum of host proteins, including immunoglobulins and complement components, effectively corrupting the innate immune system and promoting immune evasion(23). The discrepancy between phenotypic gelatinase activity (62%) and *zapA* gene detection (37.5%) suggests that additional unidentified protease genes may contribute to the overall proteolytic phenotype of these isolates (Figure 4).

Durability of *Pantoea* infection is likely a result of synergistic action between its adhesion factors and secreted proteases. *Pantoea* bloodstream infections are rare (0.4% of gram-negative isolates), but they typically occur in patients with multiple comorbid conditions as evidenced by retrospective clinical studies(17). Based on our observations regarding the capacity to form biofilm and virulence arsenal diversity in opportunistic pathogens, we highlight how these highly versatile organisms take advantage of impaired host defense. The biofilm matrix provides physical protection for the bacteria, but also acts as a microhabitat enabling secreted proteases to concentrate locally enough to effectively degrade host tissues and immune effectors(23).

These findings have important implications for infection control and therapeutic development. The recalcitrance of biofilm-mediated infections to standard antimicrobial therapy necessitates the exploration of alternative treatment strategies. Targeting biofilm-specific mechanisms, such as the chemical inhibition of fimbriae-mediated adhesion or the neutralization of secreted proteases, may represent a promising avenue for combating *Pantoea* infections in vulnerable patient populations(24- 28). Furthermore, routine surveillance and molecular characterization of *Pantoea* isolates from clinical specimens should be implemented to monitor the prevalence of virulence determinants and to guide empirical treatment protocols in nosocomial settings.

Limitations of the Study

This study expands our understanding of the virulence mechanisms used by clinical *Pantoea spp*., nevertheless, there are limitations that must be taken into account. First, the 32 *Pantoea* isolates used in this study are limited to the single institution experience and are not necessarily representative of the genetic and phenotypic diversity of *Pantoea* species both geographically within healthcare settings and qualitatively across healthcare settings. Second, the study utilized PCR for the detection of three virulence genes (*fimH*, *zapA* and *mrkD*) only which might underestimate the overall virulence potential of these isolates. Other virulence parameters such as a range of other adhesin classes, secretion systems or genes encoding presence of toxins were not tested. Third, phenotypic characterization of biofilm formation was restricted to the in vitro environment using defined laboratory media; biofilm formation under clinically relevant conditions (e.g., in human serum or on clinical substrates) can vary markedly. In addition, the lack of data on clinical correlates (patient outcomes, responses to antimicrobial therapy and severity of infection) restricted direct correlations between genotypic/phenotypic virulence profiles with clinical manifestations. Fifth, the CRA is a common method but with known limitations of lower sensitivity and specificity when compared to quantitative

molecular methods. Last, the cross-sectional design precludes making causal or temporal conclusions about virulence factor expression and infection status. *Pantoea* future studies, employing multisite sampling with whole-genome sequencing, in vivo infection models, and longitudinal clinical follow-up would not only enhance the understanding of *Pantoea* pathogenesis but also lay ground for therapeutics development.

Conclusion

Clinical *Pantoea* spp. isolate expresses an advanced multifactorial virulence mechanism comprised of universal biofilm production and co-ordinated adhesion and proteolysis. The predominance of *fimH* expression, accompanied by *ZapA* metalloprotease expression is key in enabling tissue invasion and immune evasion, creating a strong pathogenic phenotype. The ability of all isolates to form biofilm in vitro indicates that biofilm formation is a constitutive virulence factor rather than 'conditional' trait. This result fundamentally challenges this therapeutic approach, which must now employ biofilm-targeted strategies. Research in the future should clarify biofilm regulatory mechanisms and identify new antibiotic agents focused on both attachment and proteolytic pathways. Molecular surveillance combined with improved infection control practices is needed to prevent *Pantoea*-mediated nosocomial infections in these immunocompromised groups.

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Competing Interests

None.

Declaration

I hereby declare that this research is my original work, conducted ethically and independently, and has not been submitted or published elsewhere. All sources have been properly cited, and no conflicts of interest exist regarding this study.

Ethical Approval

The study was approved by the Ethical Committee FMSA .98\3 5 2025\ of Al-Iraqia College of Medicine. Patient confidentiality was maintained by anonymizing all the data before the analysis.

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